

Full Length Research Paper

Antimicrobial activity of autochthonous lactic acid bacteria isolated from Algerian traditional fermented milk “Raïb”

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Twenty samples of traditional fermented milk “Raïb” were collected in eastern Algeria from individual household. They were evaluated for the presence of autochthonous bacteriocin-producing lactic acid bacteria. From 13 of these samples 52 strains of lactic acid bacteria were isolated, and shown to exhibit inhibitory activity against the indicator strain *Listeria monocytogenes*. Five of these inhibitor-producing isolates were selected for further study on the basis of their relatively wide antimicrobial spectrum. The inhibitory spectra of activity of the selected strains were evaluated against a range of Gram-positive and Gram-negative test organisms. *Listeria monocytogenes* and *Staphylococcus aureus* were the most sensitive indicator tested. All the antimicrobial compounds produced by the selected lactic acid bacteria were fully or partially inactivated by some of the proteolytic enzymes, but were unaffected by catalase which indicates their proteinaceous nature. The compounds were heat stable up to 120°C for 20 min, and were active from pH 3.0 to 10.0. Highest bacteriocin activity was recorded under acidic conditions and activity decreased with increasing alkalinity.

Key words: Traditional fermented milk, Raïb, lactic acid bacteria, bacteriocin.

INTRODUCTION

Fermented milk is a dairy product obtained by the fermentation of milk, which may have been made from products obtained from milk with or without any modification of their composition, via the action of appropriate microorganisms and which result in a lowering of the pH with or without coagulation. Production of traditional cheeses (el-Klila, Jben) and other fermented milk products such as raib (fermented milk), lben (skimmed fermented milk), has a very long tradition in Algeria. Raib is made from the raw cow or goat milk. Milk fermentation, like many traditional fermenting processes, is spontaneous and uncontrolled and could be a valuable source of autochthonous Lactic Acid Bacteria (LAB) (Hamama, 1992; El Soda et al., 2003). The microbiological characteristics of several fermented milk have been studied in Indonesia (Hosono et al., 1989), South Africa (Beukes et al., 2001) and Morocco (Hamama, 1992).

Lactic acid bacteria play an important role in food fermentation processes. Raw foods such as milk, fruit, vegetables or meat are often preserved by lactic acid fermentation (Savadogo et al., 2006; Daeschel 1989). In such food products LAB have the capacity to perform fermentative activities, which may result in active inhibition of spoilage and pathogenic bacteria. The antimicrobial effect may be due to the production of a number of antimicrobial substances such as lactic acid, hydrogen peroxide, diacetyl and bacteriocins (Hoover, 2000; Lindgren and Doborogosz, 1990). Bacteriocins are produced by some strains of LAB; they are antimicrobial peptides with activity against strains closely related to the producer micro-organism. Some bacteriocins are also active against Gram-positive food-borne pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus subtilis* and spores of *Clostridium perfringens*. For this reason, they have received much attention for use as natural or so-called ‘biopreservatives’ in foods in recent years (Savadogo et al., 2006; Savadogo et al., 2004). Bacteriocins of LAB have been classified into four

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structural classes, namely I, II, III and IV (Nes et al., 1996). Classes I and II are small, mainly hydrophobic and heat-stable peptides. Class I, the so-called lantibiotics, are post-translationally modified, while Class II, non-lantibiotic bacteriocins, are divided into three subcategories: Class IIa are the pediocin-like bacteriocins with strong antilisterial effects; Class IIb bacteriocins consist of two peptides, both required for full antimicrobial activity and Class IIc bacteriocins are secreted by a sec-dependent mechanism. Class III are high molecular weight, heat-labile protein bacteriocins. Class IV are complex bacteriocins, composed of a protein moiety plus one or more non-proteinaceous additions, e.g. lipid or carbohydrate groups required for activity (Nes et al., 1996).

The purposes of this study were to isolate bacteriocin-producing lactic acid bacteria from traditional fermented milk 'Raïb' samples and to determine their spectrum of activity against food-borne pathogens. The antimicrobial activity of previously identified bacteriocin-producing lactic acid bacteria against these pathogens was also determined. We report that several strains produce bacteriocins active against *L. monocytogenes*, *S. aureus*, and *Escherichia coli*.

MATERIALS AND METHODS

Bacterial strains and growth media

All strains used in this study were maintained as frozen stocks in 25% glycerol at -20°C and were propagated twice in broth for 16 h before experimental use. LAB isolates were selected and cultivated on de Man Rogosa Sharpe Agar (MRS, Oxoid) at 30°C. Bacteria chosen as indicators were: *Staphylococcus aureus* ATCC 25293, *Listeria monocytogenes* ATCC 7644, *Bacillus cereus* ATCC 14578, *Bacillus subtilis* ATCC8, *Enterococcus faecalis* ATCC 19433, *Escherichia coli* ATCC 25422 and *Pseudomonas aeruginosa* 27853 were propagated in Tryptic Soy Broth at 30°C.

Fermented milk sampling

Twenty samples of traditional fermented milk (Raib) were collected from individual households of rural areas in eastern Algeria. Samples were collected in sterile small bottles and stored in laboratory under refrigeration at 5°C until they were used in experiments.

Selection procedure for LAB from fermented product

10 ml of each sample were aseptically added into 90 ml of sterile 0.9% NaCl solution and mixed thoroughly. Serial dilutions (10^{-1} to 10^{-8}) were performed and 1 ml aliquots of the appropriate dilutions were directly inoculated in triplicate on media for lactic acid bacteria, M17 (Terzaghi and Sandine, 1975) and MRS (de Man et al., 1960) adjusted to pH 5.5. After incubation at 30°C for 24 h and 3 days, representative strains of lactic acid bacteria were obtained from M17 and MRS plates of highest sample dilutions. Colonies were either randomly picked up or when the plate contained less than 10 colonies.

Detection of antagonistic activity

Isolated colonies of the assumed LAB isolates were screened for antimicrobial-producing activity essentially using the spot method as described by Spelhaug and Harlander (1989). An overnight culture of the test organism grown in MRS broth supplemented with 2.5% yeast extract (MRSY) was diluted 10-fold in 10 mmol l⁻¹ Tris HCl (pH 7.0), and 2 ml aliquots were spotted onto MRS agar. Plates were incubated for approximately 24 h, until growth was evident, and then overlaid with 5 ml Trypticase soft agar (0.7% agar) seeded with 0.1 ml of an overnight culture of *L. monocytogenes* ATCC7644. Plates were incubated for an additional 18 h, and then checked for clear zones around spots of the putative producers.

Presumptive identification of bacteriocin-producing strains

Bacteriocin producing strains were Gram stained and examined microscopically for cellular morphology and Gram-stain phenotype. Catalase activity was tested by spotting colonies with 3% hydrogen peroxide. Growth was assayed in MRS broth at 10, 15, 37 and 45°C. Salt tolerance was tested with 6.5, 7.0 and 10% (w/v) NaCl in MRS broth. Growth of the strains was also studied, at pH 4.4 and 9.6 in MRS broth (Schleifer and Kilpper-Balz, 1984). The configuration of lactic acid, hydrolysis of arginine and production of CO₂ from glucose were determined according to the methods described by Schillinger and Lucke (1987). Sugar fermentation reactions were performed using API 50CH test strips and 50CHL medium, according to the manufacturer's instructions (BioMe'rieux).

Sensitivity to heat, pH, and hydrolytic enzymes

Cell-free supernatants (CFS) from the lactic acid cultures were collected by centrifugation (7500 g, 10 min, 4°C) of overnight MRS broth cultures. The supernatant fluids were adjusted to pH 6.5 and exposed to heat treatments of 65°C for 40 min, 95°C for 20 min, and 120°C for 20 min, and then were tested for remaining antimicrobial activity. In order to determine the effect of pH on Semi-purified preparations of the bacteriocin were adjusted to various pH values in the range of 3 to 10. The pH-adjusted bacteriocin samples were incubated at 37°C for 20 min and then neutralized to pH 6 and tested for bacteriocin activity.

The following enzymes were tested for their hydrolytic activity on the antimicrobial compounds contained in the supernatants: proteinase K (2.6 U mg⁻¹), pronase E (22 U mg⁻¹), pepsin (16U mg⁻¹), catalase (adjusted to a final activity of 2600 U mg⁻¹), lipase (50 U mg⁻¹), and α -amylase (15 U mg⁻¹). The assays were performed at a final concentration of 0.5 mg ml⁻¹ and at pH 6.5, except for pepsin (pH 3.0). Samples with and without enzymes were held at 35°C for 6 h and the remaining activity was determined by well-diffusion assay as described before using *L. monocytogenes* ATCC7644 as indicator strain.

Bacteriocin spectrum of inhibitory activity

The spectrum of activity against different bacteria (Table 2) was determined by the well-diffusion assay (Schillinger and Lucke, 1989) and disk diffusion assay (Tagg and McGiven, 1971). The well-diffusion was conducted in TSA agar media overlaid with 7 ml of soft agar media which contained 4% inoculum of an overnight culture of the indicator strain wells, 4 mm in diameter, were cut into these agar plates and 300 μ l of the culture supernatant of the potential producer strain were placed into each well (Figure 1). The plates were incubated for 24 h at 37°C and subsequently examined for zones of inhibition (Barefoot and Klaenhammer, 1983).

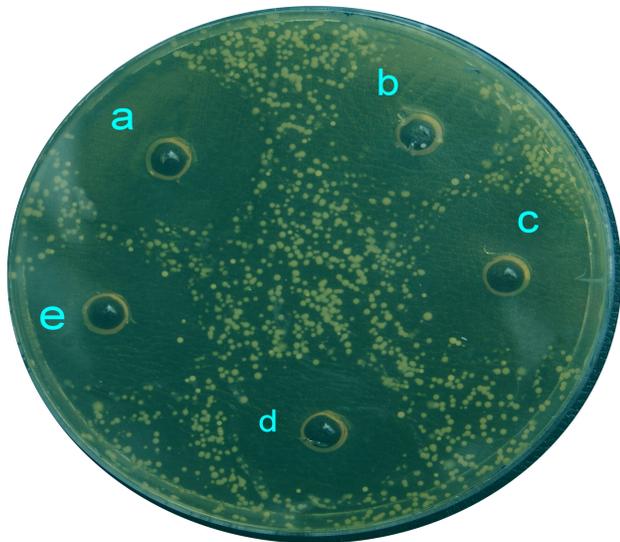


Figure 1. Inhibition of *Listeria monocytogenes* ATCC 7644 by the cell-free supernatants of the five producing isolates using the agar well-diffusion assay: *Lactobacillus plantarum* LB44a (well a), *Ln. mesenteroides* subsp. *mesenteroides* LM25a (well b), *Lactobacillus brevis* LM93b (well c), *Lactococcus lactis* subsp. *lactis* MB31a (well d) and *Lactobacillus acidophilus* LM65c (well e).

RESULTS

Detection of antimicrobial producing LAB

Putative antimicrobial-producing LAB isolated from fermented milk were detected using the spot method assay on the basis of their ability to inhibit growth of the indicator strain *L. monocytogenes* ATCC7644. According to the results, of a total of 20 different traditional fermented milk samples analysed, 13 samples presented strains of lactic acid bacteria that were found to produce bacteriocin-like substances. From each of these samples, 52 inhibitor-producing bacteria, which were presumed to be LAB, were isolated. 5 of these inhibitor-producing isolates were selected for further study on the basis of their relatively wide antimicrobial spectrum, and consistently released their activity into the CFS (Figure 1).

The five presumptive bacteriocin producers were characterised and identified to species level utilising carbohydrate fermentation profiles, biochemical and physiological characteristics (Table 1) details, these bacteriocin-producing strains were Gram-positive, catalase-negative two cocci and three rod. All strains were capable of growing at 15°C but not at 45°C, nor at pH 9.6 and nor in the presence of 10% NaCl (Table 1). Based on these characteristics, as well as on carbohydrate fermentation patterns (Table 1), the strains were presumptively identified as *Lactobacillus plantarum* (LB44a), *Lb. acidophilus* (LB65c), *Lb. brevis* (LB93b), *Leuconostoc* (LM25a) and *Lc. Lactis* (MB31a).

Sensitivity to proteolytic and lipolytic enzymes

The sensitivity of the antibacterial substances produced by lactic acid bacteria to α -chymotrypsin, trypsin, pronase E, proteinase K, pepsin, catalase, and lipase was determined in controlled and reproducible conditions shown in Table 3. All the compounds were fully or partially inactivated by some of the proteolytic enzymes, which indicate their proteinaceous nature.

In general, the inhibitory compounds produced by these strains presented different patterns of sensitivity. All of them were completely inactivated by α -chymotrypsin, pronase E, and pronase K. Only one was resistant to pepsin (strain LB44a), while the compounds produced by strains MB31a and LB93b, were partially inactivated after treatment with lipase, indicating that these inhibitory substances may have a lipid moiety in their chemical composition.

Inhibitory spectrum

The sensitivity of various Gram-positive and Gram negative bacteria to the CFS of the five producing isolates was determined using the well-diffusion assay (Table 2). The inhibitory spectrum of the CFS obtained from the five isolated bacteriocin-producing LAB tested against these bacteria included most notably *B. cereus* and *B. subtilis*, which were consistently inhibited by isolates LB44a and LB65c, although not to the same extent as some of the other bacteria tested. Whereas the CFS from the strain LB44a shown to inhibit Gram negative bacteria tested; *Escherichia coli* ATCC 25422 and *Pseudomonas aeruginosa* ATCC 27853.

CFS from bacteriocin-producer LB65c and LB44a was shown to have the broadest inhibitory spectrum of the producer strains against these bacteria, while the CFS of producers, LM25a, LB93b and MB31a exhibited a narrower spectrum.

Temperature and pH stability of bacteriocins

The stability of the secreted inhibitory compounds was tested using different temperature treatments (Table 3). The inhibitory activity was shown to be completely unaffected following heat treatments at 65 and 95°C. The inhibitory compounds produced by isolates LB65c and LB93b were seen to be the most stable to heat treatments up to and beyond 100°C. LB93b maintains its activity even after treatment at 120°C for 20 min, a property which is typical for bacteriocins. The observed protease sensitivity and stability at high temperatures therefore conclusively identifies these compounds as bacteriocins.

The stability of the inhibitory activity was tested at different pH values (Table 3). The bacteriocins produced by isolates, L65c and LB44a showed greater pH tolerance

Table 1. Phenotypic characteristics of the bacteriocin-producing strains isolated from traditional fermented milk.

Characteristic	Strain designation				
	LB44a	LM25a	LB65c	LB93b	MB31a
Gram stain	+	+	+	+	+
Morphology	R	C	R	R	C
Catalase test	-	-	-	-	-
Voges-Proskauer	+	+	+	+	+
Formation of: H ₂ S	-	-	-	-	-
NH ₃ from arginine	+	-	-	+	+
Growth at:					
10°C	n	+	+	+	-
15°C	+	+	+	+	+
45°C	-	-	-	-	-
pH 4.4	-	+	+	+	+
pH 9.6	-	-	-	-	-
Growth in:					
6.5% NaCl	+	+	+	+	n
7.0% NaCl	-	+	-	-	-
10.0% NaCl	-	-	-	-	-
Gas from glucose	-	+	-	+	-
DL-Lactic acid	n	n	D	D	n
Carbohydrates					
Arabinose	-	-	-	+	-
Cellobiose	+	+	+	-	+
Esculin	+	-	+	+	n
Galactose	+	-	+	+	+
Gluconate	+	-	-	+	+
Glycerol	-	-	-	-	-
Inulin	+	-	-	+	n
Lactose	+	-	+	+	+
Maltose	+	+	-	-	+
Mannitol	+	-	+	-	+
Melezitose	+*	-	+	-	-
Melibiose	+	-	+	+	-
Raffinose	+	-	-	+	-
Rhamnose	+*	-	-	-	-
Ribose	+	+	-	+	+
Salicin	+	-	-	-	-
Sorbitol	+	-	-	-	-
Sucrose	+	+	+	-	+
Trehalose	+	+	-	-	+
Xylose	-	-	-	-	+
Identified as	<i>Lactobacillus plantarum</i>	<i>Ln. mesenteroides</i> subsp. <i>mesenteroides</i>	<i>Lb. acidophilus</i>	<i>Lb. brevis</i>	<i>Lc. lactis</i>

+ = Growth (+), - = no growth, +* = delayed fermentation.
R, Rod; C, Cocci; n, not performed.

and stability than those secreted by isolates (LM31a), (LB93b), and (LM25a).

DISCUSSION

Isolation and screening of microorganisms from naturally

occurring processes have always been the most powerful means for obtaining useful cultures for scientific and commercial purposes. This is certainly true for lactic acid bacteria (LAB), which play an important role in a large number of various traditional food fermentations (El Soda et al., 2003; Vijai et al., 2004). Among these traditional

Table 2. Inhibitory spectrum of the pH neutralized cell-free supernatants of the LAB strains isolated from fermented milk, as determined with the well-diffusion assay.

Indicator strains	Inhibitory activity of producer strains				
	LB44a	LM25a	LB65c	LB93b	MB31a
Gram positive					
<i>Bacillus cereus</i> ATCC 14578	++	-	+	-	-
<i>Bacillus subtilis</i> ATCC8	+	-	+	-	-
<i>Staphylococcus aureus</i> ATCC 25293	++	++	+	+	+++
<i>Listeria monocytogenes</i> ATCC 7644	+++	+++	+++	+++	+++
<i>Ent. faecalis</i> ATCC 19433	+	+	++	+	+
Gram negative					
<i>Escherichia coli</i> ATCC 25422	++	-	++	-	-
<i>Pseudomonas aeruginosa</i> ATCC 27853	+	-	-	-	-

- = no inhibition zone, + = inhibition zone up to 4 mm, ++ = inhibition zone up to 10 mm; +++ = inhibition zone over 10 mm.

Table 3. Effect of heat treatment, pH and proteolytic enzymes on the antimicrobial compounds produced in the supernatant by selected lactic acid bacteria isolated from Algerian fermented milk^{ab}.

Treatment	% Activity ^c				
	LB44a	LM25a	LB65c	LB93b	MB31a
Heat Treatment					
65 °C/40 min	100	100	100	100	100
95 °C/20 min	100	100	100	100	100
100 °C/20 min	50	50	100	100	50
120 °C/20 min	50	50	50	100	50
pH					
3	50	50	50	00	50
4	50	50	100	50	50
5	100	50	100	50	100
6	100	100	100	100	100
7	100	100	100	100	100
8	100	50	100	00	50
9	50	50	50	00	00
10	50	00	50	00	00
Enzymes					
Pronase E	0	0	0	0	0
Proteinase K	0	0	0	0	0
α-Chymotrypsin	0	0	0	0	0
Pepsin	100	0	0	0	0
Lipase	100	100	100	50	50
Catalase	100	100	100	100	100

^aAll assays were conducted with *Listeria monocytogenes* ATCC 7644 as indicator strain.

^bProducer strains are termed by the numbers of collection: *Lactococcus lactis* subsp *lactis* (MB31a), *Ln. mesenteroides* subsp *mesenteroides* (LM25a), *Lactobacillus brevis* (LM93b), *Lactobacillus plantarum* (LB44a) and *Lactobacillus asidophilus* (LM65c).

^cAntimicrobial activity was expressed as the % of residual activity.

processes, fermented milk is known to be essentially fermented by LAB, although often a functional secondary flora develops. Some properties of LAB such as flavour and texture formation as well as inhibit pathogenic and

spoilage microorganisms are especially important to the food and feed industries because of their applicability for a large variety of products. In addition, a large number of bacteriocins from lactic acid bacteria have been des-

cribed recently. While bacteriocin production has been reported from bacteria in milk products, fermented foods (Onda et al., 2003) and silage (Gollop et al., 2005)

In the present study, 52 lactic acid bacteria isolated from traditional fermented milk and were screened for bacteriocin production, from which, five bacteriocinogenic strains were identified and selected for further study, representing three isolates of *Lactobacillus brevis*, *Lactobacillus acidophilus*, *Lactobacillus plantarum* one *Leuconostoc* and one *L. lactis* isolates. This would indicate that a wide variety of bacteriocin-producing LAB are present on fermented milk, which therefore represent an abundant resource of such potentially useful bacteria.

The results of the Table 3 show that antibacterial compounds produced are inactive by all the proteolytic enzymes (pepsin, trypsin, α -chymotrypsin), indicating that the inhibitory compounds are proteinaceous nature, a general characteristic of bacteriocin. No zone of inhibition was discovered after stake in the presence of our extracts with these various enzymes. It has been reported that other bacteriocins than nisine are generally inactivated by an array of proteolytic enzymes including those of pancreatic origin (trypsin and α -chymotrypsin) and sometimes of gastric origin (pepsin). This high sensitivity of lactic acid bacterial bacteriocins to metabolic proteolytic enzymes is very interesting with respect to food safety, since it means that the ingestion of bacteriocins will not alter digestive tract ecology and also will not cause risks related to the use of common antibiotics (Bromberg et al., 2004).

It is interesting to note that the compound produced by the strains LB44a and MB31c, were partially inactivated after treatment with lipase, indicating that these inhibitory substances may have a lipid moiety in their chemical composition. Some bacteriocins produced by bacteria of the genus *Lactobacillus* are sensitive to non-proteolytic enzymes. Plantaricin B is inactivated by a lipase and by an α -amylase, and plantaricin S is inactivated by glycolytic, lipolytic and phospholipolytic enzymes (Jéminez-Díaz et al., 1993). These observations indicate that the active part of bacteriocins of lactobacilli may be chemically heterogeneous, which could signify that the term bacteriocin covers a set of chemically varied substances.

The inhibitory compounds produced by the five isolates demonstrated a high resilience to heat treatments ranging in temperature from 30 to 120°C (Table 2). In the other hand the bacteriocins were shown to be stable over a broad pH range with all peptides maintaining some antimicrobial activity within the pH range of pH 3 to 10. According to Tagg et al. (1976) bacteriocins differ greatly with respect to sensitivity to pH. Many of them are considerably more tolerant of acid than alkaline pH values. In the present study bacteriocin produced by the strain LB65c exhibited the same profile and was active at pH values between 4 - 9. Maximum inhibitory activity was demonstrated at pH 4 and 5. Similar properties have

been reported for other bacteriocins including lactacin, lactacin 27, acidolin, pediocin A, and pediocin PA-1 (Hastings et al., 1996). These bacteriocins were also stable over a wide range of pH. Piard and Desmazeaud (1992) also reported that temperature stability is very convenient if the bacteriocin is to be used in food preservative, because many processing procedures involve a heating step, and cold is one of the most popular preservation procedures. Furthermore, activity at neutral pH constitutes an advantage over other bacteriocins used as food preservatives and particularly over nisine, whose maximal solubility and stability are at pH 2, with these parameters decreasing significantly as the pH increases.

Generally, the bacteriocins from LAB were shown to be ineffective against Gram negative bacteria. The partially purified bacteriocin preparations from the strain (LB44a) showed broad antimicrobial activity including against Gram-negative *Pseudomonas* and *E. coli* strains (Table 2). Earlier, Sumar et al. (1998) also reported the inhibitory action of bacteriocin of *L. plantarum* against Gram-negative strains. *Lactobacillus* bacteriocins are found within each of the four major classes of antimicrobial proteins produced by LAB and the lactobacilli produce many different bacteriocins activity (Alpay Karaoglu et al., 2003). Among the lactobacilli, there has been great interest in *L. plantarum*, due to the potential application of the microorganism as a starter bacterium for a variety of fermented foods (McKay and Baldwin, 1990). The bacteriocins produced from *L. plantarum* have been found to be inhibitory towards closely related LAB, particularly the mesophilic and thermophilic lactobacilli (Sumar et al., 1998)

Of all the indicator strains tested, *L. monocytogenes* and *S. aureus*, were the most sensitive, being inhibited by all five strains. However the cultures that produced "high" inhibition zones against *L. monocytogenes* were: LB44a, LB65c and LB93b. Therefore, the high sensitivity of the *Listeria* strain to the bacteriocins produced by our isolates is not surprising, since Daeschel et al. (1989) screened many bacteriocin-producing lactic acid bacteria for inhibition of *Listeria* species and found that some of them were able to produce an antimicrobial substance that was active against *Listeria monocytogenes*.

Conclusion

The analysis of antimicrobial activity of LAB isolated from a collection of LAB was made by isolating them from traditional fermented milk that is manufactured according to the local tradition without using any known starter culture. Analysis of LAB from the collection of natural isolates revealed that they produce bacteriocins.

The antimicrobial activity of the bacteriocins produced by the lactic acid bacteria isolated in this research could act as a barrier to inhibit food spoilage and/or growth of pathogenic microorganisms in foods. Considerable effort

has recently been focussed on the understanding of the structure, the genetic organization and the mode of action of several bacteriocins. There has been a concomitant development in the description of new bacteriocins, whose biochemical and genetic characterization should lead to the discovery of important elements for the elucidation of structure/function relationships in these substances.

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