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ORIGINAL ARTICLE

Effect of bovicin HC5 on growth and spore germination of *Bacillus cereus* and *Bacillus thuringiensis* isolated from spoiled mango pulp

A.A.T. de Carvalho, E.D. Costa, H.C. Mantovani and M.C.D. Vanetti

Department of Microbiology, Viçosa Federal University, Viçosa, Minas Gerais, Brazil

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Bacillus spp., bacteriocins, food preservation, mango pulp, spore germination.

Correspondence
H.C. Mantovani, Departamento de Microbiologia, Universidade Federal de Viçosa, Viçosa, MG 36570-000, Brazil.
E-mail: hcm6@ufv.br

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Abstract

Aims: To use bovicin HC5 to inhibit predominant bacteria isolated from spoiled mango pulp.
Methods and Results: Bovicin HC5 and nisin were added to brain heart infusion (BHI) medium (40–160 AU ml^{-1}) or mango pulp (100 AU ml^{-1}) and the growth of *Bacillus cereus* and *Bacillus thuringiensis* was monitored. Cultures treated with bovicin HC5 or nisin showed longer lag phases and grew slower in BHI medium. Bovicin HC5 and nisin were bactericidal and showed higher activity in mango pulp at acidic pH values. To determine the effect on spore germination and *D* values, mango pulp containing bovicin HC5 was inoculated with 10^6 and 10^9 spores per ml^{-1}, respectively, from each strain tested. Bovicin HC5 reduced the outgrowth of spores from *B. cereus* and *B. thuringiensis*, but thermal sensitivity was not affected.
Conclusions: Bovicin HC5 was bactericidal against *B. cereus* and *B. thuringiensis* isolated from spoiled mango pulp.
Significance and Impact of the Study: *Bacillus cereus* and *B. thuringiensis* had not been previously isolated from spoiled mango pulp and bovicin HC5 has the potential to inhibit such bacteria in fruit pulps.

Introduction

Mango (*Mangifera indica* L.) is a tropical fruit that can be consumed fresh or in the processed form. In recent years, the consumption of processed fruits and mango pulp has increased in several countries. Mango pulp has a pH of *c.* 4·0 and this factor limits the growth of some spoilage and pathogenic bacteria (Mannan *et al.* 2003). To ensure the microbiological safety of fruit products with low pH, a pasteurization treatment of 80–95°C for 20–30 min or ultra high temperatures are often used to prevent microbial growth (Azizi and Ranganna 1993; Silva *et al.* 1999). The heat treatment can inactivate vegetative cells and the surviving bacterial spores usually do not germinate under these acidic conditions (Blocher and Busta 1983; Leguerinel *et al.* 2005).

However, some bacterial strains belonging to the genera *Bacillus, Clostridium*, (Azizi and Ranganna 1993; Couvert *et al.* 1999) and *Alicyclobacillus* (Silva *et al.* 1999; Vieira *et al.* 2002) have been related to the spoilage of heat-treated acidified products, such as fruit juices and fruit pulps. In order to inactivate these micro-organisms, drastic thermal treatments would be necessary for processing, but these treatments can interfere with the sensory properties of the final product. Due to an increased demand for food products that are less processed, alternative preservation technologies have been sought (Devlieghere *et al.* 2004). Among these, the potential application of bacteriocins has been a matter of extensive work (Yamazaki *et al.* 2000; Cleveland *et al.* 2001; Grande *et al.* 2005).

Bacteriocins are ribosomally-produced, extracellularly released antimicrobial peptides, which can have a relatively narrow spectrum of antibacterial activity (Cleveland *et al.* 2001). The bacteriocins from lactic acid bacteria have shown great potential for industrial application as natural food preservatives (Cleveland *et al.* 2001). Recently, a small pore-forming peptide (bovicin HC5) produced by *Streptococcus bovis* HC5 was isolated and

characterized (Mantovani *et al.* 2002). Bovicin HC5 is stable at high temperatures and low pH (Mantovani *et al.* 2002; Houlihan *et al.* 2004), and recent work indicated that this bacteriocin has activity against some food-borne micro-organisms, including *Listeria monocytogenes* (Mantovani and Russell 2003).

The aim of this study was to isolate and identify *Bacillus* strains from spoilage mango pulp that had been commercially sterilized and to determine the antibacterial activity of bovicin HC5 against vegetative cells and spores from these micro-organisms cultivated in complex media and mango pulp.

Materials and methods

Micro-organisms and growth

Streptococcus bovis HC5 was provided by Dr James B. Russell (Cornell University, Ithaca, NY, USA) and methods of culture were described previously (Mantovani and Russell 2003). The *Bacillus* strains (see Table 1) were isolated from spoiled mango pulp that had been commercially sterilized and were grown aerobically in brain heart infusion (BHI; Acumedia, Baltimore, MD, USA) medium at 30°C. The indicator strain, *Lactococcus lactis* ATCC 19435, was cultivated in MRS broth (De Man *et al.* 1960) at 30°C.

Isolation and identification of bacterial isolates obtained from spoiled mango pulp

Samples of spoiled mango pulp (*c.* 250 g) from three different commercial lots were obtained from a fruit juice industry located at Minas Gerais state, Brazil. Samples were serially diluted (10-fold increments) from 10^{-1} to 10^{-5}. From each dilution, 100 μl aliquots were plated onto PCA media (Oxoid, Basingstoke, Hampshire, UK) and were incubated at 30°C in an anaerobic glove box (ThermoForma, Marietta, OH, USA). Isolated colonies that appeared after 48 h of incubation were picked and transferred to BHI broth and incubated at 30°C for 48 h. Cultures were re-streaked on PCA medium and tested for catalase activity and Gram-staining.

Catalase-positive isolates (isolates LMA09, LMA19 and LMA65) were characterized biochemically by standard methods (Bennett and Belay 2001). The isolates were tested for gelatin hydrolysis, motility, acid production from glucose, indole production, haemolytic activity, nitrate reduction and tyrosine hydrolysis (see Table 1). The isolates were then plated onto mannitol egg yolk polymyxin agar (Difco, Detroit, MI, USA) supplemented with egg yolk and polymyxin B (25 000 U) to determine mannitol fermentation and lecithinase activity. Isolates ($n = 3$) that showed pink colonies (unable to ferment mannitol) and lecithinase activity were presumptively identified as *Bacillus cereus*.

The *Bacillus* isolates were then identified by fatty acids methyl esters (FAME) analysis. Each isolate was plated three times onto tryptic soy agar (Difco) containing 5% (v/v) sheep blood. After the third transfer, colonies were harvested and used for fatty acid extraction. Fatty acid extracts were prepared as described by the manufacturer (Sherlock Microbial Identification System, Newark, DE, USA) and analysed on a HP 5890 Series II Gas chromatograph (Hewlett Packard, Ramsey, MN, USA) with a flame ionization detector, fitted with a Agilent Ultra 2 capillary column (length, 25 m; internal diameter, 0·2 mm; film thickness, 0·33 μm; Agilent, Palo Alto, CA, USA). The carrier gas was hydrogen at a flow rate of 30 ml h^{-1}. The oven temperature was held at 170°C. To differentiate between *B. cereus* and *Bacillus thuringiensis*, the presence of crystalline inclusion bodies was evaluated by light microscopy.

Spore production

Bacillus spp. was grown in nutrient broth containing glass beads (2-mm diameter, 8% w/v) and supplemented with

Table 1 Biochemical characteristics of the *Bacillus* strains isolated from deteriorated mango pulp

Characteristic	*Bacillus thuringiensis**	*Bacillus cereus**	*B. thuringiensis* LMA09	*B. cereus* LMA19	*B. thuringiensis* LMA65
Gram	+	+	+	+	+
Catalase	+	+	+	+	+
Motility	±	±	+	+	+
Nitrate reduction	+	+	+	+	+
Blood haemolysis	+	+	+	+	+
Tyrosine decomposition	+	+	+	+	+
Gelatin hydrolysis	+	+	+	+	+
Indole production	−	−	−	−	−
Acid from glucose	+	+	+	+	+
Crystalline inclusion bodies	+	−	+	−	+

*Typical results of *B. cereus* and *B. thuringiensis* strains as described in the Bergey's Manual of Determinative Bacteriology are also shown.

manganese sulfate (5 mg ml^{-1}) to stimulate sporulation (Collado *et al.* 2005). Cultures were incubated at 35°C and 150 rev min^{-1} in an orbital shaker. After 5 days, examination of the culture under a light microscope indicated that the majority of the bacterial cells had produced spores. The spore suspension was concentrated fourfold (1742 g/15 min/4°C – Sorvall® RT6000D, Newtown, CI, USA), re-suspended in 20 ml of sterile saline solution (0·85% NaCl) and stored at 4°C. Before use, spore suspensions were heated (70°C/20 min) to inactivate vegetative cells and germinated spores were enumerated by plating into BHI media.

Preparation and activity of bovicin HC5 and nisin

Extracts of bovicin HC5 were prepared as described by Mantovani and Russell (2003). Stationary phase *S. bovis* HC5 cultures were heat treated (70°C/30 min) and the cells were harvested by centrifugation (1742 ***g***, 15 min, 4°C). Cell pellets were washed twice in phosphate buffer (5 mmol l^{-1}, pH 6·8) and treated with 100 mmol l^{-1} NaCl (pH 2·0) for 2 h at room temperature. The cell suspensions were then re-centrifuged and the cell-free supernatants were lyophilized (Edwards, Super Modulyo, Livermore, CA, USA). The lyophilized material was re-suspended in phosphate buffer at pH 2·0 and stored at 4°C. The bovicin HC5 extracts were serially diluted in phosphate buffer (5 mmol l^{-1}, pH 2·0) and assayed for antimicrobial activity against *L. lactis* ATCC 19435 by an agar well-diffusion assay (Tagg *et al.* 1976). One arbitrary unit (AU) was defined as the reciprocal of the highest dilution that showed a zone of inhibition with at least 5 mm in diameter.

Nisin solution was prepared by adding 1 g of Chrisin C (1000 U mg^{-1}; CHL Hansen, Hørsholm, Denmark) into 10 ml of phosphate buffer adjusted to pH 2·0 with HCl (1 mol l^{-1}). The suspension was centrifuged (1742 ***g***/10 min) and the pellet was discarded. The bacteriocin activity was determined by the agar well-diffusion assay (see above), using *L. lactis* ATCC 19435 as the indicator organism.

Inhibition of the bacterial isolates in liquid media and mango pulp

Increasing concentrations of bovicin HC5 or nisin (40–160 AU ml^{-1}) were added to BHI broth and bacterial growth was monitored via changes in optical density (OD) at 600 nm in a Spectronic $20D^{+}$ (Thermal Electron, Madison, WI, USA). The tubes were incubated at 30°C and the specific growth rate, duration of lag phase and maximum OD values were determined.

The effect of bovicin HC5 on bacterial isolates growing in mango pulp was also determined. Mango pulp was diluted twofold with distilled water and the pH of the solution was adjusted to values of 4·0, 4·5, 5·0, 5·5, 6·0, 6·5 and 7·0 using NaOH (1 mol l^{-1}) or HCl (1 mol l^{-1}). Tubes containing 5 ml of the diluted mango pulp were added with 100 AU ml^{-1} of either bovicin HC5 or nisin and were inoculated with *c.* 10^{6} CFU ml^{-1} of each *Bacillus* isolate. The tubes were incubated at 30°C/150 rev min^{-1} in an orbital shaker and 100-μl samples were taken at 0, 12 and 24 h of incubation. Each sample was serially diluted (10^{-1} to 10^{-7}) and plated onto BHI media to determine the viable cell number. Control treatments were performed by inoculating the isolates in mango pulp without bacteriocin.

Effect of bovicin HC5 on spore germination

To verify the effect of bovicin HC5 on spore germination, *c.* 10^{7} spores per ml from each *Bacillus* isolate were plated on BHI media containing 80 AU ml^{-1} of bovicin HC5. Effect on spore germination was also assayed in mango pulp with pH 4·0. Tubes containing 7 ml of mango pulp were inoculated with 10^{7} spores per ml. Bovicin HC5 was added to the tubes (80 AU ml^{-1}) and samples (500 μl) were withdrawn and serially diluted at 0, 6, 12, 24, 36, 60, 84 and 122 h of incubation to determine the number of viable spores. At each time point, the samples were heated at 70°C/20 min to activate the spores. The number of germinated spores was determined by the difference between the initial and the final spore count at each time point.

Thermal treatments

Prior to inoculation, the spore suspension was heat activated and the effect of bovicin HC5 on thermal sensitivity of *Bacillus* sp. was assayed. This was performed by adding 80 AU ml^{-1} of bovicin HC5 into stainless steel tubes (AISI 304; 7·4 × 127 mm and 0·25 mm of thickness) containing sterile mango pulp (pH 4·0) inoculated with *c.* 10^{9} spores per ml. The samples were heated at 80°C for 5, 10, 15, 20 and 25 min. The viable spore number of *Bacillus* isolates was determined by plate count in BHI media after incubation at 30°C. D_t values were defined as the time in minutes at a given temperature necessary to decrease the bacterial spores number by 1 $\log_{10}$ cycle.

Statistical methods

All experimental determinations were performed in triplicate and the mean values and standard deviations are reported. When appropriate, the statistical significance of the mean values was assessed by the use of the Tukey test.

Results

Biochemical characterization of the bacterial isolates obtained from spoiled mango pulp

Three catalase-positive bacterial isolates (isolates LMA09, LMA19 and LMA65) were obtained from spoiled mango pulp. These isolates were characterized phenotypically and results indicated that they belonged to the genus *Bacillus*. FAME analysis based on C_{12} to C_{18} cell membrane fatty acids indicated that the three isolates were *B. cereus* or *B. thuringiensis*. Additional tests showed that the isolates were endospore-formers and stained Gram-positive. The isolates also reduced nitrate, were motile and could decompose tyrosine (Table 1). Two isolates (isolates LMA09 and LMA65) also showed crystalline inclusion bodies and were classified as *B. thuringiensis* (Table 1). The other isolate (isolate LMA19) was identified as a strain of *B. cereus*.

Effect of bovicin HC5 on bacterial isolates cultivated in liquid media and mango pulp

When *B. thuringiensis* LMA09, *B. cereus* LMA19 and *B. thuringiensis* LMA65 were inoculated into BHI media and incubated at 30°C, the specific growth rate was 0·60, 0·38 and 0·50 h^{-1} and the final optical density reached values of 0·87, 1·08 and 0·90, respectively (Table 2). If bovicin HC5 was added to the culture medium, the growth rate and the final optical density (OD 600 nm) of all strains were reduced. Bovicin HC5 at 80 AU ml^{-1} increased the length of the lag phase up to 60 h and concentrations of 160 AU ml^{-1} completely inhibited cell growth for at least 144 h (Table 2). When nisin was used, bacterial growth was also inhibited, but the maximal optical density was equal to or higher than control treatments for *B. thuringiensis* LMA09 and LMA65.

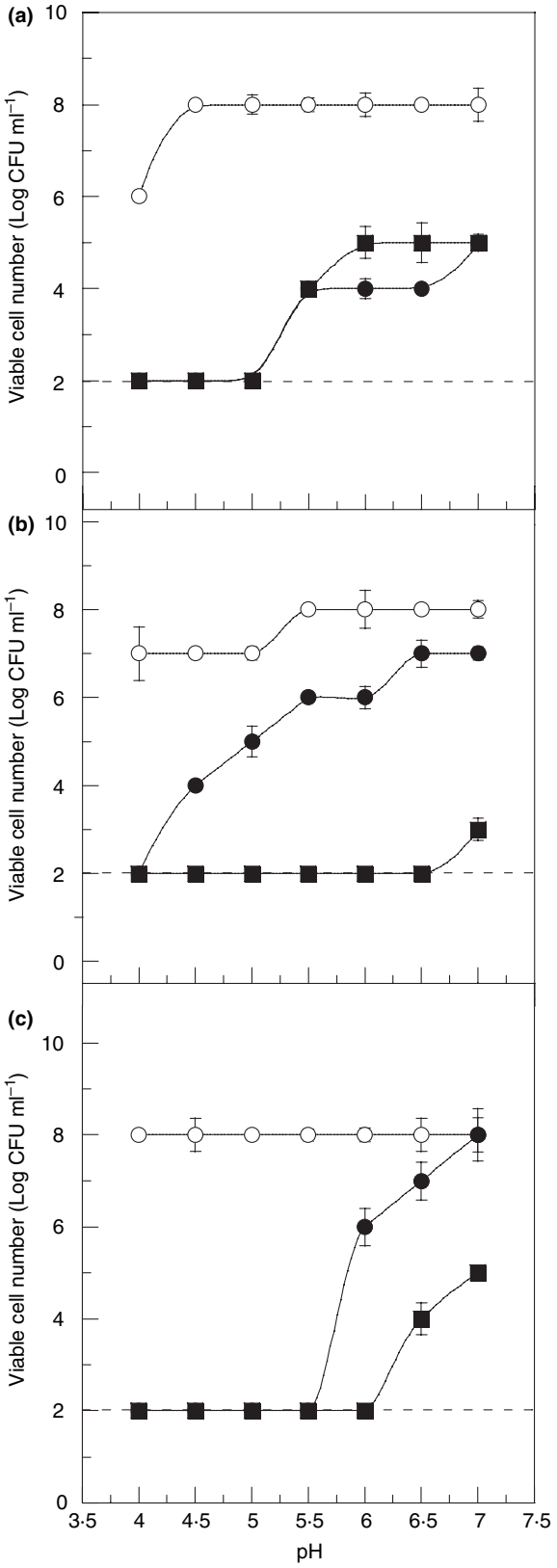
When 10^6 CFU ml^{-1} of *B. thuringiensis* LMA09, *B. cereus* LMA19 and *B. thuringiensis* LMA65 was inoculated into mango pulp with pH ranging from 4·0 to 7·0, an increase in the viable cell number occurred at pH values ≥4·5, regardless of the *Bacillus* strain used (Fig. 1). Only *B. thuringiensis* LMA09 did not increase its viable cell number in mango pulp at pH 4·0 after 12 h of incubation (Fig. 1a).

However, if bovicin HC5 (100 AU ml^{-1}) was added to the mango pulp, the growth of *B. thuringiensis* LMA09, *B. cereus* LMA19 and *B. thuringiensis* LMA65 was bellow the detection level at pH values ≤5·0, 4·0 and 5·5, respectively (Fig. 1). The viable cell number increased at higher pH values, but viability was at least 3 log cycles lower than control treatments for *B. thuringiensis* LMA09 and *B. cereus* LMA19. No inhibition by bovicin HC5 was observed after 12 h for *B. thuringiensis* LMA65 inoculated in mango pulp with pH adjusted to 7·0. If the incubation period was increased to 24 h, the viability of all strains decreased in the presence of the bacteriocin, regardless of the media pH (data not shown). Nisin also decreased the viability of *Bacillus* isolates in mango pulp, and the bactericidal activity was more pronounced under acidic conditions (Fig. 1). These results indicated that both nisin and bovicin HC5 were bactericidal against *Bacillus* strains isolated from spoiled mango pulp and the inhibitory activity was more pronounced at acidic pH.

The bactericidal effect of bovicin HC5 and nisin (100 AU ml^{-1}) was confirmed when the *Bacillus* strains were inoculated into mango pulp at pH 4·0 and the viable cell count was determined at different time intervals (Fig. 2). At this pH value, the viable cell number decreased rapidly in the presence of bovicin HC5 or nisin,

Table 2 Effect of different concentrations of bovicin HC5 and nisin on growth of *Bacillus* strains inoculated into brain heart infusion media

Micro-organism	Bacteriocin concentration (AU ml^{-1})	Specific growth rate (h^{-1})		Lag-phase duration (h)		Maximal optical density (600 nm)	
		Bovicin HC5	Nisin	Bovicin HC5	Nisin	Bovicin HC5	Nisin
Bacillus thuringiensis LMA 09	0	0·60	0·60	2·00	2·00	0·87	0·87
	40	0·42	0·37	>24	8·00	0·64	0·94
	80	0·33	0·23	>60	10·0	0·57	0·87
	160	–	0·17	>144	10·0	–	0·92
Bacillus cereus LMA 19	0	0·38	0·38	2·00	2·00	1·08	1·05
	40	0·21	0·24	10·0	12·0	0·72	0·825
	80	0·13	0·15	18·0	>12·0	0·67	0·88
	160	–	0·04	>144	24·0	–	0·88
B. thuringiensis LMA 65	0	0·50	0·50	2·00	2·00	0·90	0·89
	40	0·21	0·28	>12	7·00	0·73	0·91
	80	–	0·11	>144	10·0	0·66	0·94
	160	–	0·08	>144	12·0	–	0·88



and the viability was 7 log units lower than the untreated controls after 24 h of incubation at 30°C. This result indicated that both bovicin HC5 and nisin were effective for preventing the growth of *B. cereus* and *B. thuringiensis* in mango pulp.

Effect of bovicin HC5 on spore germination

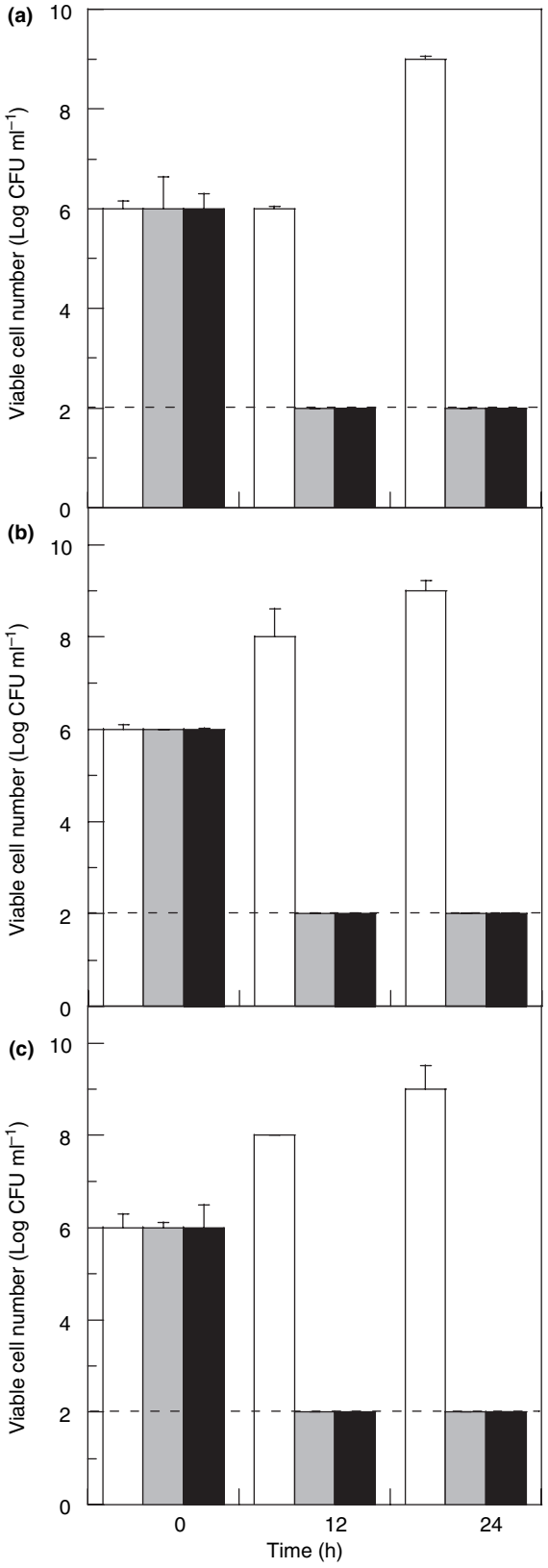
When 10^7 spores per ml from *B. thuringiensis* LMA09, *B. cereus* LMA19 and *B. thuringiensis* LMA65 were plated into BHI media containing bovicin HC5 (80 AU ml^{-1}), colony formation could not be detected even after 48 h of incubation (data not shown). If spores (10^7 ml^{-1}) were inoculated into mango pulp (pH 4·0) and heat treated, vegetative cells started to develop as early as 6 h after the beginning of the incubation (Fig. 3). The rapid decrease in spore number observed after *c.* 36 h of incubation indicated that most of the bacterial spores could germinate in mango pulp at pH 4·0 (Fig. 3). Only 0·01%, 0·1% and 0·01% of the initial number of viable spores from *B. thuringiensis* LMA09, *B. cereus* LMA19 and *B. thuringiensis* LMA65, respectively, could be counted after 122 h of incubation (Fig. 3).

However, if bovicin HC5 (80 AU ml^{-1}) was added to the mango pulp (pH 4·0) inoculated with 10^7 spores per ml from *B. thuringiensis* LMA09, *B. cereus* LMA19 and *B. thuringiensis* LMA65, *c.* 10% of the spores did not develop vegetative cells for at least 84 h of incubation. Bovicin HC5 reduced spore germination for all the strains tested and the number of non-germinated spores after 122 h of incubation was at least 100-fold greater than control treatments. These results suggest that bovicin HC5 can reduce the germination of heat-treated spores from *B. thuringiensis* LMA09, *B. cereus* LMA19 and *B. thuringiensis* LMA65 in mango pulp.

Effect of bovicin HC5 on thermal sensitivity of spores inoculated into mango pulp

Decimal reduction times (*D* values) for spores of *B. thuringiensis* LMA09, *B. cereus* LMA19 and *B. thuringiensis* LMA65 were calculated from the reciprocal of the slope obtained from spore survivor curves. The *Bacillus* strains

Figure 1 Viable cell number of *Bacillus thuringiensis* LMA09 (a), *Bacillus cereus* LMA19 (b) and *B. thuringiensis* LMA65 (c) inoculated (10^6 CFU ml^{-1}) into mango pulp at different pH values. Bovicin HC5 (closed circles) and nisin (closed squares) were added to the mango pulp at 100 AU ml^{-1} at the beginning of the experiment. After 12 h of incubation at 30°C, the viable cell number was determined. Control treatments without the bacteriocins are also shown (open circles). The bars indicate the standard deviations of the mean. The dotted line shows the detection limit of the enumeration.



were inoculated in mango pulp at pH 4·0 and exposed to thermal treatment at 80°C. *Bacillus* strains showed similar $D_{80°C}$ and bovicin HC5 did not significantly affect ($P < 0·05$) the decimal reduction time of the *Bacillus* strains (Table 3). However, nisin significantly ($P < 0·05$) increased the $D_{80°C}$ of *B. cereus* LMA19 and *B. thuringiensis* LMA65. The addition of nisin to mango pulp increased the $D_{80°C}$ of spores from *B. cereus* LMA19 and *B. thuringiensis* LMA65 by *c.* 28·5% and 26·2%, respectively (Table 3).

Discussion

Heat treatments are frequently used to eliminate thermoresistant spore-forming bacteria, but the use of high temperatures in food products can cause protein denaturation and loss of vitamins and volatile compounds important to flavour (Lado and Yousef 2002). Bacteriocins from lactic acid bacteria have been proposed as an alternative to traditional processing methods because they usually do not interfere with desirable properties of food products (Abee *et al.* 1995; Stiles 1996; Cleveland *et al.* 2001). These bacteriocins can inhibit spoilage bacteria in foods and prevent the growth of food-borne pathogens (Abee *et al.* 1995; Stiles 1996).

Several studies have demonstrated the potential use of antimicrobial peptides for food processing, but their application is still limited (Komitopoulou *et al.* 1999; Yamazaki *et al.* 2000; Grande *et al.* 2005). Grande *et al.* (2006) showed the inhibitory effect of enterocin AS-48, produced by *Enterococcus faecalis* A-48-32, against vegetative cells and endospores from *Alicyclobacillus acidoterrestris* inoculated in fruit juices. Yamazaki *et al.* (2000) also noted that nisin decreased the thermal resistance of spores from *A. acidoterrestris* in different fruit juices. As *E. faecalis* and *L. lactis* have several nutritional requirements, the production of bacteriocins using these lactic acid bacteria is often expensive.

Bovicin HC5 is a small antimicrobial peptide that has been recently characterized (Mantovani *et al.* 2002). The inhibitory effect of bovicin HC5 against *L. monocytogenes* (Mantovani and Russell 2003) and *Clostridium sporogenes* (Flythe and Russell, 2004) has been previously demonstra-

Figure 2 Bactericidal effect of bovicin HC5 and nisin against *Bacillus thuringiensis* LMA09 (a), *Bacillus cereus* LMA19 (b) and *B. thuringiensis* LMA65 (c) inoculated into mango pulp at pH 4·0. The initial inoculum was *c.* 10^6 CFU ml^{-1} for each strain. At 0, 12 and 24 h of incubation at 30°C, samples were withdrawn and the viable cell number was determined. Bovicin HC5 (grey bars) and nisin (black bars) were added at 100 AU ml^{-1}. Control treatments are also shown (open bars). The bars indicate the standard deviations of the mean. The dotted line shows the detection limit of the enumeration.

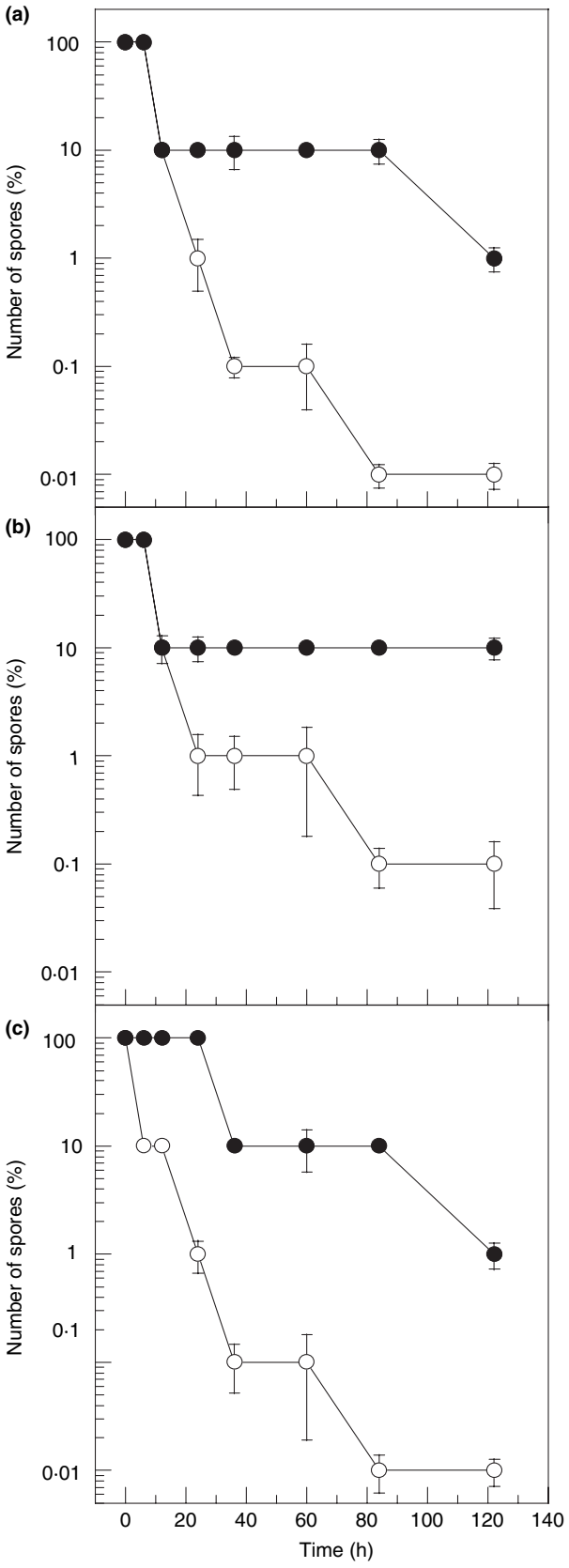


Table 3 Decimal reduction time ($D_{80°C}$) of bacterial spores inoculated into mango pulp (pH 4·0) without bacteriocin (control) and in the presence of bovicin HC5 (80 AU ml^{-1}) or nisin (80 AU ml^{-1})

	$D_{80°C}$ (min)*		
Isolate	Control	Bovicin HC5	Nisin
Bacillus thuringiensis LMA 09	5·69^{a} ± 0·18	5·46^{a} ± 0·62	7·18^{b} ± 0·84
Bacillus cereus LMA 19	5·83^{a} ± 0·05	5·69^{a} ± 0·73	7·49^{b} ± 0·91
B. thuringiensis LMA 65	5·63^{a} ± 0·63	6·32^{a} ± 0·55	6·09^{a} ± 0·90

*Results represent the average of three replicates.
$^{a,\ b}$Averages followed by the same letter do not differ from each other by the Tukey test at a significance level of 5%.

ted. As *S. bovis* is a lactic acid bacterium that shows few nutritional requirements and uses ammonia as its sole nitrogen source, bovicin HC5 could be produced at low cost by growing this bacterium in cheap and abundant substrates such as cheese whey or sugar cane molasses. Our preliminary experiments indicated that these substrates are effectively used by *S. bovis* HC5.

In this study, we tested the effect of bovicin HC5 against *Bacillus* strains isolated from spoiled mango pulp. Spores of these micro-organisms are commonly found in soil and can contaminate foods. As they can form endospores, these bacteria can pass through and survive several processing methods used in the food industry, such as heat treatment (Iurlina *et al.* 2006). These endospores can germinate after the heat treatment and resume vegetative growth, which can either lead to deterioration of the food product or result in the production of toxins that are harmful to individuals.

Generally, the low pH values (≤4·5) of acidic food products can inhibit the germination of endospores from thermoresistant organisms, such as *Clostridium botulinum* and *B. cereus* (Blocher and Busta 1983; Azizi and Ranganna 1993). However, spores from *A. acidoterresrtris* (Yamazaki *et al.* 1996), *Bacillus coagulans* (Azizi and Ranganna 1993) and *Bacilllus licheniformis* (Palop *et al.* 1996) have been able to germinate even under these acidic conditions. In this study, the *Bacillus* strains used were able to germinate in mango pulp with pH value of 4·0, and it should be noted that these strains were isola-

Figure 3 Number of viable spores of *Bacillus thuringiensis* LMA09 (a), *Bacillus cereus* LMA19 (b) and *B. thuringiensis* LMA65 (c) inoculated in mango pulp (pH 4·0) at 10^7 spores per ml in the presence (closed circles) or absence (open circles) of bovicin HC5 (80 AU ml^{-1}). The tubes were incubated at 30°C and agitated at 150 rev min^{-1}. Samples were taken at different time intervals, heat treated (70°C/20 min) to kill vegetative cells, and the number of remaining viable spores was determined.

ted from acidified mango pulp that had been heat treated.

The conversion of bacterial spores into vegetative cells involves four major stages: activation, germination, outgrowth and growth (Setlow and Johnson 2001; Moir 2003; Setlow 2003). If one of these series of events is interrupted, spores cannot germinate and will not represent a major risk to the health of consumers. Bovicin HC5 inhibited spore germination of *Bacillus* strains in synthetic media and reduced the germination in mango pulp, suggesting that this bacteriocin could reduce the growth of spoilage and pathogenic bacteria found in fruit pulp. Considering that bovicin HC5 was bactericidal against vegetative cells of *Bacillus* spp., even if the spores germinate, outgrowth and growth of the bacterial spores could be inhibited by this bacteriocin. However, additional experiments will be needed to verify the stability of bovicin HC5 over the shelf-life of the mango pulp.

Isolates obtained from spoiled mango pulp in this study belonged to the species of *B. thuringiensis* and *B. cereus*. These bacteria can not only deteriorate food products but also produce toxins. The relevance of *B. thuringiensis* as a food-borne pathogen is still questionable, but the lack of reports relating *B. thuringiensis* to foods might be due to the difficulty to distinguish this bacterium from *B. cereus* (Rosenquist *et al.* 2005). To our knowledge, this is the first report to describe the isolation of *Bacillus thuringiensis* and *B. cereus* from spoiled mango pulp. Bovicin HC5 inhibited the growth of both *Bacillus* species, especially at low pH values. As the effect was bactericidal, bovicin HC5 seems effective preventing the growth of *Bacillus* strains in acidified, heat-treated foods. Previous studies indicated that bovicin HC5 has a pronounced activity at acidic conditions (Houlihan *et al.* 2004).

In this study, bovicin HC5 was effective against strains of *B. cereus* and *B. thuringiensis*, even when the initial inoculum was as high as 10^6 CFU ml^{-1}. After 24 h of incubation in the presence of the bacteriocin, $<10^2$ CFU ml^{-1} could be counted by plating methods. The infectious dose for *B. cereus* in foods ranges from 10^3 to 10^{10} CFU g^{-1} of food (Notermans and Batt 1998). Rosenquist *et al.* (2005) isolated both *B. cereus* and *B. thuringiensis* from foods and enumeration experiments indicated that the samples usually had $<10^3$ CFU g^{-1} of food. The counts were above10^4 CFU g^{-1} in only 0·5% of the samples. These results indicate that bovicin HC5 could be effective against *Bacillus* strains, even if the contamination level was higher than its infectious dose, maintaining the microbiological safety of the product.

Some studies have demonstrated that bacteriocins such as nisin and enterocin AS-48 reduce the thermal resistance of spores from *Clostridium*, *Alicyclobacillus* and *Bacillus* (Mazzotta and Montville 1999; Yamazaki *et al.* 2000; Grande *et al.* 2006). Mazzotta and Montville (1999) reported that nisin induced the germination of spores from *C. botulinum* and increased their sensitivity to heat treatments. The authors suggested that once the spores started outgrowth, nisin induced pore formation in the cell membrane and prevented the cells to resume the vegetative growth (Mazzotta and Montville 1999).

Bovicin HC5 did not affect the thermal resistance of the *Bacillus* strains used in this study. This result could be due to a difference in the mechanism of action between nisin and bovicin HC5, the latter not being stimulatory to spore germination. Due to the presence of several layers protecting the spore core, peptides and other small substances are physically impeded from acting directly on the spore. However, if added to the mango pulp, bovicin HC5 could remain stable during the heat treatment, and could reduce the germination of spores that were not killed during processing. When we added nisin to the mango pulp, there was a significant increase in the decimal reduction time for spores from *B. thuringiensis* LMA09 and *B. cereus* LMA19. These results are contrary to the observations of Mazzotta and Montville (1999) and Yamazaki *et al.* (2000), but it should be noted that we have used a different food matrix in our experiments.

Our results indicate that bovicin HC5 and nisin were effective against *B. cereus* and *B. thuringiensis* isolated from spoiled mango pulp. Nisin is a bacteriocin that has been approved by the Food and Drug Administration to be used in food products, in concentrations ranging from *c.* 5·5 to 12·5 mg kg of food (Siragusa *et al.* 1999; Cleveland *et al.* 2001). For a bacteriocin to be used in the food industry, several factors must be considered. The peptide must be isolated, sequenced and the gene encoding the bacteriocin should be identified. In addition, its mode of action, efficacy, spectrum of activity and allergenic and toxicological effects are of special interest (Cleveland *et al.* 2001). Desirable characteristics also include stability to high temperatures and activity against food-borne pathogens such as *L. monocytogenes* and *C. botulinum* (Holzapfel *et al.* 1995; Rodríguez *et al.* 2002). Although bovicin HC5 has some of the characteristics described above, more studies are needed to see how this peptide affects other food-borne pathogens and if there is development of resistance by susceptible bacteria.

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