Brazilian Journal of Microbiology (2004) 35:137-144
ISSN 1517-8382

ISOLATION OF BACTERIOCIN-PRODUCING LACTIC ACID BACTERIA FROM MEAT AND MEAT PRODUCTS AND ITS SPECTRUM OF INHIBITORY ACTIVITY

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Submitted: November 04, 2003; Returned to authors: February 12, 2004; Approved: May 20, 2004.

ABSTRACT

A total of 285 samples of meat and meat products were evaluated for the presence of bacteriocin-producing lactic acid bacteria by the "sandwich" test. From 174 of these samples, 813 strains of lactic acid bacteria were isolated. They were able to inhibit the growth of *Staphylococcus aureus* CTC 33 and/or *Listeria innocua* Lin 11. When evaluated by the well-diffusion assay, 128 of these strains inhibited the growth of the indicator strains. The inhibitory spectra of activity of the isolates were evaluated against a range of Gram-positive and Gram-negative test organisms. *S. aureus* was the most sensitive indicator tested, whereas *Enterococcus faecalis* and *Lactobacillus plantarum* were the most resistant ones. All the compounds produced by the lactic acid bacteria were fully or partially inactivated by some of the proteolytic enzymes, which indicates their proteinaceous nature. The antimicrobial activity of the bacteriocins produced by the lactic acid bacteria isolated in this work could act as a potential barrier to inhibit the growth of spoilage bacteria and foodborne pathogens.

Key words: bacteriocins, lactic acid bacteria, meat, inhibitory activity

INTRODUCTION

Recently attempts have been made to apply biopreservation techniques to meat products (18). These have involved the introduction of a competitive microflora of lactic acid bacteria as protective cultures for meat products, including bacteriocin-producing lactic acid bacteria and purified anti-listerial bacteriocins (12). Considering that bacteriocin-producing bacteria are isolated from foods that normally contain lactic acid bacteria, such as meat and dairy products, they have been consumed for a long time. Bacteriocins produced by lactic acid bacteria are defined as extracellularly produced primary or modified products of bacterial ribosomal synthesis, which can have a relatively narrow spectrum of bactericidal activity (4). Bacteriocin-producing strains can be used as part of or adjuncts to starter cultures for fermented foods in order to improve safety and quality. In this context, bacteriocins produced by lactic acid bacteria associated with meat, such as *Pediococcus, Leuconostoc, Carnobacterium* and *Lactobacillus* spp., are likely to have a much greater potential as meat preservatives (3,31,33,38). The possibility of exploiting bacteriocins in food fermentation arises where the inhibitory spectrum includes food spoilage and/or pathogenic microorganisms, giving the producing strain a competitive advantage in the food. An important advantage of bacteriocins over classical antibiotics is that the digestive enzymes destroy them (4). This fact indicates that the ingestion of these compounds will not alter digestive tract ecology and also that will not cause risks related to the use of common antibiotics.

Bacteriocins could be applied in hurdle technology, which takes advantage of the synergies of combined treatments to preserve food more effectively (6). The use of nisin, a bacteriocin produced by *Lactococcus lactis* ssp., is currently allowed in about 50 countries. Nevertheless, some researchers concluded that nisin is not effective in meat application due to the high pH (24), difficulty to uniformly distribute the bacteriocin throughout the food and interference by meat components such as phospholipids (8) and glutathione (26). Due to the difficulties in using nisin in meat applications, the search for new bacteriocin-producing cultures should continue.

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The aim of this study was to screen a range of meat and meat products for the presence of bacteriocin-producing strains that might be valuable for use in biopreservation strategies for meat products. Thus, the potential of the isolates to inhibit food spoilage and foodborne pathogenic bacteria was evaluated.

MATERIALS AND METHODS

Bacterial strains and growth media

The strains used in this study are listed in Table 1. The bacteria chosen as indicators were *Staphylococcus aureus* CTC 33 (Instituto de Tecnologia de Alimentos - ITAL, Campinas, Brazil), *Listeria innocua Lin* 11 (Pasteur Institute, Paris, France), and *Bacillus cereus* CTC 1 (ITAL). The bacteriocin-producing *Lactobacillus casei* LC 705 (Wiesby) was used as the positive control. The stock cultures of lactic acid bacteria and the other microorganisms were maintained at -80ºC in de Man Rogosa Sharpe broth (MRS, Oxoid Ltd., Basingstoke, UK) or in Trypticase Soya broth (TSB, Oxoid) supplemented with 15% glycerol. Working cultures were prepared as slants on MRS agar for lactic acid bacteria or TSA agar with 0.6% yeast extract (Oxoid) supplement for the indicators, and stored at 4ºC. Cultures for experiments were streak-plated once a week and inoculated into media from a single colony and incubated for 24 h. Before use, the lactic acid bacteria cultures were transferred twice into the appropriated medium, and incubated according to the conditions showed in Table 1.

Table 1. Microorganisms and growth conditions.

Microorganisms	Growth conditions
Control	
Lactobacillus casei LC 705 (Wiesby[a])	MRS[g] 24 h/30ºC
Indicators	
Bacillus cereus ATCC[b] 14579	TSB[h] 24 h/30ºC
B. cereus CTC[c] 1	TSB 24 h/30ºC
Clostridium perfringens CTC 42	TSB 24 h/37ºC
Cl. sporogenes CTC 6	TSB 24-48 h/37ºC
Enterococcus faecalis ATCC 19433	TSB 24 h/30ºC
Escherichia coli ATCC 25422	TSB 24 h/37ºC
Lactobacillus helveticus (Wiesby)	MRS 24-48 h/45ºC
Lb. plantarum TECNOLAT[d] 434	MRS 24 h/30ºC
Leuconostoc mesenteroides ATCC 10830	MRS 24 h/30ºC
Listeria innocua Lin 11 (INRA[e])	TSB 24 h/37ºC
L. monocytogenes CTC 21	TSB 24 h/37ºC
Micrococcus sp. ATCC 4698	TSB 24 h/30ºC
Pseudomonas sp. CTC 32	TSB 24 h/37ºC
Salmonella typhimurium ATCC 14028	TSB 24 h/37ºC
Staphylococcus aureus CTC 33	TSB 24 h/37ºC
Streptococcus sp. ATCC 25175	TSB 24 h/30ºC
Sulphite-reducing clostridia CTC 5	TSB 24 h/37ºC
Weissella viridescens CCT[f] 849	MRS 24 h/30ºC

[a]Wiesby GmbH & Co. KG, Germany; [b]ATCC: American Type Culture Collection, Rockville, MD, USA; [c]CTC: Centro de Tecnologia de Carnes, Instituto de Tecnologia de Alimentos, Campinas, S.P., Brazil; [d]TECNOLAT: Centro de Tecnologia de Laticínios, Instituto de Tecnologia de Alimentos, Campinas, S.P., Brazil; [e]INRA: Institute National de Recherches Agronomiques, Jouy-en-Josas, France; [f]CCT: Fundação Tropical André Tosello, Campinas, S.P., Brazil. [g]MRS: de Man Rogosa Sharpe broth; [h]TSB: Trypticase Soya broth.

Samples

Two hundred and eighty five samples of a variety of meats and meat products obtained from different Brazilian manufacturers were analysed. They included fresh meat, raw, cooked, matured, dried or fermented meat products. After purchase in retail market, all the samples were stored at 3 ± 1ºC for up to a maximum of 24 h before analysis.

Isolation of bacteriocin-producing lactic acid bacteria from meat

A 25-g portion of each meat sample was aseptically transferred to a sterile stomacher bag and 225 mL of Buffered Peptone Water (BPW, Oxoid) added to obtain a 1:10 dilution. The samples were blended for 1 min using a stomacher (Model 400 - BA 7021, Seward Medical, London, UK). Serial dilutions of the samples were made in 0.1% peptone water. For detection of antagonistic activity, a "sandwich" test was used (37). For this purpose the dilutions were inoculated (pour plate) onto MRS agar supplemented with 0.01% sodium azide to inhibit Gram-negative bacteria. Inhibitory activity from the hydrogen peroxide was eliminated by the addition of catalase (Sigma Chemical Co., Dorset, UK) at a final concentration of 100 U. To rule out any inhibition due to pH reduction caused by organic acid production, 2% sodium β-glycerophosphate (Ecibra, Brazil) was added to MRS agar. The plates were overlaid with the same media to exclude inhibition due to lytic bacteriophage, which are non-diffusing entities, followed by aerobic incubation at 35ºC for 48 h to allow the colonies to develop.

After the incubation period, plates containing up to 10^2 CFU were overlaid with 4.5 mL of soft TSB (containing 0.75% agar). The overlay agar was seeded with 500 ml of *S. aureus* CTC 33 or *L. innocua* Lin 11 at a level of 10^6 to 10^7 CFU/mL. The plates were incubated at 35ºC for 24 h. Lysis of the indicator strains resulted in a clear zone. Colonies showing zones of inhibition were transferred to TSB and incubated at 30ºC for up to 72 h. The cultures were purified on MRS agar plates and incubated at 30ºC for 18 h. The purified isolates were examined by Gram-staining and catalase production, assayed according to Harrigan and McCance (10).

Detection of antagonistic activity

Bacteriocin production by the lactic acid bacteria isolated from meat and meat products was assayed by the agar well-diffusion method according to Benkerroum *et al.* (1), this being a modification of that described by Tagg and McGiven (35). The plates were examined for lysis around the wells at different time intervals for a total of 24 h. A direct comparison was made between the diameters of the zones of inhibition produced by different strains.

Bacteriocin spectrum of inhibitory activity

Bacteriocin-producing cultures isolated from meat and meat products were also tested against the strains of bacteria shown in Table 1. The well-diffusion assay was used as described before. The strains that presented a broad spectrum of activity were also assayed using the critical dilution assay of Mayr-Harting *et al.* (19). The title was defined as the reciprocal of the highest dilution showing an inhibition of the indicator strain multiplied by 100 to express the results as activity units per millilitre (AU/mL).

Sensitivity of bacteriocin-like substance to enzymes

Cell-free supernatants from the lactic acid cultures were collected by centrifugation (7,500 g, 10 min, 4ºC) of overnight MRS broth cultures. The supernatant fluids were adjusted to pH 6.5 with 10 N NaOH and exposed to heat (95ºC for 5 min) in a boiling waterbath. The supernatants were treated with the following enzymes at a final concentration of 0.2 mg/mL: ficin (Sigma Chemical Co., Dorset, England) in 20 mM sodium phosphate, pH 7.0; trypsin (Sigma) in 40 mM Tris-HCl, pH 8.2; α-chymotrypsin (Sigma) in 20 mM Tris-HCl, pH 8.0; pronase E (Sigma) in 20 mM Tris-HCl, pH 7.8; pepsin (Merck Darmstad, Germany) in 0.002 N HCl; lipase (Merck) in 0.1 M potassium phosphate, pH 6.0; papain (Sigma) in 0.05 M sodium phosphate acetate, pH 7.0. All these solutions were filter-sterilised through Millex GV 0.22 m filters (Millipore S.A., St. Quentin-en-Yvelines, France) and then added to sterile cell-free supernatants (v/v, 1/1). Controls consisted of enzyme solutions without bacteriocin and only cell-free supernatant in 0.1 M sodium phosphate buffer. The samples and controls were incubated at 37ºC for 2 h and heated in boiling water for 5 min to inactivate the enzymes. The remaining bacteriocin activity was determined by the critical dilution assay of Mayr-Harting *et al*. (19) as described before, using *B. cereus* CTC 1 as indicator strain.

RESULTS AND DISCUSSION

Bacterial isolation and screening

Since bacteriocin-producing bacteria isolated from meat and products are well adapted to these conditions, they could ensure the safety and extend the shelf life of these foods. Therefore a search was made for antagonistic activities against food spoilage and pathogenic bacteria, in isolates from a range of meat and meat products.

According to the results, of a total of 285 different fresh meat and meat products samples analysed, 174 presented strains of lactic acid bacteria that were found to produce bacteriocin-like substances by the "sandwich" test. From each of these samples, at least 4 colonies capable of inhibiting *S. aureus* CTC 33 and/or *L. innocua* Lin 11 were isolated, representing a total of 813 colonies. De Martinis *et al.* (7) screened twenty samples of Brazilian meat and meat products and isolated four bacteriocin-producing lactic acid bacteria that presented antilisterial activity.

After purification, the cultures were then checked for bacteriocin production using the well-diffusion assay. Using this method, by inoculating the wells with broth cultures from various indicator microorganisms, comparisons may be made between the bacteriocin production of different strains growing under identical conditions. Of the 813 isolates, only 128 (15.7%) produced inhibition zones on MRS agar. These bacteriocin-producing strains were all Gram-positive and catalase negative, 75.8% being cocci and 24.2% rods. Schillinger and Lücke (28) obtained similar results on checking *Lactobacillus sake* strains that were positive in the agar spot test and negative in the well-diffusion assay: of a total of 19 strains, only six produced inhibition zones on agar in the well-diffusion assay. Lewus *et al*. (16) found that only a few of the strains that tested positive using the spot-on-the-lawn method gave positive results in the well-diffusion assay. They considered that allowing some time for the bacteriocins to diffuse into the agar prior to incubation, or increasing the well size so that more sample could be applied, might increase the sensitivity of the assay. According to these authors, aggregation, non-diffusable bacteriocins, protease inactivation and concentration effects, can all lead to false negative results in the well-diffusion assay.

The results of the well-diffusion assay showed that 64.1% of the isolated strains only inhibited *S. aureus,* and 11.7% only showed inhibitory activity against *L. innocua,* whereas 24.2% of the meat isolates which inhibited *S. aureus* also inhibited *L. innocua*. According to Lewus *et al*. (16) the indicator microorganism used in the initial screening needs to reflect the final or proposed application of the bacteriocin-producing strain. *S. aureus* and *Listeria* sp. are often present in fresh tissues, because the slaughtering process does not include a bactericidal step. The growth of *S. aureus* in foods presents a potential public health hazard, since many strains of *S. aureus* produce enterotoxins that cause food poisoning if ingested. Meat and meat products are commonly associated with staphylococcal food poisoning (25,36). *Listeria* species have been found in meat and meat products (13). Foodborne transmission of *L. monocytogenes* has been implicated in human outbreaks of listeriosis involving the consumption of various foods (9,17,29). *L. innocua* is frequently isolated from meat, and often the incidence of this organism is higher than that of *L.*

monocytogenes (2). The use of *Listeria* species other than *L. monocytogenes* as indicators of the presence of this organism has been proposed (39).

Fig. 1 shows the groups of meat and meat product samples that presented bacteriocin-like producing lactic acid bacteria, according to the "sandwich" test and well-diffusion assay. However, not all the bacteria isolated by the "sandwich" test were confirmed in the well-diffusion assay. The "sandwich" test presented 174 (61.0%) positive samples for bacteriocin-type producing organisms, of which only 55 (31.1%) tested positive in the well-diffusion assay. According to the "sandwich" test, the majority of the bacteriocin-producing bacteria were isolated from matured, dried or fermented meat products (78.4%). The same was not observed in the well-diffusion assay: fresh meat had the higher numbers of bacteriocin-producing bacteria (27.5%). On the other hand, both methods showed that cooked meat products contained less samples with lactic acid bacteria positive for bacteriocin-like substances.

These negative results could show that bacteriocin production is not highly conserved in these strains. Some of the bacteriocins are plasmid-mediated proteins (34), so one should consider the possibility that some cultures could have lost their plasmids after consecutive transfers during the purification.

Spectra of inhibitory activity

The activity against 18 indicator strains, of the antibacterial compounds produced by the bacterial isolates, is shown in Table 2. These strains presented a broad inhibitory spectrum since they were able to inhibit many of the indicator strains tested. These data suggest that several different indicator microorganisms should be used in bacteriocin-screening tests, to avoid missing a producer. Of all the indicator strains tested, *S. aureus* CTC 33, *Cl. sporogenes* CTC 6, and *B. cereus* CTC 1 were the most sensitive, being inhibited by the greatest number of cultures, whereas *Ent. faecalis* ATCC 19433, *Lb. plantarum* TECNOLAT 434, sulphite-reducing clostridia CTC 5, *Leuc. mesenteroides* ATCC 10830, and *W. viridescens* CCT 849 were inhibited by a minor number of strains. Of the strains tested for bacteriocin production, none showed inhibitory activity against all the indicators. Only 4 strains (CTC 165, CTC 376, CTC 469, and CTC 484), inhibited the majority of the indicator strains tested (data not showed).

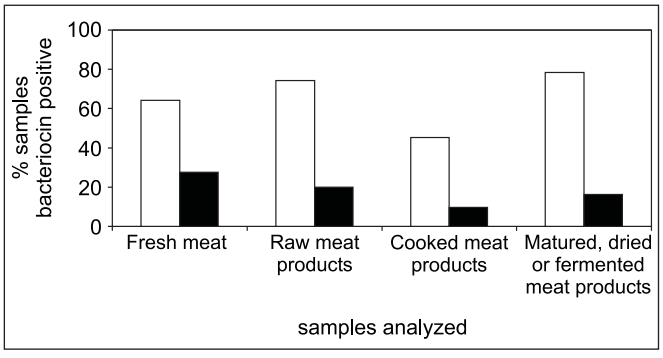

Figure 1. Meat and meat product samples positive for bacteriocin-like producing lactic acid bacteria in the (□) "sandwich" test and (■) well-diffusion assay.

The majority of the inhibition caused by lactic acid strains produced "low" inhibition zones (radius of the clearance zone was lower than 3 mm). *Lb. helveticus* (Wiesby) was a very sensitive indicator, since 90.2% of the inhibition caused by the bacteriocins tested over this bacterium produced "high" inhibition zones (radius of the clearance zone was higher than 5 mm). The cultures that produced "high" inhibition zones against indicator strains were: CTC 3, CTC 12, CTC 35, CTC 36, CTC 38, CTC 40, CTC 49, CTC 51, CTC 78, CTC 141, CTC 142, CTC 144, CTC 172, CTC 176, CTC 185, CTC 204, CTC 205, CTC 206, CTC 210, CTC 211, CTC 212, CTC 231, CTC 253, CTC 330, CTC 346, CTC 352, CTC 359, CTC 375, CTC 376, CTC 377, CTC 378, CTC 396, CTC 404, CTC 469, CTC 483, CTC 484, and CTC 485 against *Lb. helveticus*; strains CTC 78 and CTC 172 against *B. cereus* CTC 001; strain CTC 172 against *Cl. sporogenes* CTC 006; and strain CTC 332 against *Cl. perfringens* CTC 42.

Some strains produced bacteriocin-type substances which inhibited the Gram-negative bacteria tested: *Pseudomonas* sp. CTC 32 was inhibited by 61 (47.6%) strains, *E. coli* ATCC 25422 by 49 (38.3%) strains, and *Salm. typhimurium* ATCC 14028 by 48 (37.5%) strains. The target of bacteriocins is the cytoplasmic membrane, so due to the protective barrier provided by the LPS of the outer membrane of Gram-negative bacteria, bacteriocins are generally only active against Gram-positive cells (32). However, mutant strains or protoplasts of Gram-negative bacteria became sensitive to bacteriocin action after exposure to sub-lethal stress such as heating, freezing, or thawing, which disrupt the outer membrane and allow the access of bacteriocins to the cytoplasmic membrane, leading to an increased sensitivity (11,30,32).

Of the 128 cultures tested for antimicrobial activity, the 12 that inhibited the greatest number of indicators were tested using the Mayr-Harting *et al.* (19) assay (Table 3). According to the results, *B. cereus* CTC 1 and *L. monocytogenes* CTC 21 were the most sensitive microorganisms tested since the lactic acid strains analysed inhibited 100% and 91.7% of them, respectively. A variation was noted with respect to the level of inhibition amongst the indicator strains tested. *W. viridescens* CCT 849 was the most resistant one, since it presented the lowest activity value. *B. cereus* ATCC 14579, *Leuc. mesenteroides* ATCC 10830, and the sulphite-reducing clostridia CCT 5, exhibited an intermediate sensitivity pattern, whilst *S. aureus* CTC 33, *L. innocua* Lin 11, *L. monocytogenes* CTC 21, and *Cl. perfringens* CTC 42 were less resistant bacteria. The

Table 2. Sensitivity of pathogenic and spoilage bacteria to bacteriocin-producing strains isolated from meat and meat products by the agar well-diffusion method.

Indicator strains	Inhibitor producers				
	negative	positive*			
		"low"	"average"	"high"	total
B. cereus CTC 1	29 (22.6%)	85 (66.4%)	12 (9.4%)	2 (1.6%)	99 (77.3%)
B. cereus ATCC 14579	45 (35.2%)	57 (44.5%)	-	-	57 (44.5%)
Cl. perfringens CTC 42	34 (26.6%)	93 (72.6%)	-	1 (0.8%)	94 (73.4%)
Cl. sporogenes CTC 6	24 (18.6%)	94 (73.4%)	9 (7.0%)	1 (0.8%)	104 (81.1%)
Ent. faecalis ATCC 19433	121 (94.5%)	6 (46.9%)	1 (0.8%)	-	7 (5.5%)
E. coli ATCC 25422	79 (61.7%)	49 (38.3%)	-	-	49 (38.3%)
Lb. helveticus (Wiesby)	87 (68.0%)	4 (3.1%)	-	37 (28.9%)	41 (32.0%)
Lb. plantarum TECNOLAT 434	121 (94.5%)	7 (5.5%)	-	-	7 (5.5%)
Leuc. mesenteroides ATCC 10830	104 (81.2%)	23 (18.0%)	1 (0.8%)	-	24 (18.8%)
L. innocua Lin 11	77 (60.2%)	49 (38.3%)	2 (1.6%)	-	51 (39.8%)
L. monocytogenes CTC 21	79 (61.7%)	48 (37.5%)	1 (0.8%)	-	49 (38.3%)
Micrococcus sp. ATCC 4698	63 (49.2%)	57 (44.5%)	6 (4.7%)	-	63 (49.2%)
Pseudomonas sp. CTC 32	67 (52.3%)	60 (46.9%)	1 (0.8%)	-	61 (47.6%)
Salm. typhimurium ATCC 14028	80 (62.5%)	48 (37.5%)	-	-	48 (37.5%)
S. aureus CTC 33	8 (6.2%)	119 (93.0%)	1 (0.8%)	-	120 (93.8%)
Streptococcus sp. ATCC 25175	99 (77.3%)	29 (22.6%)	-	-	29 (22.6%)
Sulphite-reducing clostridia CTC 5	108 (84.4%)	20 (15.6%)	-	-	20 (15.6%)
W. viridescens CCT 849	104 (81.2%)	24 (18.8%)	-	-	24 (18.8%)

*The inhibitory activity is expressed as the radius "r" of the clearance zone. Based on these results a division into 3 groups with increasing inhibitory activity was made ("low": r < 3 mm; "average": 3 mm < r < 5 mm; "high": r > 5 mm).

Table 3. Activity spectra of bacteriocin produced by lactic acid cultures according to Mayr-Harting *et al*. (19).

Indicators	*Activity of bacteriocin-producing bacteria (AU/mL*)*											
	141	164	204	210	352	368	396	404	469	483	484	485
B. cereus CTC 1	800	800	200	200	200	400	800	400	1600	1600	200	1600
B. cereus ATCC 14578	0	0	0	0	0	0	0	0	0	0	400	400
Cl. perfringens CTC 42	800	0	200	0	0	0	0	200	0	200	200	200
Cl. sporogenes CTC 6	0	0	0	0	0	0	0	0	0	0	0	0
Ent. faecalis ATCC 19433	0	400	0	0	0	0	0	0	1600	0	200	0
E. coli ATCC 25422	0	0	0	0	0	0	0	0	0	0	0	0
Lb. helveticus (Wiesby)	800	800	800	0	800	0	800	400	800	800	800	1600
Lb. plantarum TECNOLAT 434	0	0	0	0	0	0	0	0	0	0	0	0
Leuc. mesenteroides ATCC 10830	0	400	0	400	0	0	0	0	200	200	0	0
L. innocua Lin 11	0	400	400	200	800	800	0	200	400	200	0	0
L. monocytogenes CTC 21	400	800	400	200	800	800	400	200	200	0	200	200
Micrococcus sp. ATCC 4698	0	0	0	0	0	0	0	0	0	0	0	0
Pseudomonas sp. CTC 32	0	0	0	0	0	0	0	0	0	0	0	0
Salm. typhimurium ATCC 14028	0	0	0	0	0	0	0	0	0	0	0	0
S. aureus CTC 33	200	200	400	0	800	800	0	0	200	200	200	0
Streptococcus sp. ATCC 25175	0	0	0	0	0	0	0	0	0	0	0	0
Sulphite-reducing clostridia CTC 5	0	0	0	0	0	0	0	0	400	0	0	0
W. viridescens CCT 0849	0	200	0	0	0	200	0	0	0	0	0	0

* AU/mL: results expressed as activity units per millilitre.

most sensitive microorganisms, *B. cereus* CTC 1, *Ent. faecalis* ATCC 19433, and *Lb. helveticus* (Wiesby) showed the highest sensitivities against some of the producers. Among the producers, strains CTC 210 and CTC 404 exhibited lower inhibition activity values, whilst strains CTC 469, CTC 483, and CTC 485 showed the highest activity. Of the strains tested, CTC 164 and CTC 469 showed the widest activity spectra, since they were able to inhibit 44.4% of the indicator cultures.

Some of the microorganisms that exhibited sensitivity towards the strains tested using the well-diffusion assay, such as the Gram-negative species (*E. coli* ATCC 25422*, Pseudomonas* sp. CTC 32*,* and *Salm. typhimurium* ATCC 14028), *Cl. sporogenes* CTC 6*, Micrococcus* sp. ATCC 4698*, Lb. plantarum* TECNOLAT 434, and *Streptococcus* sp. ATCC 25175, were not inhibited in the Mayr-Harting *et al.* (19) assay. Only *B. cereus* CTC 1 exhibited the same pattern of sensitivity when analysed by both methods.

Indicator culture inhibition was greater using the well-diffusion assay. Since both the producer cultures and the indicators grew at the same time, the inhibition could be caused by a competition for nutrients from the culture media. The use of cell-free supernatants in the Mayr-Harting *et al.* (19) assay could avoid this problem.

Sensitivity to proteolytic and lipolytic enzymes

The sensitivity of the antibacterial substances produced by lactic acid bacteria to α-chymotrypsin, trypsin, pronase E, ficin, pepsin, papain, and lipase was determined in controlled and reproducible conditions shown in Table 4. All the compounds were fully or partially inactivated by some of the proteolytic enzymes, which indicates 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 ficin. Only one was resistant to trypsin (strain CTC 141), while the substance produced by strain CTC 204 lost 75% of its activity after treatment with this enzyme. Some authors differentiate nisin from other lactococcal bacteriocin by the fact that α-chymotrypsin is the only proteolytic enzyme to which nisin is sensitive (14,22). Nevertheless, this property is to be regarded with caution, since other authors have reported that nisin can also be inactivated by other enzymes such as pronase E (15,23), and ficin (5,21).

Pepsin inhibited the antagonistic activity of 75% of the strains (CTC 141, CTC 164, CTC 210, CTC 368, CTC 396, CTC 404, CTC 483, CTC 484, and CTC 485). Sensitivity to pepsin was shown in other bacteriocins: plantaricin 35d (20), sakacin A (28), and enterocin 416KI (27). Papain did not affect the activity of the antibacterial substances produced by strains CTC 141 and CTC 404, while this enzyme inactivated the other ones.

The compounds produced by strains CTC 164, CTC 210, CTC 368, CTC 483, CTC 484, and CTC 485, were fully or partially inactivated after treatment with lipase, indicating that these inhibitory substances may have a lipid moiety in their chemical composition.

It is interesting to note that the compounds produced by these strains were inactivated by an array of proteolytic enzymes, including those of pancreatic origin (trypsin and α-chymotrypsin) and many times of gastric origin (pepsin). On the basis of the definition of bacteriocins of Gram-positive bacteria given by Tagg *et al*. (34) and Klaenhammer (14) and by using the properties observed in bacteriocins from lactic acid bacteria (22), the antibacterial compounds produced by the present strains can be classified as bacteriocins. The pattern of protease sensitivity indicates the singularity of the bacteriocins produced by the isolates and that the strains are different. Although some of the bacteriocins presented similar pattern of protease sensitivity, as between CTC 210 and CTC 484 or CTC 352 and CTC 469, they did not share an identical spectrum of activity.

Table 4. Sensitivity of bacteriocins produced by lactic acid strains to treatment with proteolytic and lipolytic enzymes.

Enzymes	Strains											
	141	164	204	210	352	368	396	404	469	483	484	485
Control*	800	800	800	200	200	400	800	400	1600	1600	200	1600
α-Chymotrypsin	0	0	0	0	0	0	0	0	0	0	0	0
Trypsin	800	0	200	0	0	0	0	0	0	0	0	0
Pronase E	0	0	0	0	0	0	0	0	0	0	0	0
Ficin	0	0	0	0	0	0	0	0	0	0	0	0
Pepsin	0	400	800	0	200	0	0	200	1600	400	0	400
Papain	800	0	0	0	0	0	200	400	0	200	0	0
Lipase	800	400	800	0	200	200	800	400	1600	400	0	400

* Phosphate buffer + cell-free supernatants.

The results showed that 61% of the meat samples analysed presented bacteriocin-producing lactic acid bacteria with potential to inhibit harmful microorganisms, such as *L. monocytogenes, S. aureus*, and sporulated bacteria. 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. Further work to evaluate the nature of these substances and their applicability in biopreservation techniques for meats is in progress.

ACKNOWLEDGEMENTS

The authors thank FAPESP (Fundação de Amparo a Pesquisa do Estado de São Paulo) for financial support (Process 99/12314-0).

RESUMO

Isolamento de bactérias lácticas produtoras de bacteriocinas a partir de carnes e produtos cárneos e seu espectro de atividade inibitória

Um total de 285 amostras de carnes e produtos cárneos foi avaliado para detecção de culturas produtoras de bacteriocinas pelo método do "sanduíche". A partir de 174 destas amostras, 813 linhagens de bactérias lácticas com atividade inibitória sobre *Staphylococcus aureus* CTC 033 e/ou *Listeria innocua* Lin 11 foram isoladas. Quando examinadas pelo método de antagonismo simultâneo em poços, 128 destas linhagens inibiram o crescimento dos microrganismos indicadores. O espectro de atividade das linhagens isoladas foi avaliado com diversos microrganismos Gram-positivos e Gram-negativos. De um modo geral, *S. aureus* foi o microrganismo indicador mais sensível, enquanto *Enterococcus faecalis* e *Lactobacillus plantarum* foram os mais resistentes. Todos os compostos antimicrobianos produzidos pelas bactérias lácticas testadas foram completa ou parcialmente inativados por enzimas proteolíticas, o que indica sua natureza protéica. A atividade antimicrobiana das bacteriocinas produzidas pelas linhagens de bactérias lácticas isoladas neste trabalho pode atuar como uma barreira potencial para inibir o crescimento de bactérias deterioradoras e patogênicas de origem alimentar.

Palavras-chave: bactérias lácticas, bacteriocinas, carne, atividade inibitória

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