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Contribution of hydrogen peroxide to the inhibition of *Staphylococcus aureus* by *Lactococcus garvieae* in interaction with raw milk microbial community

Céline Delbes-Paus*, Géraud Dorchies, Zineddine Chaabna, Cécile Callon, Marie-Christine Montel

Unité de Recherches Fromagères UR545, INRA, 20 Côte de Reyne, F-15000 Aurillac, France

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ABSTRACT

The response of *Staphylococcus aureus* growth inhibition by *Lactococcus garvieae* to catalase and milk lactoperoxidase, and its efficiency in raw milk cheese were evaluated. *S. aureus* and *L. garvieae* were co-cultivated in broth buffered at pH 6.8, and in raw, pasteurized and microfiltered milk, in presence and absence of catalase. Although H_2O_2 production by *L. garvieae* was detected only in agitated broth, the inhibition of *S. aureus* by *L. garvieae* was reduced by catalase both in static and shaking cultures by 2.7 log, pasteurized milk (~0.7 log), microfiltered milk (~0.6 log) and raw milk (~0.2 log). The growth of *S. aureus* alone in microfiltered milk was delayed compared with that in pasteurized milk and inhibition of *S. aureus* by *L. garvieae* was stronger in microfiltered milk. The inhibition of coagulase-positive staphylococci (CPS) by *L. garvieae* in raw milk cheese was similar to that in raw milk (~0.8 log), but weaker than that in pasteurized and microfiltered milks. *L. garvieae* also had an early antagonistic effect on the growth of several other microbial groups, which lastingly affected populations levels and balance during cheese ripening.

1. Introduction

Staphylococcus aureus strains can produce staphylococcal enterotoxins (SEs), which have frequently been responsible for food poisoning associated with raw milk cheese in France (De Buyser et al., 2001), in other European countries (Haeghebaert et al., 2002) and in Brazil (Simeao do Carmo et al., 2002), and with reconstituted milk in Japan (Ikeda et al., 2005). To ensure the safety of raw milk cheeses the development of food borne pathogens must be controlled, in compliance with European regulations laying down the obligation to determine the presence of enterotoxins in cheese if the level of *S. aureus* is over 10^5 $CFUg^{-1}$.

Safety has been greatly improved at the milk production stage by controlling mastitis in herds and applying hygienic practices (Sommerhauser et al., 2003; Chassagne et al., 2005). Bio-preservation strategies based on the addition of inhibitory bacterial strains may also be used to inhibit the growth of pathogens in cheese. Some members of raw milk complex microbial communities such as lactic-acid bacteria (e.g., *Lactococcus lactis*, *Lactococcus garvieae*, *Lactobacillus* spp., *Leuconostoc* spp., and *enterococci*) could contribute to the control of *S. aureus* in cheese, not only by decreasing pH but also by producing bacteriocins (Arques et al., 2005) or H_2O_2 (Haines and Harmon, 1973; Ocana et al., 1999; Otero and Nader-Macias, 2006). *L. garvieae* is commonly isolated from raw milk and dairy products. *L. garvieae* strains have been reported to produce bacteriocin (garviecin; Villani et al., 2001) and bacteriocin-like substances that inhibit indicator strains of *Listeria monocytogenes* and *L. innocua* but also *Lactobacillus sakei*, *Enterococcus faecium* and *E. mundtii* (Corsetti et al., 2008), *L. lactis* and *Pediococcus acidilactici* (Sanchez et al., 2007). Alomar et al. (2008a,b) showed that *L. garvieae* inhibited *S. aureus* in microfiltered milk and in cheese made with pasteurized milk. Inhibition of *S. aureus* by *L. garvieae* in microfiltered milk has been shown to be due neither to acidification nor to competition for amino acids. A preliminary study in brain–heart infusion (Alomar, 2007) suggested that hydrogen peroxide may play a role in the inhibition of *S. aureus* by *L. garvieae*, although the determination of hydrogen peroxide production by *L. garvieae* using ABTS (2,2-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid) (Yang and Ma, 2005) gave inconsistent results. If hydrogen peroxide is involved in the inhibition of *S. aureus* by *L. garvieae*, its efficiency in milk may be affected by the lactoperoxidase system (LPS), an antimicrobial system found in milk (Piard and Desmazeaud, 1991). To be functional, lactoperoxidase and thiocyanate, which occur naturally in milk, must be combined with a source of H_2O_2 (Kamau et al., 1990). The efficiency of *S. aureus* inhibition by *L. garvieae* may also depend on the interactions of both these organisms with the indigenous microflora of raw milk, which, among others, includes catalase-positive bacteria.

* Corresponding author. Tel.: +33 4 71 45 64 19; fax: +33 4 71 45 64 13.
E-mail address: celine.delbes@clermont.inra.fr (C. Delbes-Paus).

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The present work had two aims. One was to investigate the role of H_2O_2 in the inhibition of *S. aureus* growth by *L. garvieae* and the interaction with milk lactoperoxidase. The other, with a view to exploiting the antistaphylococcal activities of *L. garvieae* in raw milk cheese, was to examine the efficiency of inhibition during cheese-making and ripening. In particular, the mutual effect of *L. garvieae* and the indigenous microbiota of raw milk on the inhibition was investigated.

2. Material and methods

2.1. Strains and culture conditions

L. garvieae N201 and *S. aureus* SA15, isolated from raw milk, were cultured as described by Alomar et al. (2008a). Cell concentrations in broths were determined by microscopy using a Petroff–Hausser cell.

2.2. Co-culture of S. aureus and L. garvieae in brain–heart infusion (BHI) and sensitivity of S. aureus to hydrogen peroxide

S. aureus SA15 (150 CFU ml^{-1}) was co-cultivated with *L. garvieae* N201 (10^8 CFU ml^{-1}) in 40-ml portions of BHI (Biokar Diagnostics, Pantin, France) in 100-ml flasks incubated at 30 °C for 24 h, either under aerobic static or shaking (150 rpm) conditions (Table 1). BHI was buffered at pH 6.8 with phosphate buffer KH_2PO_4/K_2HPO_4 0.1 mol l^{-1} (KH_2PO_4, Prolabo; K_2HPO_4, Merck) to avoid the effect of decreasing pH values on the growth of *S. aureus*. Catalase from bovine liver (ref. C100, Sigma) was added at a final concentration of 4000 IU ml^{-1}. Samples constituted by the whole content of one flask for each experimental condition were taken at time 0 and after 3, 6, 9 and 24 h of incubation to determine *S. aureus* and *L. garvieae* counts and hydrogen peroxide concentrations.

In order to determine the sensitivity of *S. aureus* SA15 to hydrogen peroxide, this strain was inoculated at 150 CFU ml^{-1} in BHI containing from 0.5 to 5 mmol l^{-1} of hydrogen peroxide and incubated at 37 °C as the optimal temperature for growth of *S. aureus*, for 18 h. *S. aureus* SA15 count after 18 h was determined and compared to that in a control assay without hydrogen peroxide.

2.3. Co-culture of S. aureus and L. garvieae in raw, high-temperature pasteurized and microfiltered milk

Bulk tank milk (120 L) was divided into 3 portions. The first part (1 L) was not treated (raw milk). The second part (40 L) was pasteurized at 80 °C for 15 s. The third part (70 L) was separated into cream and skimmed milk. The raw cream was pasteurized at 80 °C for 15 s. The skimmed milk was processed at 50 °C through the microfiltration unit equipped with a 1.2 μm-pore size ceramic membrane. The pasteurized cream was blended with the microfiltration permeate to obtain a protein:fat ratio equal to that in the raw whole milk.

Raw, pasteurized and microfiltered milks were each distributed in 40-ml portions in 100-ml flasks and allowed to settle at 33 °C, the average temperature of milk in the vat when starter cultures are added at the begining of the cheese-making process, in a non-agitated water bath. *S. aureus* (SA15) was added to all flasks at a concentration of 150 CFU ml^{-1} of milk. *L. garvieae* (N201) was added at a concentration of 10^8 CFU ml^{-1}, as previously determined (Alomar et al., 2008a), to half of the flasks (Table 2). Catalase from bovine liver (ref. C100, Sigma, Saint-Louis, Missouri, USA) was added at final concentration of 4000 IU ml^{-1}. Milk samples constituted by the whole content of one flask for each experimental condition were taken at time 0 and after 6 and 24 h of incubation. Subsamples were stored at −20 °C for microbiological analysis and to determine lactoperoxidase activity. The entire experimental design was repeated three times.

Table 1
Production of hydrogen peroxide by *L. garvieae* N201 and effect of catalase on the inhibition of *S. aureus* SA15 by *L. garvieae*, in buffered Brain Heart Infusion (pH 6.8). ND, not determined.

	L. garvieae (log CFU/ml)	Catalase (IU/ml)	*S. aureus* (log CFU/ml) Time (h)				H_2O_2 (mmol/l) Time (h)			
			3	6	9	24	3	6	9	24
Static	0	0	2,83	4,48	5,77	7,28	0	0	0	0
	8	0	2,48	3,00	4,04	4,57	0	0	0	0
	8	4000	ND	ND	ND	7,30	0	0	0	0
Shaking (150 RPM)	0	0	2,72	4,32	5,70	8,36	0	0	0	0
	8	0	2,48	2,30	3,60	4,60	0	1	1,4	1,6
	8	4000	ND	ND	ND	7,34	0	0	0	0

2.4. Co-culture of S. aureus and L. garvieae in high-temperature pasteurized milk with or without lactoperoxidase

Bulk tank milk was pasteurized at 80 °C for 15 s, distributed in 20-ml portions in 100-ml flasks and allowed to settle at 33 °C in a non-agitated water bath. *S. aureus* (SA15) and *L. garvieae* (N201) were added respectively at a concentration of 150 CFU ml^{-1} and of 10^8 CFU ml^{-1} (see Table 3 for experimental design). Lactoperoxidase from bovine milk (ref. L2005, Sigma, Saint-Louis, Missouri, USA) was added at final concentration of 37 mg l^{-1}, similar to what was found in raw milk in the previous assays. Three flasks for each experimental condition were taken at time 0 and after 6 and 24 h of incubation. Subsamples were stored at −20 °C for microbiological analysis and to determine lactoperoxidase activity.

2.5. Inoculation of L. garvieae in raw milk and cheese making

Raw farm milk was collected and transferred to the experimental cheese plant (INRA, URF, Aurillac) within 15 min. It was used to make 3 assay cheeses (600 g) inoculated with *L. garvieae* and 3 control cheeses without *L. garvieae* using an uncooked pressed cheese technology. Milk was placed into six 6-L vats and warmed to 33 °C. The commercial MY800 starter culture (*Streptococcus thermophilus*, *Lactobacillus delbruekii* subsp. *lactis*, and *L. delbruekii* subsp. *bulgaricus*) (Rhodia S.A., Paris La Défense, France) was inoculated in all vats (0.6%, vol/vol). *L. garvieae* (N201) was added to half the vats (Table 3) at a concentration of 10^8 CFU ml^{-1} of milk. Rennet was added to all vats at 1.51 g l^{-1} (Beaugel 520 mg/L of chymosin). Coagulation proceeded for about 45 min and then the curd was cut, prepressed and placed in molds. Salt (26 g per cheese) was added and draining was completed under 3-bar pressure for 24 h. The cheeses were coated with wax containing 1000 ppm of natamycin (Coquard, Villefranche sur Saône, France) to prevent the development of moulds and yeasts on the cheese surface. Then they were ripened in a ripening room at 10 °C, 96% relative humidity for 32 days. Samples were taken aseptically from the raw milk in each vat prior to the addition of the rennet, and from the cheeses (one quarter of each cheese) on days 1, 8, 16 and 32. Cheese rind (5 mm) was discarded. Subsamples were stored at 4 °C and analysed within 24 h for their coagulase-positive staphylococci count and the remainder stored at −20 °C for further microbiological tests. The entire experimental design was repeated twice with raw milk from two different farms.

2.6. Microbial analyses

Milk and cheese samples were emulsified in sterile phosphate buffer 2% (wt/vol) and blended with a Stomacher Lab Blender (Seward Medical, London, UK) for 4 min. All counts were performed

Table 2
Evolution of pH values and growth of coagulase-positive staphylococci (CPS) in cocultures with *L. garvieae* in raw, microfiltered and pasteurized milks, in presence or absence of catalase. CPS 0–6 h: log count at 6 h − log count at time 0; CPS 6–24 h: log count at 24 h − log count at 6 h, etc. Counts in log CFU per milliliter. Values are means of 3 batches. SD, Standard deviation. Letters a, b, and c in the same column indicate homogeneous statistical processing groups that were significantly different according to the Newman Keuls statistical test, with A < B < C. *, P, 0.05; **, P, 0.01; ***, P, 0.001; NS, not significant.

Milk	Catalase (IU/ml)	*L. garvieae* (log CFU/ml)	pH 6 h			pH 24 h			CPS 0–6 h			CPS 6–24 h			CPS 0–24 h	
			Mean	SD		Mean	SD		Mean	SD		Mean	SD		Mean	SD
Raw	0	0	6,78	0,03	b	4,57	0,33	a	2,42	1,33	d	1,09	1,06	b	3,51	1,12
Raw	0	8	6,71	0,06	b	4,73	0,38	a	1,41	0,22	b	1,22	1,06	b	2,63	1,24
Raw	4000	0	6,76	0,01	b	4,55	0,33	a	1,86	0,75	c	1,55	0,92	b	3,41	1,09
Raw	4000	8	6,66	0,07	ab	4,69	0,39	a	1,20	0,54	b	1,64	1,43	b	2,84	1,05
Pasteurized	0	0	6,74	0,06	b	6,03	0,33	c	1,79	0,41	b	1,81	1,30	b	3,59	1,34
Pasteurized	0	8	6,51	0,20	ab	5,60	0,20	bc	−0,05	1,13	a	1,25	1,10	b	1,20	2,22
Pasteurized	4000	0	6,73	0,05	b	5,99	0,27	c	1,69	0,33	b	1,15	0,84	b	2,83	0,56
Pasteurized	4000	8	6,45	0,22	a	5,40	0,26	b	0,45	0,58	ab	1,42	1,01	b	1,87	1,10
Microfiltered	0	0	6,77	0,02	b	6,62	0,02	e	0,44	0,94	ab	3,78	0,38	c	4,21	0,55
Microfiltered	0	8	6,61	0,09	ab	6,00	0,22	c	0,45	0,31	ab	0,76	0,53	a	1,21	0,64
Microfiltered	4000	0	6,75	0,04	b	6,20	0,35	d	0,82	0,77	b	2,61	1,67	b	3,43	1,37
Microfiltered	4000	8	6,59	0,08	ab	5,92	0,19	bc	0,34	0,33	ab	1,50	0,90	b	1,84	0,89
			**			**			**			**			NS	

in duplicate either by manual plating for milk samples or using a Spiral plate system for cheese samples. Coagulase positive staphylococci were enumerated on Rabbit Plasma Fibrinogen Agar (RPFA) (EN ISO 6888-2). Mesophilic bacterial populations and thermophilic bacterial populations were enumerated on M17 agar (Terzaghi and Sandine, 1975) incubated at either 30 °C or 42 °C. Lactococci were enumerated on Turner–Sandine–Elliker (TSE) agar (Turner et al., 1963) with nalidixic acid, facultatively heterofermentative (FH) lactobacilli on agar as described by Isolini et al. (1990), dextrane-producing leuconostocs on Mayeux–Sandine–Elliker (MSE) agar (Mayeux et al., 1962), enterococci on SB agar (Slanetz and Bartley, 1957), *Pseudomonas* on cetrimide-fucidin-cephalosporin (CFC) agar, Enterobacteriaceae on violet–red–bile–glucose (VRBG) agar and yeasts and moulds on oxytetracyclin–glucose agar (OGA). Samples were also spread on non selective media: Cheese Ripening Bacteria Medium (CRBM) described by Denis et al. (2001), Plate Count Agar supplemented with 1% milk (PCAM) and Plate Count Agar supplemented with 1% milk, 5 mg/l vancomycin and 5 mg/l crystal violet as inhibitors of Gram-positive bacteria (PCAI). Both PCA media were incubated at 30 °C for 48 h. All media were purchased from Biokar Diagnostics (Pantin, France).

The pH values of milk and cheese were determined with a 926 VTV pH meter with an Ingold 406 MX penetration electrode (Mettler-Toledo S.A., Viroflay, France).

2.7. Quantification of *L. garvieae* in milk and cheese by quantitative PCR

2.7.1. Primer design − specificity

The sequences of 16S rRNA genes from *L. garvieae* (9 strains), 5 species of *Lactococcus* and 2 species of *Enterococcus* were aligned using Clustalw software. The *L. garvieae* 16S rDNA sequence was then examined for the presence of specific regions for the detection of *L. garvieae*. Two primers having a size of less than 25 nucleotides, 20–80% of GC, less than 2 G or C in the last five nucleotides in 3′-end, for which there were at least 3 mismatches with sequences of other species, and which gave an amplicon size of less than 200 pb were determined. Regions corresponding to gene positions 30 to 52 (rtLgFw 5′-GCAAGTCGAGCGATGATTAAAGA- 3′) and to gene position 160 to 185 (rtLgRev 5′-TTCAAATAAGAATCATGCGATTCTC-3′) amplifying a fragment of 140 pb were selected. These primers were validated using Primers Express software (Applied Biosystems).

The specificity of primer sequences was initially evaluated *in silico* using the Probe Check program of the Ribosomal Database Project (Cole et al., 2003). It was then confirmed experimentally by classical PCR on 15 species of *Lactococcus*, *Leuconostoc*, *Enterococcus*, *Lactobacillus* and *Pediococcus*. The primers were synthesized by Eurogentec.

2.7.2. Estimation of the sensitivity of the method

L. garvieae genomic DNA isolated from an overnight culture in 10 ml of broth was serially diluted 10-fold in water and 5 µl of the dilution were then subjected to real-time PCR amplification (conditions described below). All assays were performed in duplicate and the average values were used to determine the threshold cycle (Ct). There was a linear relationship between the Ct and the logarithm of CFU/g (correlation coefficient R^2 of the standard curves was 0.993). The amplification efficiency calculated ($E = 10^{-1/\text{slope}}$) was 1.98. The detection limit of the method was close to 10^3 CFU/g and the amplification was linear over 5 log. Analysis of the melting curve showed a specific amplified product and no peak was observed with the controls in which all required reagents but no

Table 3
Evolution of pH values and growth of coagulase-positive staphylococci (CPS) in cocultures with *L. garvieae* in pasteurized milk in presence or absence of lactoperoxidase. CPS 0–6 h: log count at 6 h − log count at time 0; CPS 6–24 h: log count at 24 h − log count at 6 h, etc. Counts in log CFU per milliliter. Values are means of 3 batches. SD, Standard deviation. Letters a, b, and c in the same column indicate homogeneous statistical processing groups that were significantly different according to the Newman Keuls statistical test, with A < B < C. *, P, 0.05; **, P, 0.01; ***, P, 0.001; NS, not significant.

Lactoperoxidase (mg l^{-1})	*L. garvieae* (log CFU ml^{-1})	pH 6 h			pH 24 h			CPS 0–6 h			CPS 6–24 h			CPS 0–24 h		
		Mean	SD		Mean	SD		Mean	SD		Mean	SD		Mean	SD	
0	0	6,75	0,01	b	6,39	0,01	b	2,33	0,10	c	3,48	0,08	b	5,82	0,08	c
0	8	6,35	0,01	a	5,69	0,02	a	−1,20	0,35	a	0,80	0,35	a	−0,40	0,35	a
37	0	6,73	0,01	b	6,16	0,33	b	2,29	0,04	c	3,04	0,46	b	5,33	0,50	c
37	8	6,36	0,01	a	5,57	0,16	a	0,14	0,05	b	1,22	0,26	a	1,36	0,24	b
		**			**			**			**			**		

DNA were added. The method was then validated by quantifying *L. garvieae* inoculated at different concentrations in microfiltered milk. A good match was observed between real-time PCR quantification and theoretic values in milk.

2.7.3. DNA extraction from milk and cheese samples

10 ml of milk or 1g of cheese were subjected to DNA extraction according to protocols described by Callon et al. (2007) for the milk, and by Duthoit et al. (2003) for the cheeses, using Phase-Lock tubes (Eppendorf) for the phenol/chloroform extraction steps.

2.7.4. Real-time PCR conditions

SYBR Green PCR amplifications were performed using an ABI Prism 7700 (Applied Biosystems) in a 25 µl final volume containing 5 µl of DNA sample or standard, 2 mM $MgCl_2$, 200 mM of each primer, 2 mM dNTP mix, Sybr Green PCR buffer 1×, Ampli Taq Gold polymerase 5 U/µl. Amplification consisted of an initial hold at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 61 °C for 1 min. Standard and 1/10 diluted samples were run on the same plate and analysed in duplicate. Threshold cycle (Ct) values were determined by the SDS software (Applied Biosystems).

2.8. Determination of hydrogen peroxide production in BHI

The concentration of H_2O_2 during cell growth in buffered BHI was determined as described by Batdorj et al. (2007) with slight modifications. First a 100-µl aliquot of culture supernatant (9600 g, 4 °C, 10 min) was mixed with a solution composed of 750 µl of phosphate buffer Na_2HPO_4/NaH_2PO_4 (0.1 M, pH 7), 100 µl of 4-aminoantipyrine solution (4 mg ml^{-1}, amino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one, Sigma) and 20 µl of water-saturated phenol. Then 30 µl of horseradish peroxidase type VI-A (Sigma, 1000 U ml^{-1} solution in sodium phosphate buffer pH 6) were added, the reaction was allowed to proceed for 2 min and the change in absorbance was measured at 505 nm. The hydrogen peroxide was quantified using a standard curve determined with H_2O_2 solutions in sodium phosphate buffer at concentrations ranging from 1.5 to 100 µmol l^{-1}.

2.9. Determination of hydrogen peroxide in milk

The method based on 4-aminoantipyrine (Batdorj et al., 2007) gave inconsistent results for hydrogen peroxide analysis in milk. Instead, the method used was derived from that described by Ferrier et al. (1970). Hydrogen peroxide standards (0, 125, 250, 500 and 1000 µM) were prepared by adding 1 ml of water containing the appropriate amount of hydrogen peroxide to 9 ml of whole milk previously heated to 80 °C for 5 min. For H_2O_2 determination, 4 ml of milk were added to 4 ml of 2% trichloro-acetic acid, well mixed and filtered through Whatman no. 42 filter paper. One hundred µl of Titanium (IV) chloride solution 0.09 M in 20% HCl (Aldrich) were added to a 1-ml aliquot of the filtrate and the absorbance was measured against a reagent blank (filtrate + $TiCl_4$) at 415 nm on a Hitachi U-2000 spectrophotometer. The detection level for hydrogen peroxide in milk using this method was 100 µM.

2.10. Determination of lactoperoxidase enzyme activity in milk

The LP activity of the milk samples was determined using the ABTS (Sigma, ref. A 9941) assay as described by Kumar and Bathia (1998). Since several of the milk samples coagulated during incubation, all milk samples were homogenized by vigorously shaking for 10 s, diluted to 1/250 in phosphate buffer (0.1 M, pH 6) containing 0.1% gelatin (PBSG), and filtered through a Whatmann n°42 filter paper. The change in the absorbance readings at 412 nm for 2 min was recorded using a Hitachi U-2000 spectrophotometer. The enzyme concentration was calculated using a standard curve determined with solutions of commercial lactoperoxidase (Sigma, ref. L2005) in boiled milk at concentrations ranging from 5 to 50 mg l^{-1}.

2.11. Determination of galactose, lactose, lactic and acetic acid contents of cheese

Galactose, lactose, L-Lactate, D-lactate and acetic acid contents were determined using enzymatic method as recommended with R-Biopharm kits (R-Biopharm AG, Darmstadt, Germany). Results were expressed in g per kg of cheese dry weight.

2.12. Statistical analyses

Standard analyses of variance were performed to assess the effect of the milk treatments, the inoculation with *L. garvieae* and the addition of catalase on pH values and microbial counts using Statistica software (Statsoft, version 6, Maisons-Alfort, France). Where the differences were significant, a Newman–Keuls test was performed. Statistical correlations were carried out by the Pearson's correlation coefficient. A stepwise multiple linear regression was performed for milks inoculated with *L. garvieae* to rank the parameters (milk treatment, pH, catalase) which can explain the growth of coagulase-positive staphylococci between 0 and 6 h, and between 6 and 24 h of incubation.

3. Results and discussion

3.1. Potential role of hydrogen peroxide in the inhibition of S. aureus by L. garvieae in brain heart infusion (BHI)

S. aureus growth was inhibited by *L. garvieae* in buffered BHI in both static and shaking conditions (Table 1). *S. aureus* count after 24 h in broth inoculated with *L. garvieae* was lower than the corresponding control cultures by ~2.7 log in static cultures and by ~3.8 log in shaken cultures. This inhibition could not be explained by pH values since they remained above 6.7 in the buffered broth. The detection level for hydrogen peroxide in buffered BHI using the method described by Batdorj et al. (2007) was 1.5 µM. Hydrogen peroxide was only detected in aerated, shaken cultures where it reached up to 1.6 mM after 24 h of incubation. The addition of catalase together with *L. garvieae* released inhibition by 2.7 log both in static cultures (100% reduction) and shaking cultures (72%).

The sensitivity of *S. aureus* SA15 to concentrations of hydrogen peroxide from 0.5 to 5 mM was determined in buffered BHI incubated at 37 °C. The *S. aureus* SA15 count after 18 h was 7.3 log CFU ml^{-1} in the control assay. It was 0.3 log lower in presence of 0.5 mM of hydrogen peroxide and 2 log lower in presence of 1 mM of hydrogen peroxide. *S. aureus* populations were below the detection level (10 CFU ml^{-1}) after 18 h with hydrogen peroxide concentrations of 2–5 mM.

It is striking that *S. aureus* growth was inhibited by *L. garvieae* in static cultures although no H_2O_2 was detected in these conditions. Results from literature show that *Lactobacillus crispatus* produced up to 2.8 mM of H_2O_2 in agitated culture but no detectable amount of this metabolite in aerobic non-agitated cultures (Ocana et al., 1999). On the other hand, cells of *Streptococcus gordonii* and *Lactobacillus delbrueckii* subsp. *bulgaricus* still synthetized H_2O_2 when grown statically, although at a lower rate than cells which were aerated during growth (Marty-Teysset et al., 2000; Barnard and Stinson, 1999). These results suggest that the need for agitating LAB cultures in order to detect H_2O_2 production in culture medium could be dependent on species or strain. Moreover, hydrogen peroxide at low concentrations is quite unstable and may react with

other microbial metabolic end products or intermediates (Gilliland and Speck, 1969), making the determination of H_2O_2 production difficult. Therefore, addition of catalase is commonly used as an indirect method of determining the involvement of H_2O_2 in inhibition (Regev-Yochay et al., 2006). In our study, inhibition of *S. aureus* by *L. garvieae* was reduced when catalase was added to BHI, both in static and shaking conditions. These results suggested that H_2O_2 was involved, either directly or via other oxygen derivatives or reaction products, in the inhibition occuring in BHI both in static and shaking conditions.

3.2. Response of the inhibition of S. aureus by L. garvieae in milk to catalase and lactoperoxidase

The aims of this part of the work were to confirm the role of H_2O_2 in the inhibition of *S. aureus* growth by *L. garvieae* in milk and to evaluate it's interaction with milk enzymatic activities and microbiota. For that purpose, milk was either heated to eliminate heat-sensitive enzymatic activities and microbial populations, or microfiltered to eliminate the latter while preserving enzymatic activities. Co-cultures of *S. aureus* and *L. garvieae* in treated and untreated milks, in presence or absence of catalase were then studied.

Microfiltration did not affect lactoperoxidase (LP) activity, which was similar to that in raw milk ($\sim$37 mg l^{-1}), whereas high-temperature pasteurization inactivated LP.

Total flora counted on PCA medium was below the detection limit (20 CFU ml^{-1}) both in microfiltered and pasteurized milks. In raw milk, total flora count was 4 $\pm$ 0.25 log CFU ml^{-1} and coagulase-positive staphylococci count was 2 $\pm$ 0.4 log CFU ml^{-1}.

Table 2 shows the growth of coagulase-positive staphylococci (CPS) in cocultures with *L. garvieae* N201 in raw, microfiltered, pasteurized milks, in presence and absence of catalase. Coagulase-positive staphylococci growth in absence of *L. garvieae* and of catalase was delayed in microfiltered milk, compared to that in pasteurized and raw milks.

In absence of catalase, CPS growth was inhibited by *L. garvieae* in all milks but inhibition differed with time and milk treatment. Between 0 and 6 h, the strongest inhibition of *S. aureus* growth by *L. garvieae* was observed in pasteurized milk ($\sim$1.8 log difference with the control). Over 24 h, growth of CPS ($\sim$4.2 log) and relative inhibition ($\sim$3 log) were the strongest in microfiltered milk, whereas relative inhibition by *L. garvieae* ($\sim$1 log) was the weakest in raw milk.

No significant difference between raw, pasteurized and microfiltered milks was found in the *L. garvieae* counts, which ranged from 8.5 to 9 log CFU ml^{-1} after 6 h and from 8.6 to 9.3 log CFU ml^{-1} after 24 h.

No hydrogen peroxide was detected in milk inoculated with *L. garvieae* or in control milk at any time. However, *S. aureus* growth between 0 and 6 h in pasteurized milk supplemented with *L. garvieae* and catalase was significantly higher (0.5 log) than in pasteurized milk inoculated with *L. garvieae* alone. Similarly, *S. aureus* growth between 6 and 24 h in microfiltered milk supplemented with *L. garvieae* and catalase was significantly higher (0.7 log) than in microfiltered milk inoculated with *L. garvieae* alone. Over 24 h, the addition of catalase partially released inhibition of *S. aureus* by *L. garvieae* in pasteurized milk (by $\sim$0.7 log, 28%), in microfiltered milk ($\sim$0.6 log, 21%) and in raw milk ($\sim$0.2 log, 24%). These results could not be explained by a direct positive effect of catalase on *S. aureus* growth since in absence of *L. garvieae*, catalase reduced *S. aureus* growth over 24 h in pasteurized and microfiltered milks by 0.7 log compared to the control milks (Table 2). Alternatively, they suggested that H_2O_2 or derivatives could be involved in the inhibition of *S. aureus* by *L. garvieae* in milk.

At time 0, no significant difference in pH values was observed between raw, pasteurized and microfiltered milks inoculated or not with *L. garvieae* and/or catalase (data not shown). After 6 h of incubation, average pH values in all milks inoculated with *L. garvieae* were significantly lower (by 0,1 to 0.4 pH units) than those in control milks (Table 2). They differed with milk treatment and ranged between 6.45 in pasteurized milk and 6.78 in raw milk. However, data from the literature (Le Marc et al., 2009; Sutherland et al., 1994) as well as data inferred from the model described by Alomar et al. (2008a) suggest that such pH values would not markedly affect the growth of *S. aureus* and could not fully explain the inhibition of *S. aureus* growth in milk within the first 6 h. After 24 h, pH values were lowest in raw milk (below 4.8) irrespective of the presence or absence of *L. garvieae*. Average pH values in pasteurized and microfiltered milks ranged between 5.3 and 6.6 and were significantly lower (by 0,1 to 0.5 pH units) in inoculated milks than in controls.

In the present study the microfiltered and raw milks had similar LP activities and differed mainly by the presence of natural milk flora in raw milk. In absence of *L. garvieae* N201, coagulase-positive staphylococci grew more rapidly between 0 and 6 h in raw milk than in microfiltered milk. Since no differentiation was made between the native CPS populations present in raw milk and the inoculated strain, this result may be due to a better adaptation of wild populations of CPS present in raw milk than the inoculated SA15 strain. Inhibition of CPS growth by *L. garvieae* was observed both in microfiltered and raw milks, but was less important in raw milk. Addition of catalase also reduced inhibition less effectively in raw milk. These results may be due to a lower sensitivity of native CPS populations to H_2O_2 or to an antagonistic effect of the raw milk microbial community on the inhibitory activity of *L. garvieae*. Finally, coagulase-positive staphylococci growth between 6 and 24 h, in absence of *L. garvieae* N201, was weaker in raw milk than in microfiltered milk. This result suggested that the indigenous microbial community of raw milk had an antagonistic effect against CPS, possibly via acidification, since pH values were below 4.8 in raw milk after 24 h.

Both HT pasteurization and microfiltration drastically reduced the indigenous milk flora. HT pasteurized and microfiltered milks may differ in terms of heat-sensitive activities such as the natural lactoperoxidase or catalase activity of milk. Lactoperoxidase was completely deactivated after HT pasteurization of milk at 80 °C for 15 s, as found by Barrett et al. (1999). In our study, whereas the evolution of *L. garvieae* counts did not differ between the pasteurized and microfiltered milks, the delay in *S. aureus* growth alone in microfiltered milk but not in pasteurized milk may be due to a heat-sensitive bacteriostatic compound present in microfiltered milk and absent in pasteurized milk. Furthermore, inhibition of *S. aureus* by *L. garvieae* was observed both in pasteurized and in microfiltered milks but was stronger in microfiltered milk. To determine whether these differences may result from lactoperoxidase activity, the response of the inhibition of *S. aureus* by *L. garvieae* in HT-pasteurized milk to addition of commercial lactoperoxidase was then studied. Table 3 shows the growth of *S. aureus* alone or in coculture with *L. garvieae*, in presence or absence of LP (37 mg l^{-1}). Growth of *S. aureus* alone and inhibition by *L. garvieae* (Table 3) in absence of LP were higher than those observed in the first set of experiment with pasteurized milk (Table 2). This may result from differences in the biochemical composition of the milk used. *S. aureus* growth in absence of *L. garvieae* was not significantly affected by the addition of LP during the first 6 h but was reduced by 0.5 log over 24 h compared to the control milks. In co-cultures with *L. garvieae*, addition of LP kept *S. aureus* counts fairly stable between 0 and 6 h whereas they droped down below the initial inoculum (~ -1.2 log) in pasteurized milk inoculated with *L. garvieae* alone. Over 24 h, the addition of LP in pasteurized milk partially released inhibition of *S. aureus* by *L. garvieae* by $\sim$1.7 log (27%). This assay did not

Table 4
Microbial counts in raw milk cheeses with or without *L. garvieae*. Counts in log CFU per milliliter in milk and in log CFU per gram in cheese. Values are the means of 6 batches. SD, Standard deviation. Letters a, b, and c in the same column indicate homogeneous statistical processing groups that were significantly different according to the Newman Keuls statistical test, with A < B < C. *, *P*, 0.05; **, *P*, 0.01; ***, *P*, 0.001.

L. garvieae (log CFU/ml)	Time (days)	RPF C+			RPF C−			SB			MSE D+			CFC			Turner		
		Coagulase positive staphylococci			Gram+ catalase+ bacteria			Enterococci			Dextrane-producing leuconostoc			*Pseudomonas*			*Lactococcus*		
		Mean	SD		Mean	SD		Mean	SD		Mean	SD		Mean	SD		Mean	SD	
0	0	2,92	0,29	a	3,49	0,15	a	2,24	0,10	a	1,70	0,45	a	2,38	0,14	a	3,56	0,27	a
8	0	2,95	0,30	a	3,39	0,22	a	2,11	0,33	a	1,48	0,28	a	2,50	0,38	a	7,31	0,53	d
0	1	5,35	0,73	c	4,75	1,03	b	5,22	0,33	c	5,36	0,42	c	2,75	0,49	a	6,55	0,20	b
8	1	4,35	0,66	b	4,17	0,32	ab	4,55	0,23	b	4,60	0,30	b	2,31	0,19	a	8,17	0,60	ef
0	8	4,28	0,49	b	4,24	0,27	ab	5,13	0,25	c	7,23	0,20	e	2,61	0,89	a	7,97	0,28	e
8	8	3,46	0,25	a	3,82	0,25	a	4,43	0,14	b	6,49	0,59	d	2,34	0,07	a	8,29	0,33	ef
0	16	3,96	0,51	b	3,98	0,24	ab	5,19	0,22	c	7,91	0,22	g	2,89	0,76	a	8,17	0,08	ef
8	16	3,17	0,21	a	3,74	0,53		4,51	0,15	b	7,37	0,63	ef	2,34	0,07	a	8,62	0,26	f
0	32	3,39	0,50	a	3,73	0,70	a	5,32	0,14	c	7,79	0,22	f	3,89	0,81	b	6,89	0,35	c
8	32	3,23	0,27	a	3,72	1,12	a	4,67	0,12	b	7,66	0,18	f	3,29	1,07	a	8,45	0,25	ef
		**			**			**			**			**			**		
L. garvieae (log CFU/ml)	Time (days)	FH			PCA + I			CRBM			OGA M			OGAL			VRBG		
		Facultative heterofermentative Lactobacilli			Gram-negative bacteria			Gram + catalase + bacteria and Enterococci			Moulds			Yeasts			Enterobacteriaceae		
		Mean	SD		Mean	SD		Mean	SD		Mean	SD		Mean	SD		Mean	SD	
0	0	2,52	0,14	a	1,96	0,65	ab	3,27	0,28	a	1,67	0,64	a	2,07	0,29	a	1,00	0,00	a
8	0	2,51	0,21	a	1,84	0,70	a	3,30	0,29	a	1,64	0,71	a	1,94	0,21	a	1,00	0,00	a
0	1	5,05	0,42	c	3,71	0,44	d	4,96	0,27	d	2,97	0,34	b	3,30	0,35	b	2,63	0,38	d
8	1	4,22	0,52	b	2,67	0,43	bc	4,41	0,12	bc	2,86	0,36	b	3,10	0,33	b	1,51	0,65	ab
0	8	6,45	0,70	d	3,59	0,53	d	5,20	0,72	de	3,11	0,83	b	4,28	0,65	d	2,23	0,27	c
8	8	6,28	0,56	d	2,63	0,48	bc	4,20	0,14	b	2,83	0,44	b	3,20	0,28	b	1,38	0,25	ab
0	16	7,15	0,22	e	2,70	0,21	bc	5,35	0,43	e	3,21	0,45	b	4,09	0,49	cd	1,59	0,54	b
8	16	6,66	0,24	d	2,83	0,40	c	4,96	0,37	d	3,33	0,06	b	3,61	0,29	bc	1,08	0,19	a
0	32	7,95	0,29	f	2,54	0,49	bc	4,60	0,22	cd	3,67	0,83	b	4,11	0,35	cd	1,00	0,00	a
8	32	7,79	0,24	f	2,77	0,81	bc	4,76	0,79	cd	3,44	0,92	b	3,94	0,66	cd	1,00	0,00	a
		**			**			**			**			**			**		

reproduce the results previously obtained with microfiltered milk. The results differed from those of Fweja et al. (2008), who reported the bacteriostatic effect against *S. aureus* of H_2O_2 combined with LP was 30% more effective than that of H_2O_2 alone. Lactoperoxidase has been found to exert a bacteriostatic effect on lactic acid bacteria, and a bactericidal effect on Gram-negative bacteria (Piard and Desmazeaud, 1991). *S. aureus* growth was delayed in milk with concentrations of LP from 5 mg l^{-1} (McLay et al., 2002; Fweja et al., 2008). In our assay, limiting amount of indigenous thiocyanate in milk may have hampered the biocidal activity of LP lying in the production of hypothiocyanite. On the contrary, LP reduced the inhibition of *S. aureus* by *L. garvieae* in pasteurized milk in a similar way as catalase did. Indeed, several studies have shown that in the presence of an excess of hydrogen peroxide, lactoperoxidase may react as a pseudo-catalase (Boots and Floris, 2006).

Thus, inhibition of CPS by *L. garvieae* in milk did not only depend on production of H_2O_2 by *L. garvieae* and was affected by synergistic or antagonistic factors such as milk indigenous lactoperoxidase system or catalase activities. Acidification was likely involved in the inhibition between 6 and 24 h, especially in raw milk. Involvement of bacteriocins or competition for nutrients in the inhibition of *S. aureus* by *L. garvieae* cannot be excluded. However, Alomar et al. (2008b) did not find any antistaphylococcal substances in the concentrated supernatants of cultures of *L. garvieae* N201. Also, competition for amino acids was not involved as addition of valine, isoleucine, threonine, methionine and phenylalanine did not suppress the inhibition of *S. aureus* by *L. garvieae* N201.

3.3. Inhibition of CPS by L. garvieae and interactions with the microbial community in raw milk cheese

Considering the potential antagonistic effect of raw milk microbial community on coagulase-positive staphylococci and *L. garvieae*, the efficiency of the inhibition of CPS by *L. garvieae* in raw milk cheese during cheese-making and ripening was examined.

Growth of CPS between 0 and 24h in raw milk cheeses was 1 log lower in presence of *L. garvieae* than in control cheeses (Table 4). Afterwards, CPS counts decreased and remained lower in cheeses inoculated with *L. garvieae* than in control cheeses at least up to 16 days of ripening.

The lactococcus count (Turner medium) was about 7.3 log CFU ml^{-1} in milk inoculated with *L. garvieae* (Table 4). It reached 8.2 log CFU ml^{-1} in cheese on day 1 and remained between 8.2 and 8.6 log CFU ml^{-1} up to the end of ripening. *L. garvieae* population levels determined by quantitative PCR were higher than the counts on Turner medium, especially in milk with 8.7 log CFU ml^{-1}, and to a lesser extent in cheese (8.6 log CFU ml^{-1} on day 1 and 9 log CFU ml^{-1} on day 32). These discrepancies may be due to the fact that the QPCR analyses were performed on genomic DNA, allowing the detection of viable non-culturable cells and dead cells. *L. garvieae* was absent or below the detection level of the QPCR assay (10^4 CFU ml^{-1}) in control milk and control cheeses.

L. garvieae only grew on Turner medium, therefore counts on all other media were not affected by the presence of colonies of *L. garvieae*. Between the milk stage and 24 hold cheese, growth of *Enterococci* (SB), dextrane-producing *Leuconostocs* (MSE), *Pseudomonas* (CFC), mesophilic heterofermentative *Lactobacilli* (FH), Gram-positive catalase-positive bacteria and *Enterococci* (CRBM), and Enterobacteriaceae (VRBG) were all significantly lower in presence of *L. garvieae*. After that, counts of *Enterococci* remained by at least 0.6 log lower in presence of *L. garvieae* up to day 32. Counts of Gram-positive catalase-positive bacteria, dextrane-producing *Leuconostocs*, mesophilic heterofermentative *Lactobacilli*, total Gram-negative bacteria (PCAI) and Enterobacteriaceae remained lower in presence of *L. garvieae* up to day 16. Counts of yeasts (OGAL) were significantly lower in presence of *L. garvieae* on days 8 and 16.

The pH values in cheese were around 5.6 after 6 h, and around 5.2 after 24 h. No significant difference was found in presence or absence of *L. garvieae*. On day 1, concentrations of D-lactate and acetate were very low (<0.1 g kg^{-1}) and no significant difference was found in L-lactate concentrations (around 9 g kg^{-1}) in presence v. absence of *L. garvieae*. Therefore, *L. garvieae* had no significant effect on acidification in cheese.

Despite the higher CPS count in raw milk used to make cheese (~3 log CFU ml^{-1}) compared with that in raw milk used for milk assays (~2 log CFU ml^{-1}), the inhibition of CPS growth by *L. garvieae* in raw milk cheese over the first day was similar to that in raw milk (~0.8 log), but lower than that in pasteurized and microfiltered milks. Again, this may be due to a lower sensitivity of native CPS populations to H_2O_2. Alternatively, or in addition, it may be due to the activity of catalase-positive bacteria from raw milk, such as ripening bacteria (coryneforms, coagulase-negative staphylococci), which reached 4 to 5 log CFU g^{-1} in cheese on day 1. Although lactic acid bacteria are catalase-negative and generally sensitive to H_2O_2 (Rochat et al., 2006), most of them demonstrated various degrees of reactive oxygen species' scavenging abilities (Lin and Yen, 1999). Most LAB, such as *Lactococcus lactis,* express superoxide dismutase activity (Miyoshi et al., 2003).

L. garvieae had an early antagonistic effect not only on coagulase-positive staphylococci but also on the growth of several other microbial groups. This lastingly affected population levels and balance during ripening. These results support the hypothesis of a non-specific inhibition mechanism, as could be a nutritional competition or the production of H_2O_2 by *L. garvieae*. Hydrogen peroxide is known to have a rapid bacteriostatic/bactericidal effect on microbial populations, especially Gram-negative bacteria (Bjorck et al., 1975).

4. Conclusion

This study has not fully resolved the question of the nature of the inhibition of *S. aureus* by *L. garvieae* but the results support the hypothesis of a mechanism involving hydrogen peroxide or derivatives, which was effective within the first 6 h of culture. Acidification was likely involved in the inhibition between 6 and 24 h in raw milk. Inhibition of CPS by *L. garvieae* in milk may also be affected by milk indigenous hydrogen peroxide scavenging activities. In particular, the potential synergistic or antagonistic interaction of milk lactoperoxidase system on the inhibition, depending on H_2O_2 and thiocyanate concentrations, remains a hypothesis which deserves further investigations. Further work is also needed to understand the role of the microbial community of raw milk which may at the same time reduce *S. aureus* growth and interact with the inhibition by *L. garvieae* for example by producing catalase. Conversely, *L. garvieae* clearly reduced counts in several microbial groups in cheese on the first day and affected their dynamics during cheese ripening. In view of exploiting the antistaphylococcal activities of *L. garvieae* for the production of raw milk cheese, further investigation is needed to evaluate the consequences for the sensory properties of the cheese. However, the safety of *L. garvieae* N201 as a biopreservative strain in food should first be determined (Casalta and Montel, 2008). *L. garvieae* is a well-recognized fish pathogen (Vendrell et al., 2006) and is regarded as a rare, opportunistic pathogen of low virulence in human infections, responsible for scattered endocarditis (Li et al., 2008). However, Foschino et al. (2008) found a slight genetic relatedness between Italian dairy isolates and fish isolates. Fortina et al. (2007) showed that strains from dairy origin were characterized by a weak lactose acidifying

capacity, which is absent in strains from fish origin, and generally by a low incidence of known virulence factors. Future research will explore genomic pathways for production of oxygen derivatives by *L. garvieae* and their potential role in *S. aureus* inhibition.

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