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Behavior of *Listeria monocytogenes* and *Staphylococcus aureus* in Yogurt Fermented with a Bacteriocin-Producing Thermophilic Starter

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ABSTRACT

Streptococcus salivarius subsp. *thermophilus* B producing a bacteriocin active against *Listeria monocytogenes* ATCC 7644 and *Staphylococcus aureus* SAD 30 was isolated from bakery yeast. The bacteriocin was partially purified by an adsorption/desorption technique, and its spectrum of action was compared to that of a neutralized cell-free supernatant (CFS). Although the CFS inhibited a number of gram-positive and -negative bacteria of health and spoilage significance, the spectrum of action of the partially purified bacteriocin was limited to gram-positive bacteria. *L. monocytogenes* was the most sensitive to both preparations. The bacteriocin-producing streptococcal strain was used in combination with a Bac^- *Lactobacillus delbrueckii* subsp. *bulgaricus* CY strain isolated from commercial yogurt to assess the effectiveness of the resulting thermophilic starter in controlling *L. monocytogenes* and *S. aureus* in yogurt during fermentation and storage at refrigeration (ca. 7°C) or abuse (ca. 22°C) temperature. Yogurt samples were contaminated with *L. monocytogenes* or *S. aureus* to the approximate levels of 10^3 and 10^6 CFU/ml of milk, respectively. The results showed that in situ bacteriocin production was more active against *L. monocytogenes* than against *S. aureus* in vitro and in contaminated samples. While *L. monocytogenes* leveled off below the detectable limit in a 1-ml sample of yogurt within 24 h of processing, *S. aureus* survived in Bac^+ and Bac^- samples during 10 days of storage at room temperature (ca. 22°C). Use of a Bac^+ starter resulted in a 5-day extension of the shelf life.

Dairy products have been associated with foodborne diseases. Recurrent cases of listeriosis *(11, 27)* and staphylococcal intoxications *(11)* due to the consumption of contaminated milk and milk derivatives have been reported throughout the world whenever accurate records were available for audit.

A recent survey revealed that *Listeria monocytogenes* and *Staphylococcus aureus* were involved in 22 and 15%, respectively, of recorded foodborne illnesses caused by dairy products in eight developed countries *(11).* According to the same report, *S. aureus* was responsible for more than 85% of the dairy-borne diseases in France. Moreover, *L. monocytogenes* was responsible for ca. 25% of the estimated foodborne disease-related deaths in the United States *(27).* Therefore, a tremendous effort has been made during the last two decades to control these pathogens in foods.

In this regard, yogurt received the least attention among dairy products. Its high acidity and milk pasteurization were often thought to be effective barriers against the product's being subjected to *L. monocytogenes* and *S. aureus* contamination. However, it is now well established that both pathogens can occur in yogurt as a result of their survival during processing or postcontamination *(4, 8, 29, 31). L. monocytogenes* is naturally resistant to environmental stress including heat, acidity, and reduced water activity *(13).* Such resistance may even increase in stressful conditions, leading to unusual acid *(14, 26)* or heat *(19, 23)* tolerance.

Most of the recent strategies for controlling the growth of undesirable microorganisms in foods tend to apply the "hurdle concept," advocating the combination of more than one barrier having different modes and spectra of action. The aim of this approach is to increase the potential of eliminating a broader range of pathogens and spoilage microorganisms and thus to enhance the safety and keeping quality of foods. Bacteriocins or bacteriocin-producing strains have often been suggested as part of the hurdle combination to reduce the prevalence of *L. monocytogenes* and *S. aureus* in yogurt and other food products *(22, 35, 37).* Nisin has first been suggested because of its generally recognized as safe status in many countries and because of its efficacy in removing the pathogens from dairy products *(6, 10).* Nonetheless, the bacteriocin has been shown to inhibit the thermophilic strains used in yogurt starters *(21)* and hence may seriously impede the fermentation. Other bacteriocins of anti-*Listeria* class IIa have also been suggested *(22),* but they still need to be legally approved for such use. Furthermore, thermophilic starter cultures commonly used in dairy fermentations were shown to be particularly sensitive to the class I lantibiotics nisin A and Z, as well as to other class II bacteriocins *(28).* Therefore,

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TABLE 1. *Sensitivity of selected gram-positive and -negative bacteria to the cell-free supernatant (CFS) or to the partially purified bacteriocin (PPB) of* Streptococcus salivarius *subsp.* thermophilus

Indicator bacteria	Origin[a]	Extent of inhibition[b]	
		CFS	PPB
Gram-positive bacteria			
Listeria monocytogenes 7644	ATCC	++++	++++
L. monocytogenes 2	CHU	++++	++++
Staphylococcus aureus SAD 30	IAV HASSAN II	++	++
Micrococcus flavus NCIB8166	OSU	−	ND
Bacillus sp. TE1	Our collection	+	ND
Streptococcus faecalis SF1	IAV Hassan II	++	ND
Enterococcus faecium A_2	Our collection	−	ND
S. salivarius subsp. *thermophilus* B	This study	−	−
Lactobacillus delbrueckii subsp. bulgaricus CY	This study	−	−
E. faecius SPH_1	Our collection	−	−
S. thermophilus SPH_2	Our collection	−	−
Lactococcus lactis subsp. *lactis* M	Our collection	−	−
L. lactis S	Our collection	−	−
L. lactis H	Our collection	−	−
Gram-negative bacteria			
Salmonella Typhimurium STP1	IAV Hassan II	+	−
Salmonella Typhi ST20	IAV Hassan II	++	−
Salmonella Hadar SH4	IAV Hassan II	+	−
Pseudomonas putida PPOSU	OSU	++	−
Pseudomonas aeruginosa PAOSU	OSU	++	−
Escherichia coli EC4	Our collection	+	−
Enterobacter sp. EI3	HMI MV	+	−
Proteus mirabilis HM10	HMI MV	+	−
Escherichia coli V517	OSU	−	−
Klebsiella pneumoniae E30PIP135	HMI MV	++	−
Enterobacter cloacae B703	HMI MV	++	−
Shigella sp. SI1	Our collection	++	−

[a] ATCC, American Type Culture Collection; OSU, Oregon State University; HMI MV, Hôpital Militaire d'Instruction Mohamed V, Rabat; CHU, Centre Hospitalier Universitaire Mohamed V, Rabat; IAV Hassan II, Institut Agronomique et Vétérinaire Hasssan II, Rabat.

[b] ++++, diameter of the inhibition zone (ø) > 20 mm; +++, 16 < ø < 20 mm; ++, 12 < ø < 16 mm; +, ø < 12 mm; −, no inhibition; ND, not determined. Data are averages of three determinations, each one in duplicate.

the search for new bacteriocin-producing strains normally used in thermophilic starters that can efficiently enhance the safety and extend the shelf life of dairy products seems worthwhile.

The objective of the present study was to isolate and characterize a bacteriocin-producing strain of *Streptococcus salivarius* subsp. *thermophilus* and to evaluate its potential to protect yogurt from the survival and growth of *L. monocytogenes* and *S. aureus* during processing and storage at refrigeration (ca. 7°C) or abuse (ca. 22°C) temperature.

MATERIALS AND METHODS

Bacterial strains and media. *S. salivarius* subsp. *thermophilus* B, a bacteriocin-producing strain (the "producer"), was isolated in this study by the method described previously *(5)*. It was identified by morphological and physiological tests *(9)*. A nonbacteriocin-producing *Lactobacillus delbrueckii* subsp. *bulgaricus* strain CY was isolated from commercial yogurt and identified according to Kandler and Weiss *(20)*.

Bacteria used in the present study (Table 1) were stored at −20°C in appropriate broths containing 25% glycerol. Before use, lactic acid bacteria (LAB) were propagated in deMan Rogosa Sharpe (MRS) broth *(12)*, and the other microorganisms were propagated in tryptic soy broth (Biokar, France).

A commercial lyophilized yogurt starter (Redi-set, Hansen Laboratories, Inc., Milwaukee, Wis.) was used for control purposes. Before use, this starter culture was activated in sterile nonfat dry milk according to the manufacturer's recommendations and tested for inhibitory activity against *L. monocytogenes* ATCC 7644 by the spot test *(33)* to confirm its suitability as a control (Bac^-).

Confirmation of the bacteriocinogenic nature of the inhibitory substance produced by *S. thermophilus* B. To exclude the effect of organic acids, the pH of an overnight culture of the producer strain in MRS broth fortified with 2.5% yeast extract was adjusted to pH 6.0 with 3 M NaOH solution and assayed for inhibitory activity against *L. monocytogenes* ATCC 7644 with the well diffusion test *(36)*. Sensitivity tests for catalase, pronase E, trypsin, and α-chymotrypsin were performed to exclude the effect of hydrogen peroxide (catalase treatment) and to confirm the pro-

teinaceous nature (protease treatment) of the inhibitory substance produced by *S. thermophilus* B. All enzymes were obtained from Sigma Chemical Company (St. Louis, Mo.), except for catalase, which was purchased from Serva (Heidelberg, Germany). Buffer preparations and enzyme treatments were conducted as described previously *(5)*.

Stability to pH and heat. Two series of cell-free supernatant (CFS) samples were prepared. In the first series, aliquots of the crude CFS samples were made and exposed to heat treatments of increasing severity. In the second, the pH was adjusted to different values ranging from 2.0 to 11.0 with concentrated HCl or 10 M NaOH solutions and was heat treated (121°C for 15 min). The supernatants of both series were tested for inhibitory activity against *L. monocytogenes* ATCC 7644 by the well diffusion assay. Nonheat-treated CFS samples of each series were tested simultaneously to serve as controls.

Partial purification of the bacteriocin produced by *S. thermophilus* B. The pH-dependent adsorption/desorption technique described by Yang et al. *(39)* was performed with some adjustments to partially purify the bacteriocin.

Before purification, the effect of the pH on the adsorption of the bacteriocin to the cell wall of the producer organism was studied. *S. thermophilus* B was grown overnight in 500 ml of MRS broth fortified with 2.5% yeast extract at 30°C. The culture was then heated at 70°C for 25 min to kill producer cells and to inactivate any proteolytic enzymes that may have been present. Arbitrary units, defined as the reciprocal of the highest dilution showing a distinct zone of inhibition of the indicator strain, were determined (AU_T). The heated culture was then aseptically dispensed in sterile test tubes (10 ml each) and adjusted to different pH values ranging from 1.5 to 8.0 by adding 1 M HCl or 3 M NaOH solutions. Tubes were kept in the refrigerator for 30 min to promote desorption of the bacteriocin from the producer cell wall. Each suspension was then adjusted to pH 6.0, and cells were removed by centrifugation in a microfuge (235C; Fisher Scientific, Fairlawn, N.J.) at maximum speed for 10 min. Bacteriocin activity was assayed in the supernatant (AU_S). The following terms were used to determine the percentage of adsorbed bacteriocin: AU_T = the total bacteriocin activity (AU/ml) determined in the culture; AU_S = the unbound bacteriocin activity (AU/ml) determined in the CFS. The amount of bacteriocin adsorbed to the cell wall could be calculated by the formula: Adsorbed Activity (%) = $[(AU_T - AU_S)/AU_T] \times 100$.

Once the adsorption profile of the bacteriocin to the producer cell wall as a function of the pH was known, the purification proceeded as described by Yang et al. *(39)*. A culture of *S. thermophilus* B was prepared by inoculation of MRS broth (1 liter) and overnight incubation at 37°C. The pH of the culture was adjusted to 6.0 with 3 M NaOH. The culture was then heated at 70°C for 25 min, and cells were harvested by centrifugation at 14,000 × *g* for 15 min. The cell pellets were washed twice with phosphate buffer (5 mM, pH 6.0) and resuspended in 50 ml of NaCl solution (100 mM) at pH 1.5. The cell suspension was stirred with a stir bar for 1 h at 7°C, and cells were removed by centrifugation at 14,000 × *g* for 20 min. The supernatant was exhaustively dialyzed and concentrated at 7°C in a dialysis membrane (500 molecular weight cutoff) against NaCl solution (100 mM, pH 1.5). Preliminary assays showed that a dialysis membrane with an 8,000-molecular weight cut-off limit did not retain the bacteriocin. Preparations were then freeze dried and stored at 7°C. In each step of the purification procedure, arbitrary units were determined to estimate bacteriocin yields and losses.

Spectrum of action of the partially purified bacteriocin. The activity of the CFS or the partially purified bacteriocin (PPB) against different gram-positive and -negative pathogenic or spoilage bacteria (Table 1) was tested by the well diffusion test *(36)*.

Yogurt trials. Nonfat dry milk was added to raw milk to adjust fat and dry matter contents to approximately 17 and 150 g/liter, respectively. After a thorough mixing, milk was pasteurized in a water bath at 80°C for 30 min, and then cooled to 40°C in ice water. Pasteurized milk was divided into two batches of 6 liters each, one of which was inoculated with *S. thermophilus* B and *L. bulgaricus* CY (1.5% each). The other (control) was inoculated with the commercial Bac⁻ starter (Redi-set, Hansen). Each batch was further divided into two parts. The first part was contaminated with an overnight culture of *L. monocytogenes* ATCC 7644 or *S. aureus* SAD 30 (0.03 or 3.0 ml) to achieve an approximate inocula of 10^3 and 10^6 CFU/ml of milk, respectively. The second part was not contaminated and hence served as a negative control. Inoculated milk was then dispensed into plastic cups containing 100 ml, incubated at 40°C until coagulation, and then transferred to the refrigerator (ca. 7°C). At 24 h, half of the yogurt cups of each trial were withdrawn from the refrigerator to be stored at ambient temperature (ca. 22°C).

Enumeration of microorganisms and pH measurement. Microbial enumerations were performed at regular intervals during processing and storage until alteration was perceivable. LAB were enumerated on MRS agar *(12)* after 24 to 48 h of incubation at 40°C, *L. monocytogenes* was enumerated on Al-zoreki-Sandine Listeria Medium *(3)* after 48 to 72 h of incubation at 37°C, and *S. aureus* was enumerated on Chapman agar (Oxoid, Hampshire, UK) after incubation at 37°C for 24 to 48 h. The pH was measured every hour until coagulation, and then at 24 h, with a Jenway pH meter (Model 3310, Felsted, Dunmow, Essex, UK).

Statistical analysis. Experiments were repeated three times for *L. monocytogenes* and twice for *S. aureus*. All determinations were done in duplicate. Analysis of variance ($\alpha = 0.05\%$) and Student's *t* test were performed for comparison of means.

RESULTS

Detection of an anti-*Listeria* bacteriocin-producing strain of *S. thermophilus*. Among several bacteriocin-producing strains previously isolated from different Moroccan products, one isolated from bakery yeast and identified as *S. thermophilus* was inhibitory to *L. monocytogenes,* to *S. aureus,* and to some gram-negative bacteria (Table 1). Such results suggest that this strain has a good potential to enhance the safety and keeping quality of yogurt. Therefore, further study focused on the characterization of the inhibitory substance it produced and on the assessment of its inhibitory performance against these specific pathogens in yogurt during processing and storage at refrigeration (ca. 7°C) or ambient (ca. 22°C) temperature.

The inhibitory substance produced by *S. thermophilus* B possesses the main criteria of a bacteriocin. It retained its activity at pH 6.0, resisted catalase, was hydrolyzed by proteolytic enzymes (Table 2), and was bactericidal to sensitive strains (data not shown). Unlike the most frequently produced bacteriocins by LAB, it seems to have a broad spectrum of action that extends to gram-negative organisms. It is worth mentioning, however, that the inhibition zones of sensitive gram-negative bacteria were usually

TABLE 2. *Sensitivity of* Listeria monocytogenes *ATCC 7644 to the cell-free supernatant of* Streptococcus salivarius *subsp.* thermophilus *B exposed to various enzymes and treatments*[a]

Treatment	Diameter of inhibition zone (mm)[b]	
	Treated sample	Control (untreated sample)
Enzymes		
Catalase	19.5 A[c] (±1.3)[d]	19.7 A (±1.5)
Pronase E	NI[e]	19.3 (±0.6)
Trypsin	NI	19.7 (±0.6)
α-Chymotrypsin	NI	19.0 (±1.7)
Pepsin	9.3 B (±1.2)	18.0 A (±2)
Heat		
60°C pendant 30 min		
80°C pendant 30 min	19.3 A (±2.1)	19.0 A (±1.7)
100°C pendant 30 min	20.3 A (±2.1)	19.5 A (±1.3)
Autoclaving (121°C,	19.0 A (±3.5)	19.3 A (±2.1)
15 min)	20.3 A (±1.5)	19.7 A (±1.2)

[a] *Listeria monocytogenes* ATCC 7644 was used as the indicator strain for sensitivity testing by the well diffusion assay *(36).*
[b] Means from three experiments. Values include the diameter of the well (7 mm).
[c] Means with the same letter are not significantly different (α = 0.05%).
[d] Standard deviation.
[e] NI, no inhibition.

smaller and less clear than those observed with gram-positive bacteria. *Listeria* strains were the most sensitive.

Stability of bacteriocin to pH and heat. Stability of the bacteriocin produced by *S. thermophilus* B was assessed by exposing the crude CFS to different heat treatments. The activity of the crude CFS was not affected ($P > 0.05$) by any of the heat treatments tested (Table 2). Therefore, the combined effect of pH and heat was studied by autoclaving the CFS adjusted to different pH values ranging from 2.0 to 11.0. The results showed that such resistance was pH-dependent. Although the bacteriocin could withstand autoclaving at acidic pH values (maximum resistance between pH 4.0 and 6.0), it was readily destroyed at pH values less acidic than 8.0 by the same treatment. In fact, the bacteriocin was unstable in alkaline conditions even without heating, as evidenced by the drastic reduction in its inhibitory activity at pH 8.0 and its complete loss of activity at pH values less acidic than 10.0 (Table 3).

Extraction and partial purification of bacteriocin. Adsorption of the bacteriocin onto the producer cell wall was dependent on the pH of the medium. Approximately 100% of the bacteriocin was attached to the cells at pH 6.0. Conversely, virtually all of the activity produced was found in the supernatant at pH 1.5 (Fig. 1). Recovered activity in the CFS was reduced at a pH less acidic than 6.0. Therefore, the respective pH values of 6.0 and 1.5 were used for the adsorption and desorption steps in the purification procedure. Such pH adjustment allowed the recovery of 96.2% of PPB from the CFS, which could be considered good performance if a high purity level was not required.

TABLE 3. *Combined effect of heat treatment (121°C, 15 min) and pH on the stability of the bacteriocin produced by* Streptococcus salivarius *subsp.* thermophilus *B*[a]

pH	Mean diameter of the inhibition zone (mm)[b]	
	Control (nonheated)	Heated (121°C, 15 min)
2.0	19 A[c] (±2.6)[d]	13 B (±2)
4.0	16 A (±2)	14 A (±1.7)
6.0	18 A (±2)	17 A (±2.6)
8.0	16 A (±1)	8 B (±0)
10.0	9 (±1.7)	NI
11.0	NI[e]	NI

[a] *Listeria monocytogenes* ATCC 7644 was used as the indicator strain for sensitivity testing by the well diffusion assay *(36).*
[b] Means from three experiments. Values include the diameter of the well (7 mm).
[c] Means in a row with the same letter are not significantly different (α = 0.05%).
[d] Standard deviation.
[e] NI, no inhibition.

Yogurt manufacture. Evolution of the pH of yogurt samples was monitored during fermentation and storage at refrigeration (ca. 7°C) or ambient (22°C) temperature. The pH decreased in standard yogurt fermentation. No significant difference ($P > 0.05$) was observed between Bac^+ and Bac^- samples during fermentation and storage. At ambient temperature, the Bac^- samples were altered by the growth of yeasts and molds on the surface at day 15, while in the Bac^+ samples, such alteration marks were perceivable 5 days later.

Effect of in situ bacteriocin production on *L. monocytogenes.* The in situ effect of bacteriocin production by

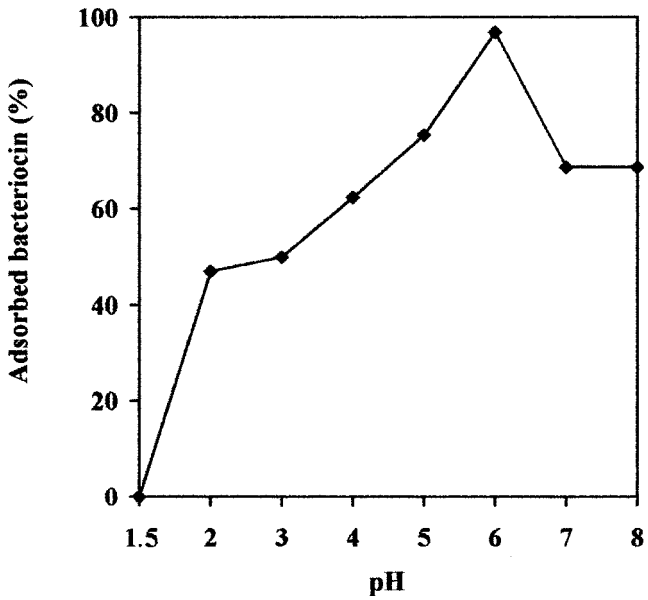

FIGURE 1. *Percentage of bacteriocin adsorbed onto the producer cells of* Streptococcus salivarius *subsp.* thermophilus *B as a function of the pH.*

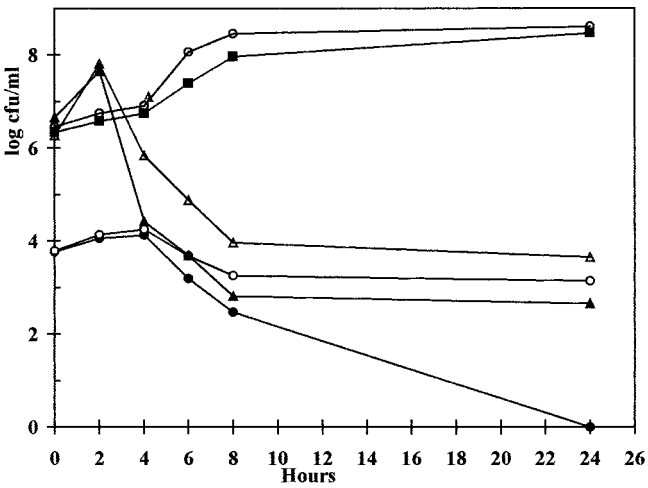

FIGURE 2. *Growth of lactic acid bacteria (—■—, —□—),* Listeria monocytogenes *(—●—, —○—), and* Staphylococcus aureus *(—▲—, —△—) during fermentation of yogurt with Bac⁺ (—■—, —●—, —▲—) or Bac⁻ (—□—, —○—, —△—) thermophilic starter.*

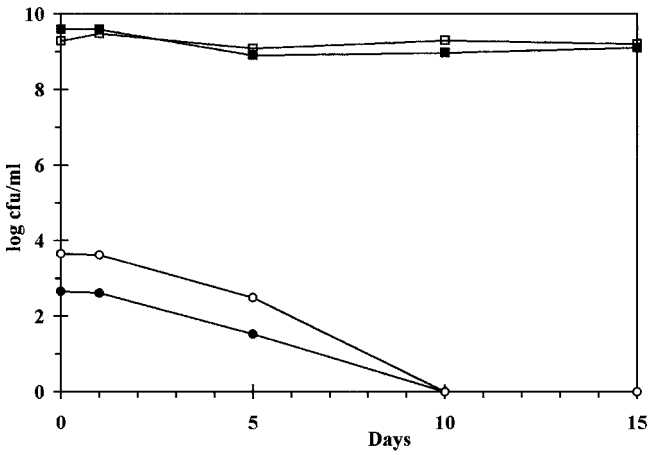

FIGURE 3. *Growth of lactic acid bacteria (—■—, —□—) and* Staphylococcus aureus *SAD 30 (—●—, —○—) in yogurt fermented with Bac⁺ (—■—, —●—) or Bac⁻ (—□—, —○—) thermophilic starter during storage at refrigeration temperature (ca. 7°C).*

S. thermophilus on the growth and survival of *L. monocytogenes* in yogurt made from milk contaminated with ca. 10^3 CFU/ml of *Listeria* was studied during fermentation and storage. The growth of LAB was monitored simultaneously. The regular increase in numbers of LAB during fermentation (Fig. 2), along with the steady increase in acidity, indicates the normal processing of yogurt fermented either with the Bac⁺ or Bac⁻ starter culture. In both cases, counts of LAB were ca. 10^8 CFU/ml at 8 h of fermentation (Fig. 2), and no significant difference ($P > 0.05$) was observed in their count during processing.

L. monocytogenes fell below the detectable level in 1 ml in the Bac⁺ samples between 8 and 24 h, whereas in the Bac⁻ samples, only a 1-log unit reduction in the *Listeria* counts was observed at 24 h (Fig. 2). Moreover, the pathogen survived in the Bac⁻ samples throughout the storage period at both storage temperatures.

Effect of in situ bacteriocin production on *S. aureus* in yogurt. The behavior of *S. aureus* SAD 30 in yogurt fermented with Bac⁺ or Bac⁻ starter was studied in the same manner as for *L. monocytogenes* ATCC 7600, except that a higher initial inoculum (10^6 CFU/ml) was used. *Staphylococci* are indeed usually encountered in milk and milk derivatives in elevated numbers. Furthermore, their minimum infectious dose is higher than that of *Listeria,* for which many countries apply the status of zero tolerance in a 25-g (or ml) sample. The results of Figure 2 show that numbers of *S. aureus* increased during the first 2 h by 0.9 and 1.5 logarithmic units in Bac⁺ and Bac⁻ samples, respectively. Afterward, a sharp decrease in CFUs was observed in both types of yogurt samples. At 24 h, *Staphylococcus* counts were reduced to 6.6×10^2 and 9.3×10^3 CFU/ml in Bac⁺ and Bac⁻ samples, respectively. Overall, in situ bacteriocin production by *S. thermophilus* B appears to give moderate protection against *S. aureus* SAD 30 compared to *L. monocytogenes* ATCC 7644, since the former pathogen survived for 10 days of storage at refrigeration temperature (7°C) in Bac⁺ and Bac⁻ samples (Fig. 3).

DISCUSSION

The strain of *S. thermophilus* B isolated in this work produces a bacteriocin that resists autoclaving under acidic conditions (Table 3). In that respect, it differs from thermophilin 347 *(38)* and thermophilin T *(2),* which are inactivated in these conditions. The main physicochemical characteristics of the bacteriocin produced by *S. thermophilus* B are shared by most class IIa bacteriocins. It is a thermostable small peptide that passes through a dialysis membrane of an 8,000-molecular weight cut-off limit, it has a narrow spectrum of action (PPB), and it is strongly inhibitory to *L. monocytogenes.* Further characterization at the molecular level is as yet needed to determine whether or not it is a novel bacteriocin and to confirm its relatedness to the bacteriocins of class IIa.

Neutralized CFS samples of the producer strain inhibited gram-positive as well as some gram-negative bacteria (Table 1). Although it is well known that bacteriocins of LAB have a narrow spectrum of action essentially directed toward gram-positive species related to the producer organism, some exceptions have been reported to inhibit gram-negative bacteria also *(7, 24, 32).* In fact, all of these authors reported on the inhibitory activity of the spent broth or the spot-on-lawn rather than the purified bacteriocin or PPB. In the present study, spectra of action of the CFS and the PPB were compared. The results showed that the PPB did not inhibit the gram-negative bacteria sensitive to the CFS (Table 1). The spent broth may contain a mixture of constituents derived from bacterial metabolism or from the culture medium, which would act synergistically with the bacteriocin to extend its spectrum of action to gram-negative bacteria. Different substances and ecological factors have been demonstrated to interfere with the integrity of the lipopolysaccharide layer and to sensitize gram-negative bacteria to bacteriocins *(15, 34).* Also, *S. thermophilus* B

may produce more than one bacteriocin having different spectra of action, as has been shown for other LAB *(30).* The putative bacteriocin specifically inhibiting gram-negative bacteria would not be recovered by the purification procedure used. In this regard, Gomez et al. *(17)* also reported on a strain of the *Streptococcus* genus, which lost inhibitory activity against gram-negative bacteria upon partial purification.

Application of the adsorption/desorption method for the purification of the bacteriocin produced by *S. thermophilus* B allowed 96.2% recovery of anti-*Listeria* activity. In fact, such a level of recovery is usual when this method is applied *(39).* Some authors even reported yields of recovered activity exceeding 100% *(18).* Although this procedure typically does not provide a high degree of purity, it has proven useful for a partial purification and for bacteriocin concentration as the first step in any purification strategy. Partial purification of the bacteriocin produced by *S. thermophilus* B was carried out essentially to confirm inhibitory action against gram-negative bacteria. Restricted inhibitory activity of the PPB compared to the crude CFS suggests that in food systems, in situ production of the bacteriocin would inhibit a broader range of undesirable microorganisms than the purified bacteriocin. Therefore, use of the producer strain in the starter culture appears to be more advantageous to food preservation and thus has a greater potential to enhance the safety and extend the shelf life of the food product. Nevertheless, the producer strain should normally be a part of the starter culture and, under these conditions, be able to produce sufficient bacteriocin in the particular food product. In fact, a study on the dynamics of bacteriocin production by the strain in reconstituted milk showed that activity was produced in a detectable amount at 5 h of incubation and reached ca. 550 AU/ml at 24 h (unpublished data). Preliminary in situ experiments need to be carried out when the bacterium is to be used for such purposes.

In this regard, our results suggest that the use of bacteriocinogenic *S. thermophilus* B in yogurt fermentation will enhance the prevention of survival and growth of *L. monocytogenes* and, to some extent, *S. aureus* during processing and storage. In a similar work, Zuniga-Estrada et al. *(40)* have shown that *L. monocytogenes* was killed in yogurt fermented with a bacteriocinogenic starter in 8 h, while Massa et al. *(25)* have shown that a 48-h period was necessary for complete removal of the pathogen when the same contamination level was used (i.e., 10^3 CFU/ml). The discrepancy in the time needed to reduce the colony-forming units of *L. monocytogenes* below the undetectable level appears to be dependent on the strain of *Listeria* and on the synergistic action between the bacteriocin and the acidity. The fact that the activity of the bacteriocin produced by *S. thermophilus* B is optimum at low pH (Table 3) may explain the removal of *Listeria* cells from yogurt before exposure at the sale point (i.e., 24 h). The pH may also act indirectly by stimulating bacteriocin production *(16)* or by increasing the amount of available bacteriocin.

S. aureus is another pathogen of concern in dairy products. Its presence in raw milk in relatively high numbers and its incidence on human health make it worthwhile to investigate its behavior in yogurt fermented with a bacteriocin-producing starter. In this regard, the present study showed that, although reported to be more sensitive to acidity than *L. monocytogenes (4),* it survived yogurt processing and 10 days of storage at refrigeration temperature (Fig. 3). A significant increase in numbers of *Staphylococci* was even noted in the first 2 h of fermentation (Fig. 2). Similar behavior of *S. aureus* has been reported previously in yogurt *(4)* and Domiati cheese *(1).* Extrinsic and intrinsic growth parameters during the first hours of fermentation are encouraging to the growth of this organism. Neither the acidity nor the bacteriocin was produced in sufficient amounts to exert the inhibitory effect on the pathogen. The pH was still ca. 6.0, and bacteriocin is not produced in a detectable amount during the early hours of fermentation. Moreover, milk obviously provides an adequate culture medium from the nutritional standpoint, and the temperature is not a limiting factor.

The bacteriocin produced by *S. thermophilus* B was less active in removing *S. aureus* than *Listeria* from yogurt. Nonetheless, accelerated decreases in *Staphylococci* numbers during processing in Bac^+ compared to Bac^- samples (Fig. 2) suggest that bacteriocin production inhibits, to some extent, the growth of *S. aureus* in yogurt. The high number of inoculum used to contaminate yogurt with *S. aureus* SAD 30 certainly accounts for the persistence of the pathogen in yogurt samples. It is well documented that the quantity of the initial inoculum has an effect on the inhibition of pathogens in foods *(5, 22, 35).* In addition, in vitro tests show that *S. aureus* is less sensitive than *L. monocytogenes* to the bacteriocin produced by *S. thermophilus* B (Table 1).

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