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Bacteriocinogenic activity from starter cultures used in Spanish meat industry

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Abstract

Thirty-nine bacterial strains, obtained from commercial starter cultures and commonly used by the meat industry in Spain, have been examined for their ability to produce bacteriocins. Fourteen (35%) of them showed antagonism against at least one of the indicator strains, by the agar spot test. The strains showing an inhibitory action against pathogenic indicator strains were identified as *Pediococcus acidilactici*, *Lactobacillus curvatus*, *Lactobacillus pentosus* and *Lactobacillus plantarum*, which showed an inhibitory action against a wide range of Gram-positive bacteria. The only strain which showed reliable inhibitory activity against pathogenic indicator strains, by the well diffusion assay, was *P. acidilactici*. This strain produces an inhibitory compound, which reaches its maximum activity at the beginning of the stationary phase of growth. This antimicrobial substance (bacteriocin) has a proteinaceous nature, is stable over a broad range of pH, resistant to heat and shows a bactericidal action.

Keywords: Pediococci; Biopreservation; Bacteriocin; Meat industries; *Pediococcus acidilactici*

1. Introduction

The starter cultures used in the meat industry start and propagate the fermentation process, extend the shelf life of the product, improve its hygienic quality and increase the acceptability of the final product. In the manufacture of fermented meat products the bacterial starter cultures used are essentially, lactic acid bacteria from genera *Lactobacillus* (Hugas, Garriga, Aymerich, & Monfort, 1993; Rovira, Nieto, Rodríguez, Reguera, & González, 1997) and *Pediococcus* (Landvogt & Fischer, 1991) and *Micrococcaceae* from genera *Micrococcus* (Geisen, Lücke, & Kröckel, 1992) and *Staphylococcus* (Nychas & Arkoudelos, 1990).

Pediococci are frequently associated with the fermentation of meat products and are one of the starter cultures used in the manufacture of fermented sausages in Spain (Cintas et al., 1995; Lizaso, Chasco, & Beriain, 1999) and elsewhere.

Some lactobacilli and pediococci strains are able to produce substances with antimicrobial activity (Garriga, Hugas, Aymerich, & Monfort, 1993; Schillinger & Lücke, 1990). Bacteriocin-producing *Pediococcus acidilactici* strains have been reported (Bhunia, Johnson, & Ray, 1988; Bhunia, Johnson, Ray, & Kalchayanand, 1991; Cintas et al., 1995; González & Kunka, 1987). Unlike many bacteriocins produced by other lactic acid bacteria, the *Pediococcus* genus ones have a very broad spectrum of action, which include strains and species from genera *Pediococcus*, *Lactobacillus*, *Leuconostoc*, *Enterococcus* and *Micrococcus*, as well as pathogenic Gram-positive bacteria from genera *Listeria, Staphylococcus*, *Bacillus* and *Clostridium*. Several studies have shown the usefulness of bacteriocins and their producer strains (starter cultures) in meat and meat products (Aymerich, Hugas, & Monfort, 1998; Hugas, 1998).

The aim of this study was to isolate bacteria, from commercial starter cultures, commonly used in Spain,

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which show bacteriocinogenic activity against several pathogenic microorganisms, and to determine the biochemical characteristics, mode of action and inhibitory spectrum of the potential bacteriocins.

2. Materials and methods

2.1. Strains

Thirty-nine bacterial strains isolated from commercial starter cultures were used: six strains of *Lactobacillus plantarum*, five of *Lactobacillus curvatus*, five of *Lactobacillus sake*, two of *Lactobacillus pentosus*, two of *Lactobacillus carnis*, seven of *P. acidilactici*, four of *Pediococcus pentosaceus*, four of *Staphylococcus carnosus*, three of *Staphylococcus xylosus* and one of *Micrococcus varians*, obtained from Lactolabo, Chr. Hansen, Lamirsa, Microlife, Roger and Rudolf-Müller.

Forty-three indicator strains were also used: 12 lactobacilli, seven *Bacillus* spp., five clostridia, four staphylococci, three *Listeria* spp., two *Carnobacterium* spp. and *Pediococcus* spp. and one from genera *Citrobacter*, *Escherichia*, *Hafnia*, *Leuconostoc*, *Proteus*, *Pseudomonas*, *Salmonella* and *Streptococcus*, obtained from the Spanish Type Culture Collection (CECT), Valencia, Spain; from the Meat Technology Center, Institute of Food Research and Technology (CTC-IRTA), Monells, Spain; from M. Daeschel, North Carolina State University, Raleigh, USA and from our own strain collection.

2.2. Culture, isolation, identification and storage

The lactic acid bacteria from the commercial starter cultures were grown in MRS (Oxoid Ltd, Basingstoke, UK) broth and agar under microaerophilic conditions in a carbon dioxide incubator (B-5060BK, Heraeus, Germany) and the *Micrococcaceae* of these starter cultures in BHI broth (Oxoid) and Chapman agar (Difco, Detroit, MI, USA) under aerobic conditions, at 30 °C for 16–18 h in broth or 48 h on agar. The lactic acid bacteria indicator strains were grown in MRS broth and agar, the *Micrococcaceae* ones in BHI and TSB (Oxoid) broth and BHI and TSA (Oxoid) agar and the other indicator strains in BHI broth.

The identification and characterization of the lactic acid bacteria was performed according to the schemes of Schillinger and Lücke (1987), as reported in Hugas et al. (1993) and Rovira et al. (1997), which include: morphology and Gram stain, mobility, catalase activity, growth profile, growth at different temperatures, tolerance to sodium chloride, growth at pH 3.9, production of hydrogen peroxide from glucose, deamination of arginine, carbohydrate fermentation using API systems 50 CHL (bioMérieux, Marcy L'Etoile, France), acetoin production, lactic acid production and pH determination. The *Micrococcaceae* identification was carried out using API systems STAPH (bioMérieux).

Strains were stored at −40 °C in 10 ml of MRS (lactic acid bacteria) or BHI broth, using glycerol as cryoprotector at 40% (v/v) final concentration. The inoculum used was 10% (v/v) from a culture of each strain grown at 30 °C for 16–18 h. Lactic acid bacteria strain was stored refrigerated at 4 °C for up to a month maximum, inoculating 1 ml of the culture in litmus milk medium (Oxoid) and MRS broth, in 10-ml tubes, filling most of the tubes in order to improve the microaerophilic conditions.

2.3. Detection of inhibitory activity

MRS broth with a low glucose concentration was used for lactobacilli and pediococci while BHI broth was used for *Micrococcaceae*. Two consecutive cultures (transfers) were performed at 30 °C for 16–18 h. In order to establish the effect of the composition of the culture medium on the inhibitory activity, MRS broth was used, containing yeast extract, meat extract and different glucose concentrations: 0.2, 1 and 5% (w/w).

Media, inoculated at 1% (v/v) were incubated under microaerophilic or aerobic conditions according to the type of strain. Bacterial cells were collected by centrifugation (Sepatech Biofuge 28 RS, Heraeus, Germany) at 5000×***g*** for 15 min at 4 °C. The pellet was washed twice with sterile physiological saline solution (0.85% NaCl) and resuspended in 5 ml of the same medium.

The supernatants of the lactic acid bacteria cultures that showed inhibitory activity from the corresponding pellets were filtered through 0.22 μm pore-size filters (Millipore, Bedford, MA, USA) and neutralized to pH 6.5 with 1 M NaOH, in order to exclude a potential inhibitory effect caused by a decrease in pH. To exclude the inhibitory effect from the possible production of hydrogen peroxide, supernatants were treated with catalase (Sigma, USA), dissolved in a phosphate buffer at pH 7.0 at 1 mg/ml final concentration, and incubated for 30 min at room temperature. Supernatants were then concentrated ten times in a rotary evaporator (RE111, Buchi) at 80 °C.

Pellets and supernatants so obtained were used to detect inhibitory activities, the former by the agar spot test and the latter, when its corresponding strains showed inhibition with one or more indicator strains, by the well diffusion assay (Schillinger & Lücke, 1989).

2.4. Viability studies

MRS broth (10 ml), inoculated with 2 ml of a *P. pentosaceus* FBB 61 indicator strain culture, grown for 16–

18 h and with 2 ml of the concentrate (obtained from each potential bacteriocinogenic strain, as described previously), were incubated at 30 °C for 8 h, recording absorbance values at 600 nm every 0.5 h (Spectronic 301, Milton Roy). The degree of lysis was calculated as the time required to reduce turbidity by a factor of 2.

2.5. Sensitivity to heat

Five millilitres of the concentrate obtained from potential bacteriocin-producing strain cultures, were treated separately in a water bath at 80 °C for 2 h and 90 °C for 1 h, and also in an autoclave (Autotester G-dry, Selecta, Spain) at 100 °C for 30 min and 115 °C for 15 min. Immediately after, samples were cooled to 4 °C and the residual activity determined by the well diffusion assay. Untreated samples were included as controls.

2.6. Sensitivity to pH

The concentrates were dispensed in tubes and the pH was adjusted by lactic acid (1% w/v) or 1 M NaOH in the range was 2.0–10.0. After 2 h at room temperature the residual activity was determined by the well diffusion assay. The concentrates of unadjusted pH were used as the controls.

2.7. Sensitivity to proteolytic enzymes

Of the trypsin (0.1 ml) (Boehringer, Mannheim, Germany), proteinase K (Boehringer), pepsin (Boehringer) and chemotrypsin (Merck, Germany) enzymes at a 10 mg/ml concentration, in 0.01 M phosphate buffer at pH 7.0, were added to 0.1 ml of the concentrates, to give a 5 mg/ml enzyme final concentration. The samples were filtered through 0.22 μm pore-size filters (Millipore) and incubated for 2–4 h at 37 °C. Immediately after, the residual activity was determined by the well diffusion assay. Untreated concentrates were used as controls.

2.8. Spectrum of antimicrobial activity

The indicator strains used to determine the spectrum of antimicrobial activity were 43: 37 Gram-positive and 6 Gram-negative strains (see "strains").

2.9. Antimicrobial active compound titre

Antimicrobial activity was quantified against *P. pentosaceus* FBB 61, obtained from M. Daeschel, North Carolina State University, Raleigh, USA; in a microtitre plate assay system (Geis, Singh, & Teuber, 1983). Each well of the microtitre plate contained 200 μl of MRS broth, bacteriocin fractions at twofold dilutions and the indicator strain ($O.D._{600}$ 0.1). The microtitre plate cultures were incubated for 3 h at 30 °C, after which growth inhibition of the indicator strain was measured spectrophotometrically at 600 nm (Microplate Reader, Dynatech). One bacteriocin unit (BU) was defined as the amount of bacteriocin which inhibited growth of the indicator strain by 50% (50% of the turbidity of the control culture without bacteriocin).

3. Results

The 39 studied commercial starter culture strains were Gram-positive, with a bacillary or coccoid morphology, catalase-negative (although some pediococci showed limited catalase activity) and did not produce gas from glucose.

Out of these 39 strains 14 (35%) showed an evident antagonism against at least one of the indicator strains by the agar spot test. In Tables 1 and 2 the inhibitory effect produced by one or several starter culture strains against the indicator strains, by the agar spot test, is shown. At the bottom of Table 2, the strains responsible for the produced inhibition are shown. The *P. acidilactici* strain (Hansen) showed the broadest spectrum and the main inhibitory activity (this strain was not the Bactoferm F-LC starter culture).

The proportion of antagonistic strains did not vary with the culture medium used for growth. However, when 2% yeast extract was added to the medium used to determine activity by the agar spot test, the diameter of the zone of inhibition against the indicator strains increased by 30–40%.

The inhibition produced against the indicator strains by the concentrates from the starter culture strains, by the well diffusion assay, is shown in Tables 1 and 2. As also shown by the agar spot test, the strain that showed the best inhibitory results was *P. acidilactici*.

Good inhibitory results were obtained against *Listeria monocytogenes*, *Clostridium perfringens*, *Bacillus cereus* and other species of the *Bacillus* genus (*B. licheniformis* and *B. subtilis*), as well as *Brochothrix thermosphacta*, *Listeria innocua* (zones of inhibition larger than 1 cm diameter), *Carnobacterium divergens* and *P. pentosaceus*. Of the lactobacilli strains showing inhibition by the agar spot test, by the well diffusion assay, only *L. plantarum* against *Streptococcus faecalis* and *Staphylococcus aureus*, as well as *L. curvatus* against *L. monocytogenes*, showed evident inhibition. *L. pentosus* only showed a weak inhibition against *C. perfringens*, the results being difficult to read and interpret.

The inhibitory activity of the *L. plantarum* and *L. curvatus* strains was not maintained, therefore, we decided not to carry on studying these strains but to study the inhibitory substance produced by the *P. acidilactici* strain, since more reliable inhibitory results were obtained with this strain.

Table 1
Inhibition of indicator strains by the bacterial starter cultures[a]

Indicator strains	Origin	Agar spot test	Well diffusion assay
Lactobacillus bulgaricus	CECT[b] 4005	+	+
L. bavaricus	CECT 980	+	+
L. brevis	CECT 216	+	–
L. sake	Our strain collection	+	–
Pediococcus acidilactici	Our strain collection	+	+
P. pentosaceus	FBB 61[c]	+	+
Bacillus cereus	CECT 148	+	+
B. pumilus	Our strain collection	+	–
B. licheniformis	Our strain collection	+	+
B. subtilis	Our strain collection	+	+
Brochothrix thermosphacta	CECT 847	+	+
Carnobacterium divergens	CTC-IRTA[d]	+	+
Clostridium sporogenes	CECT 485	+	–
C. difficile	CECT 531	+	–
Listeria innocua	Our strain collection	++	++
L. welshimeri	Our strain collection	+	–

[a] One or more than one.
[b] CECT, Spanish Type Culture Collection, Valencia, Spain.
[c] Obtained from M. Daeschel, North Carolina State University, Raleigh, USA.
[d] CTC-IRTA, Meat Technology Center, Institute of Food Research and Technology, Monells, Spain.

3.1. Production and characterization of the inhibitory compound

Fig. 1 shows the growth profile of the inhibitory *P. acidilactici* strain (obtained from Chr. Hansen Laboratories, Denmark), grown in the usual medium, MRS containing 0.2% (w/w) glucose, at 30 °C. The stationary phase of growth was reached after 16–18 h of incubation.

Fig. 1 shows the inhibition produced by the concentrate prepared at different stages of growth of the *P. acidilactici* producer strain (Hansen), against *L. monocytogenes* CECT 4031 by the well diffusion assay. No evident inhibition was detected until 12 h, reaching a maximum after 16 h of incubation, with zones of inhibition larger than 2 cm. This inhibition was maintained up to approximately 20 h, and gradually reduced thereafter with zones of inhibition around 1 cm and less than 0.5 cm after 24 and 48 h, respectively.

These results confirm that maximum bacteriocin production occurs both at the end of the logarithmic phase and at the beginning of the stationary phase of growth. At these times, the inhibitory activity was of 10 000 BU/ml.

The inhibitory *P. acidilactici* strain (Hansen) maintained 90% of its antimicrobial activity after 6 months at 4 °C in litmus milk medium. This potential bacteriocin-producing strain, as well as its concentrate, main-

Table 2
Inhibition of indicator strains by the bacterial starter cultures

Indicator strains	Origin	Starter cultures							
		(1)[a]		(2)[b]		(3)[c]		(4)[d]	
		A[e]	B[f]	A	B	A	B	A	B
Listeria monocytogenes	CECT[g] 4031	–	–	–	–	++	+	++	++
Streptococcus faecalis	CECT 481	+	+	–	–	++	–	++	++
Staphylococcus aureus	our strain collection	++	+	–	–	–	–	+	+/-
Clostridium perfringens	CECT 376	–	–	++	+/-	–	–	++	++
Pseudomonas aeruginosa	CECT 532	–	–	–	–	–	–	–	–
Salmonella spp.	CECT 545	–	–	–	–	–	–	–	–
Escherichia coli	CECT 99	–	–	–	–	–	–	–	–

[a] (1), *Lactobacillus plantarum* (Rudolf-Müller).
[b] (2), *L. pentosus* (Hansen).
[c] (3), *L. curvatus* (Lactolabo).
[d] (4), *Pediococcus acidilactici* (Hansen).
[e] A, by agar spot test.
[f] B, by well diffusion assay.
[g] CECT, Spanish Type Culture Collection, Valencia, Spain.

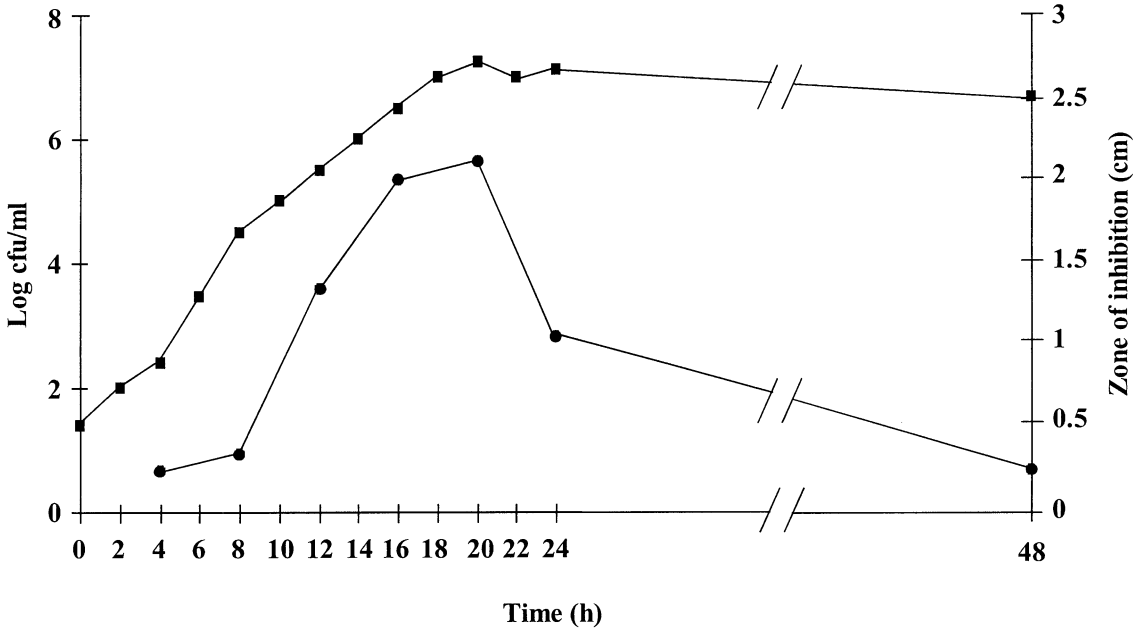

Fig. 1. Growth (■) of the inhibitory *Pediococcus acidilactici* strain (Hansen), in MRS medium at 30 °C and zone of inhibition (●) produced by the concentrate prepared at different stages of growth of the *P. acidilactici* producer strain (Hansen) against *Listeria monocytogenes* CECT 4031, by the well diffusion assay.

tained 98% of its inhibitory activity after 12 months at −40 °C.

3.1.1. Viability studies

The bactericidal activity of the concentrate from a culture of the inhibitory *P. acidilactici* strain (Hansen) against the culture of *P. pentosaceus* FBB 61 (control), in MRS medium at 30 °C, is shown in Fig. 2. After half an hour of incubation, inhibition of the control strain occurred, from an initial value of 7×10^5 cfu/ml to 2×10^4 cfu/ml after 1.5 h and by four log cycles after 3 h of incubation. Two millilitres of concentrate from a culture of a non-bacteriocinogenic *P. acidilactici* strain (Roger) were added to the control culture. After 3 h incubation, the counts reached 8×10^6 cfu/ml while in the one that showed the bactericidal effect the count was 2×10^2 cfu/ml.

3.1.2. Heat effect

In all cases, the concentrate from a culture of the inhibitory *P. acidilactici* strain (Hansen) maintained its inhibitory activity after thermal treatment, although

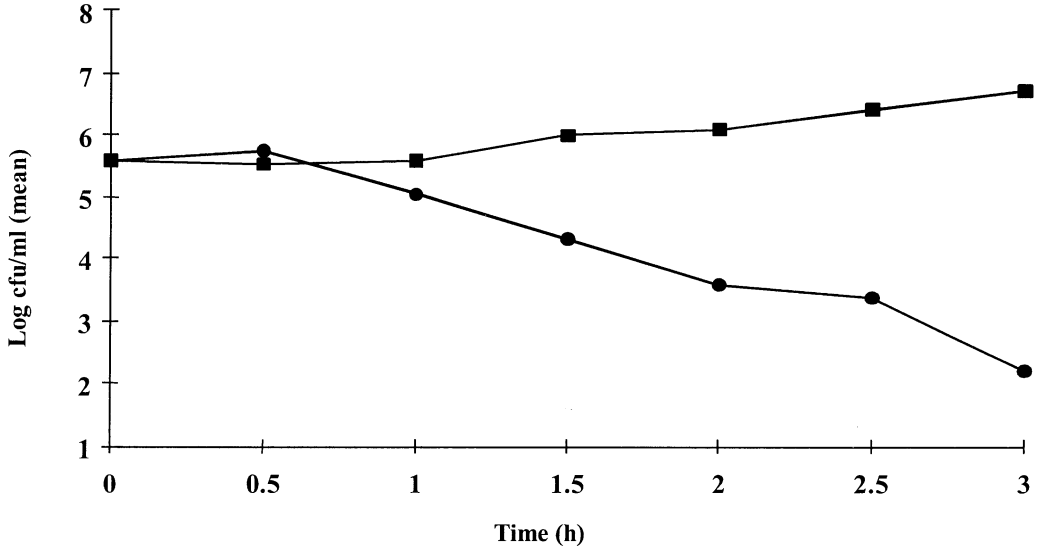

Fig. 2. Bactericidal effect (●) of the concentrate from a culture of the inhibitory *Pediococcus acidilactici* strain (Hansen) against the culture of *P. pentosaceus* FBB 61 (control, ■), grown in MRS medium at 30 °C.

after treatment at 115 °C for 15 min, it was 4000 BU/ml, against *P. pentosaceus* FBB 61.

3.1.3. pH effect

Adjustment of the pH of the concentrate from *P. acidilactici* strain (Hansen), showed optimum activity against *P. pentosaceus* FBB 61 between pH 4.0 and 7.0, with inhibition zones greater than 2 cm, while at other pH values, the zones of inhibition were equal to or less than 1 cm. No inhibitory activity was detected after treatment at pH 10.0.

3.1.4. Sensitivity to proteolytic enzymes

When the concentrate from a culture of the inhibitory *P. acidilactici* strain (Hansen) was previously treated with trypsin, proteinase K, pepsin or chemotrypsin, no inhibition was detected on *P. pentosaceus* FBB 61.

4. Discussion

The pediococci isolated from meat and meat products cannot be adequately identified following the common schemes for lactic acid bacteria (Rogosa, 1970). In 1987 Schillinger et al. developed a rapid identification scheme for lactic acid bacteria from meat, based easily determined physiological and morphological characteristics These have been mainly used for the identification of lactic acid bacteria in this study.

Indirect antagonism assays, such as the agar spot test, are often more sensitive than direct ones (Tagg, Dajani, & Wannamaker, 1976). The results obtained by different researchers with respect to the antimicrobial activity of lactic acid bacteria by such tests are quite variable (Barefoot & Klaenhammer, 1983; Garriga et al., 1993).

Differences in inhibitory activity have been reported with regard to culture medium composition (Geis et al., 1983). In our work, the most pronounced differences seen using the agar spot test were obtained when supplementing the culture medium of the potential bacteriocin-producing strains with 2% yeast extract, yielding greater antimicrobial activity. The initial pH of the culture medium influenced the inhibition, the best results were obtained when the pH was adjusted between 4.0 and 7.0, especially between 5.5 and 6.5.

The concentrates from a culture of the inhibitory*P. acidilactici* strain (Hansen) showed the best results against *L. monocytogenes*, *L. innocua*, *C. perfringens*, *B. cereus*, *B. licheniformis* and *B. subtilis*, as it did against *Pediococcus*, *Brochothrix* and *Carnobacterium* strains. No inhibition was detected against Gram-negative bacteria. Although many bacteriocin from lactic acid bacteria have a narrow inhibitory spectrum, the ones from *Pediococcus* generally produce bacteriocin against many bacteria, including pathogenic bacteria (Bhunia et al., 1988; Daeschel & Klaenhammer, 1985; González et al., 1987). These pediococci are frequently used in the manufacture of fermented meat products, which increases the interest in them as protective cultures.

4.1. Production of the inhibitory compound

The growth of the inhibitory *P. acidilactici* strain (Hansen) at 30 °C in MRS medium, of low glucose concentration, is the same as observed by other authors with *P. acidilactici* and *P. pentosaceus* strains, as well as the growth of other lactic acid bacteria from meat (Bhunia et al., 1988; Piard & Desmazaud, 1992).

The bacteriocin-production by lactic acid bacteria can occur both during the logarithmic phase and the stationary phase of growth (Piard et al., 1992). The inhibition of *L. monocytogenes* CECT 4031 by the concentrate from a culture of the inhibitory*P. acidilactici* strain (Hansen) was detected in our study at the end of the logarithmic phase of growth. The production of the inhibitory compound is therefore related to good cellular growth. This agrees with Biswas, Ray, Johnson, and Ray (1991) findings with respect to the pediocin from *P. acidilactici* H.

The growth temperature of the lactic acid bacteria is an important consideration for the choice of starter cultures. The strains in this work, showed good growth between 25 and 35 °C, as is common with other pediococci.

4.2. Characterization of the inhibitory compound

In this work, we observed the bactericidal effect of the concentrate from a culture of the inhibitory *P. acidilactici* strain (Hansen) against the culture of *P. Pentosaceus* FBB 61. According to the criteria of Tagg et al. (1976), the inhibitory substance can be defined as a bacteriocin. This bactericidal effect agrees with that observed by other authors with pediocin PA-1 (González & Kunka, 1987), pediocin AcH (Bhunia et al., 1991) and pediocin SJ-1 (Schved, Lalazar, Hensis, & Juven, 1993).

The results of our thermal treatment experiments show that the inhibitory substance is resistant to heat. This resistance is common to the other bacteriocin produced by lactic acid bacteria: lactacin B (Barefoot et al., 1983), lactacin F (Muriana & Klaenhammer, 1987), sakacin A (Schillinger & Lücke, 1989), pediocin PA-1 (González & Kunka, 1987), pediocin AcH (Bhunia et al., 1988), pediocin SJ-1 (Schved et al., 1993) and nisin (Bailey & Hurst, 1971), among others. This resistance to heat is a very common characteristic among bacteriocins (Tagg et al., 1976).

The lack of inhibition, of the concentrate, from a culture of the potentially bacteriocin-producing strain when treated with proteolytic enzymes, shows that the inhibitory compound is proteinaceous, another general

characteristic of bacteriocins. Bacteriocins are generally inactivated by proteolytic enzymes of pancreatic (trypsin), gastric (pepsin) or other origins (Piard et al., 1992; Tagg et al., 1976). The sensitivity to these enzymes, is similar to that reported by other researchers with bacteriocins produced by pediococci (Bhunia et al., 1988; Daeschel & Klaenhamer, 1985). The sensitivity of the antimicrobial substance, produced by the inhibitory *P. acidilactici* strain (Hansen), to proteases from the digestive tract, makes its use in meat products acceptable since, it will be inactivated in the stomach,

In conclusion, the bacteriocinogenic strain produces an inhibitory compound (bacteriocin), which reaches maximum activity at the beginning of the stationary phase of growth. This antimicrobial substance a proteinaceous, stable over a broad range of pH, resistant to heat and with a bactericidal mode of action. This bacteriocin is a 44-amino acid peptide, which has been purified and sequenced (Nieto-Lozano, Nissen-Meyer, Sletten, Peláez, & Nes, 1992). Further studies will reveal whether the addition of this producer strain or the bacteriocin itself, contributes to the hygienic-sanitary quality of meat and meat products and thus be an alternative to chemical preservatives.

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