

ORIGINAL ARTICLE

Bovicin HC5 reduces thermal resistance of *Alicyclobacillus acidoterrestris* in acidic mango pulp

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Abstract**Aims:** To test the effect of bovicin HC5 against vegetative cells and endospores of *Alicyclobacillus acidoterrestris* DSMZ 2498 in synthetic media and in acidic mango pulp.**Methods and Results:** *Alicyclobacillus acidoterrestris* was grown in synthetic medium at 40°C and pH 4.0. The effect on vegetative cells was assayed by adding bovicin HC5 to synthetic medium (40–160 AU ml⁻¹) or to mango pulp (100 AU ml⁻¹) at various pH values and determining the effect on growth (OD_{600nm}) and viable cell number, respectively. The effect of bovicin HC5 on spore germination and thermal sensitivity of *A. acidoterrestris* was tested in mango pulp (pH 4.0) containing 80 AU ml⁻¹ of bovicin HC5. Bovicin HC5 was bactericidal against vegetative cells of *A. acidoterrestris* at different pH values and showed sporicidal activity against endospores of this bacterium. When spores of *A. acidoterrestris* were heat treated in the presence of bovicin HC5, D-values decreased 77% to 95% compared to untreated controls at temperatures ranging from 80 to 95°C.**Conclusion:** Bovicin HC5 was bactericidal and sporicidal against *A. acidoterrestris* DSMZ 2498.**Significance and Impact of the Study:** These results indicated that bovicin HC5 has potential to prevent spoilage of acidic fruit juices by thermocidophilic spore-forming bacteria.**Introduction**

Alicyclobacillus acidoterrestris is a thermoacidophilic, aerobic spore-forming bacterium, that has been proposed as a model organism for heat resistance in bacteria (Yamazaki *et al.* 1996; Walls and Chuyate 1998). *Alicyclobacillus acidoterrestris* is frequently found in soil and can contaminate the exterior of fruits and processing lines (Deinhard *et al.* 1987; Gouws *et al.* 2005; Chen *et al.* 2006; Siegmund and Pollinger-Zierler 2007). *Alicyclobacillus acidoterrestris* was first isolated from spoiled apple juice (Cerny *et al.* 1984), but several reports indicate that this bacterium can deteriorate other acidic fruit juices (Yamazaki *et al.* 2000; Vieira *et al.* 2002; Gouws *et al.* 2005; Grande *et al.* 2006).

Mango (*Mangifera indica* L.) is an economically important crop fruit worldwide that is susceptible to contamination with bacterial endospores during harvest and

handling of the fruits. Mango pulp is often processed using heat treatments and acidification (pH 4.0) (Azizi and Ranganna 1993), and our previous work indicated that spore-forming bacteria could deteriorate canned acidified mango pulp (de Carvalho *et al.* 2007a, 2007b). Gouws *et al.* (2005) recently isolated and identified *A. acidodarius* from UHT mango products and concluded that the preventative measures of low pH, pasteurization of mango juice and the subsequent use of aseptic packaging were not sufficient to prevent the outgrowth of *Alicyclobacillus*.

To satisfy consumer demands for fresh-tasting and better-preserved juices and drinks, alternative methods to control thermoresistant bacteria in fruit products are warranted. This need is especially important if less intense processing methods are to be implemented (Grande *et al.* 2006). Some authors have suggested the use of bacteriocins as an additional hurdle in food processing (Yamazaki

et al. 2000; Grande *et al.* 2006). Bacteriocins are ribosomally produced, extracellularly released antimicrobial peptides that can have a relatively narrow spectrum of antibacterial activity (Cleveland *et al.* 2001).

Bovicin HC5, a bacteriocin from *Streptococcus bovis* HC5, was able to inhibit several Gram-positive bacteria, including spoilage and pathogenic bacteria commonly found in foods (Mantovani and Russell 2003; Flythe and Russell 2004; de Carvalho *et al.* 2007a). Bovicin HC5 is stable to high temperatures and low pH (Mantovani *et al.* 2002; Houlihan *et al.* 2004) and these characteristics are suitable to control thermoacidophilic spoilage bacteria in acidic drinks that have been heat-treated. This work describes the antimicrobial activity of bovicin HC5 on growth, spore germination and thermoresistance of *A. acidoterrestris* in mango pulp.

Materials and methods

Micro-organisms and growth conditions

Alicyclobacillus acidoterrestris DSMZ 2498 was grown at 40°C in *A. acidoterrestris* medium (AAM), described by Yamazaki *et al.* (2000). The medium was composed of (per litre): yeast extract, 1.0 g, (NH₄)₂SO₄, 0.2 g, MgSO₄·7H₂O, 0.5 g, CaCl₂·2H₂O, 0.25 g, KH₂PO₄, 1.0 g, glucose, 1.0 g, and distilled water (1000 ml, pH 4.0). Solid medium for enumeration (AAM agar) was prepared mixing twofold concentrated AAM broth (500 ml) with a 4% (w/v) agar stock solution (500 ml) prepared with distilled water. Solutions were sterilized separately (121°C/15 min), mixed and dispensed in sterile Petri plates while the medium was still hot. Isolation of *S. bovis* HC5 and methods of culture were previously described (Mantovani and Russell 2003). The indicator strain, *Lactococcus lactis* ATCC 19435, was routinely cultivated in MRS broth (De Man *et al.* 1960) at 30°C.

Preparation and activity of bovicin HC5

Extracts of bovicin HC5 were prepared as described by Mantovani and Russell (2003). Stationary-phase *S. bovis* HC5 were harvested by centrifugation and the cells were washed in sodium phosphate buffer (5 mmol l⁻¹, pH 6.7). The cell pellet was re-suspended in acidic NaCl (100 mmol l⁻¹, pH 2.0, 2 h, room temperature), the suspensions were centrifuged to remove cells and the cell-free supernatant was lyophilized (Edwards; Super Modulyo, Livermore, CA, USA). The lyophilized material was suspended in sterile phosphate buffer (5 mmol l⁻¹) with pH adjusted to 2.0 using HCl (1 mol l⁻¹). The activity of bovicin HC5 was determined as described by Hoover and Harlander (1993). Bovicin HC5 was serially

diluted into phosphate buffer (5 mmol l⁻¹, pH 2.0) and tested for antimicrobial activity against *L. lactis* ATCC 19435. One arbitrary unit (AU) was defined as the reciprocal of the highest dilution (2ⁿ) that showed a zone of inhibition with at least 5 mm in diameter.

Spore production

Spores from *A. acidoterrestris* DSMZ 2498 were obtained in the sporulation broth described by Yamazaki *et al.* (2000), containing (per litre): yeast extract, 2.0 g, (NH₄)₂SO₄, 0.4 g, MgSO₄·7H₂O, 1.0 g, CaCl₂·2H₂O, 0.5 g, KH₂PO₄, 1.2 g, MnSO₄·4H₂O, 0.5 g and glucose, 2.0 g (final pH adjusted to 4.0). The culture was incubated at 40 °C for 5 days and examination of the culture in a light microscope indicated that the majority of the bacterial cells had spores. The spore suspension was washed four times (1742 g/15 min/4°C – Sorvall® RT6000D, Newtown, CT, USA) in sterile saline solution (0.85% NaCl), resuspended in 10 ml of the same solution and stored at 4°C. Before use, spore suspensions were heated (80°C/10 min) to inactivate vegetative cells and spores were enumerated by plating into AAM agar.

Effect of bovicin HC5 against *A. acidoterrestris* DSMZ 2498 in AAM broth and in mango pulp

To verify the effect of bovicin HC5 on growth of *A. acidoterrestris* DSMZ 2498, AAM broth was added with increasing concentrations of bovicin HC5 (40–160 AU ml⁻¹) and inoculated with *c.* 10⁶ CFU ml⁻¹ of the micro-organism. Bacterial growth was monitored via changes in optical density (OD) at 600 nm in a Spectronic 20D⁺ (Thermal Electron, Madison, WI, USA) and the tubes (18 × 150 mm) were incubated at 40°C. The specific growth rate, lag phase duration and maximum OD values were 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 bovicin HC5 and were inoculated with *c.* 10⁴ CFU ml⁻¹ of *A. acidoterrestris* DSMZ 2498. The tubes were incubated at 40°C and 100 µl samples were taken at 0, 12 and 24 h of incubation. Each sample was serially diluted (10⁻¹–10⁻⁷) and plated into AAM agar to determine the viable cell number. Controls without bacteriocin were also made.

Minimal inhibitory concentrations (MIC) of bovicin HC5 were determined by an agar spot assay. Bovicin HC5 (2560 AU ml⁻¹, stock solution) was serially diluted (two-fold increments) and 5 µl samples from each dilution were spotted onto a lawn of *A. acidoterrestris* cells

(c. 10^4 CFU ml⁻¹) previously inoculated into AAM agar. The plates were incubated at 40°C for 48 h and the MIC (AU ml⁻¹) was determined from the highest dilution that still inhibited growth (halo of inhibition ≥ 5 mm).

Effect of bovicin HC5 on germination of spores inoculated in mango pulp

The effect of bovicin HC5 on spore germination was tested by inoculating c. 10^6 spores ml⁻¹ from *A. acidoterrestris* DSMZ 2498 into tubes containing 7 ml of mango pulp (pH 4.0). Bovicin HC5 was added to the tubes at the concentration of 80 AU ml⁻¹ and samples (500 μ l) were withdrawn and serially diluted at 0, 12, 24, 36, 48, 72 and 168 h of incubation to determine the viable spore number. In each time point, the samples were heated at 80°C/10 min to inactivate vegetative cells. The number of germinated spores was determined by the difference between the initial and the final spore count at each time point. Controls without bacteriocin were also made.

Thermal treatments

The effect of bovicin HC5 on thermal sensitivity of *A. acidoterrestris* DSMZ 2498 was assayed by adding 80 AU ml⁻¹ of bovicin HC5 into stainless steel tubes (AISI 304 – 7.4 \times 127 mm and 25 mm of thickness) containing sterile mango pulp (pH 4.0) inoculated with c. 10^6 spores ml⁻¹. Thermal treatments were carried out at 80, 85, 90 and 95°C at several times. After being heated, the tubes were cooled in an ice water bath as quickly as possible and the viable spore number of *A. acidoterrestris* DSMZ 2498 was determined by plate count in AAM agar after incubation at 40°C. *D*-values for *A. acidoterrestris* DSMZ 2498 were calculated from the slope of the regression line obtained from the linear portion of the survival curve. The survival curve was obtained from the plot of viable spore number (log₁₀ values) vs heating time (minutes). *D*-value was defined as the time in minutes at a given temperature necessary to decrease one log₁₀ cycle of the bacterial spore number. *Z*-value was defined as the variation in temperature that reduced 10 times the *D*-value (reduction in one log cycle). *Z*-values were determined from the regression lines obtained by plotting log₁₀ *D*-values vs the corresponding temperatures.

Results

Inhibition of *A. acidoterrestris* growth in AAM broth and in mango pulp

Cultures of *A. acidoterrestris* DSMZ 2498 grew in AAM broth with a growth rate of 0.08 h⁻¹ and approached a

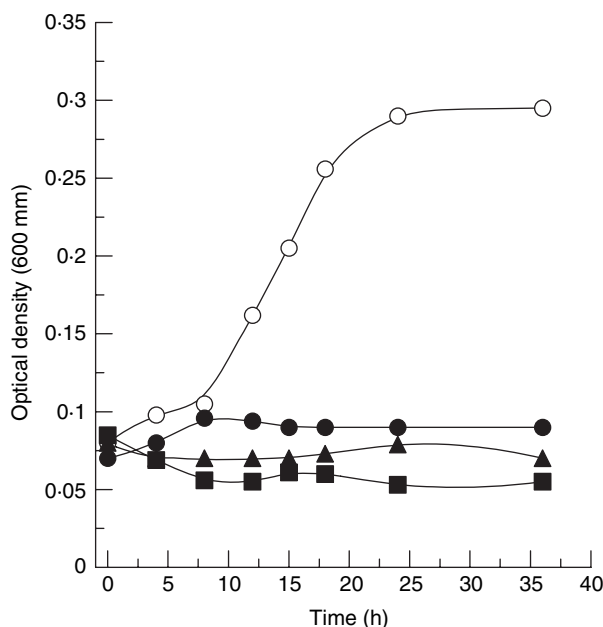


Figure 1 Effect of bovicin HC5 on growth of *Alicyclobacillus acidoterrestris* DSMZ 2498 in AAM broth at 40°C. Approximately 10^6 CFU ml⁻¹ of stationary-phase cultures were inoculated in AAM broth with bovicin HC5 in the concentrations of 40 (closed circles), 80 (closed triangles) and 160 AU ml⁻¹ (closed squares). Growth of controls without bacteriocin is also shown (open circles).

maximum OD of 0.3 after 24 h of incubation (Fig. 1). If bovicin HC5 (40–160 AU ml⁻¹) was added to AAM broth, growth was completely inhibited (Fig. 1), even if the cultures were incubated at 40°C for as long as 15 days (data not shown). When aliquots (100 μ l) were taken from the cultures treated with bovicin HC5 (40 AU ml⁻¹) and plated into AAM agar, colony formation was not observed, even after 48 h of incubation at 40°C (results not shown). When the MIC of bovicin HC5 was determined for vegetative cells and spores of *A. acidoterrestris* values of 5 and 2.5 AU ml⁻¹ were obtained, respectively.

Alicyclobacillus acidoterrestris (10^5 CFU ml⁻¹) that was inoculated into mango pulp with pH ranging from 4.0–7.0, increased viable cell number in one log cycle after 12 h of incubation at 40°C, regardless of the mango pulp pH (Fig. 2). After 24 h of incubation, *A. acidoterrestris* reached a population of 10^7 CFU ml⁻¹ (results not shown). If bovicin HC5 (100 AU ml⁻¹) was added to the mango pulp, the viable cell number of *A. acidoterrestris* was below the detection level in the range of pH 4.5 to 7.0 (Fig. 2). When the mango pulp pH was 4.0, treated cultures showed 100-fold reduction in viable cell number compared to controls. Even greater reduction in viability was observed at longer incubation periods (data not shown). These results indicated that bovicin HC5 was bactericidal against *A. acidoterrestris*.

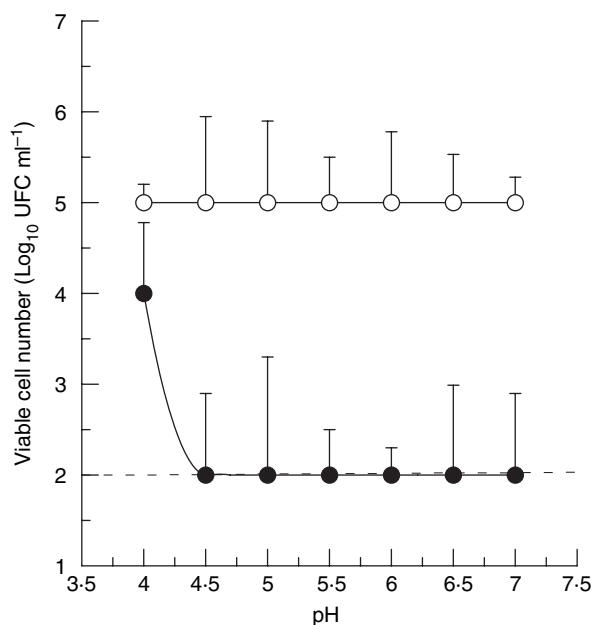


Figure 2 Viable cell number of *Alicyclobacillus acidoterrestris* DSMZ 2498 inoculated into mango pulp (c. 10^4 CFU ml^{-1}) at different pH values. Bovicin HC5 (closed circles) was added to the mango pulp at 100 AU ml^{-1} at the beginning of the experiment. After 12 h of incubation at 40°C , the viable cell number was determined. Control treatment without the bacteriocin is also shown (open circles). The bars indicate the standard deviations of the mean. The dotted line shows the detection limit of the enumeration.

Effect of bovicin HC5 on spore germination in mango pulp

When 10^6 spores ml^{-1} of *A. acidoterrestris* DSMZ 2498 were inoculated in mango pulp, most of the spores germinated after 36 h of incubation (Fig. 3). When bovicin HC5 was added in mango pulp (80 AU ml^{-1}), no viable spores could be detected in mango pulp after 36 h of incubation (Fig. 3). The same result was obtained if aliquots were taken from treated tubes and plated without the heat treatment to eliminate vegetative cells. Based on these results, it appeared that bovicin HC5 was sporicidal against spores of *A. acidoterrestris* DSMZ 2498 inoculated into mango pulp.

The sporicidal activity of bovicin HC5 was confirmed when c. 10^6 spores ml^{-1} were resuspended in phosphate buffer (pH 6.5) added with 20 AU ml^{-1} of bovicin HC5. A decrease of four log cycles in viable spores was observed after 12 h of incubation. If the spore suspension was incubated for 24 h, the viable spore number was <10 per millilitre (Fig. 4). Control treatments without bacteriocin maintained spore viability for at least 24 h of incubation (Fig. 4). These results supported the idea that bovicin HC5 could kill spores of *A. acidoterrestris* under the conditions used in our studies.

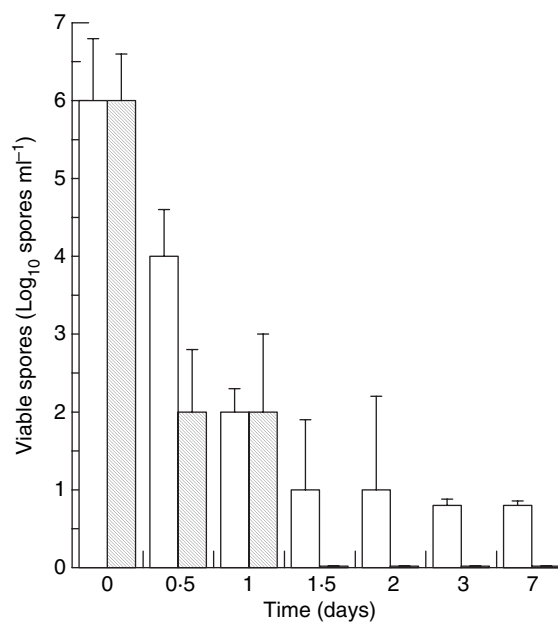


Figure 3 Effect of bovicin HC5 on spore germination of *Alicyclobacillus acidoterrestris* DSMZ 2498 in mango pulp. Approximately 10^6 spores ml^{-1} were inoculated into mango pulp added with bovicin HC5 at 100 AU ml^{-1} (grey bars) and viable spore number over time was determined. Control treatment without bacteriocin is also shown (white bars).

Effect of bovicin HC5 on thermal sensitivity of *A. acidoterrestris*

When spores from *A. acidoterrestris* were heat treated in mango pulp (pH 4.0), the time required to reduce one log cycle in viable spores counts varied from 40 min (80°C) to 8.33 min (95°C) (Table 1). If spores were heated in the presence of bovicin HC5 (80 AU ml^{-1}), the heat resistance of *A. acidoterrestris* DSMZ 2498 decreased at all temperatures tested. At 80°C , treated spores showed a *D*-value 77% lower than untreated controls. At higher temperatures, a more pronounced effect of bovicin HC5 was observed on thermal resistance of *A. acidoterrestris* spores. The *D*-value of spores decreased about 80%, 90% and 95% at temperatures of 85, 90 and 95°C respectively (Table 1).

The *Z*-value for spores of *A. acidoterrestris* inoculated into mango pulp (pH 4.0) was equal to 21.27°C , but bovicin HC5 (80 AU ml^{-1}) reduced by 48.7% the temperature needed to decrease 10-fold the *D*-values (Table 1).

Discussion

Alicyclobacillus acidoterrestris can grow in several fruit juices and has been recognized as an important spoilage bacterium in pasteurized acidic drinks (Cerny et al. 1984;

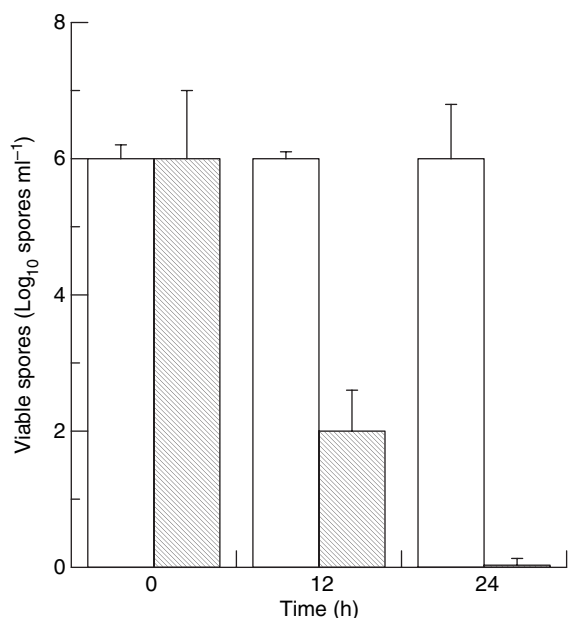


Figure 4 Sporicidal effect of bovicin HC5 against *Alicyclobacillus acidoterrestris* DSMZ 2498 in phosphate buffer. Approximately 10^6 spores ml^{-1} were inoculated into phosphate buffer (5 mmol l^{-1} , pH 6.5) added with 20 AU ml^{-1} of bovicin HC5 (grey bars). At times 0, 12 and 24 h of incubation at 40°C, samples were withdrawn and the viable spore number was determined. Control treatment without bacteriocin was also made (white bars). The bars indicate the standard deviation of the mean.

Table 1 Thermal sensitivity of spores from *Alicyclobacillus acidoterrestris* DSMZ 2498 that were heat-treated in mango pulp at pH 4.0

Treatment	D-values (minutes)				Z-value (°C)
	80°C	85°C	90°C	95°C	
Control	40 ± 1.5	25 ± 0.1	11.66 ± 1.8	8.33 ± 2.0	21.27
Bovicin HC5	9.2 ± 0.1	5 ± 0.1	1.16 ± 1.3	0.36 ± 0.8	10.36

Splittstoesser *et al.* 1994; Chen *et al.* 2006; Siegmund and Pollinger-Zierler 2007). In recent years, *A. acidoterrestris* has been suggested as the main target for pasteurization treatments applied to acidic canned products (Chen *et al.* 2006; Bahçeci and Acar 2007). The heat treatment generally applied in acidic fruit products is not able to eliminate spores from this micro-organism and act as a stimulus to germination, which follows outgrowth of the organism (Gouws *et al.* 2005). *Alicyclobacillus* species can survive in acidic fruit juices and grow at temperatures greater than 20°C. Due to these characteristics, *Alicyclobacillus* species have the potential to spoil shelf stable products (Chen *et al.* 2006).

The spoilage caused by *A. acidoterrestris* is often associated with the production of flavour taints, such as

guaiacol and 2,6-dibromophenol (Borlinghaus and Engel 1997; Pettipher *et al.* 1997; Siegmund and Pollinger-Zierler 2007). Visible detection of spoilage is often difficult, as *A. acidoterrestris* does not produce gas during growth (Silva *et al.* 1999; Gouws *et al.* 2005). Previous study indicated that $<10^5$ cells of *Alicyclobacillus* per millilitre of juice are needed before the taste threshold is reached (Gouws *et al.* 2005). As spores of *A. acidoterrestris* have high heat resistance and can germinate and grow even at pH values lower than 4.5 (Murakami *et al.* 1998; Silva and Gibbs 2001; Vieira *et al.* 2002), there is a need for methods to prevent spoilage caused by *A. acidoterrestris* in acidic fruit products (Yamazaki *et al.* 2000).

Some studies have demonstrated the effect of bacteriocins from lactic acid bacteria, such as nisin and enterocin AS-48, against *A. acidoterrestris* (Yamazaki *et al.* 2000; Grande *et al.* 2006; Peña and Massaguer 2006). In this study, we tested the effect of bovicin HC5, a bacteriocin produced by *S. bovis* HC5, against *A. acidoterrestris* in synthetic media and in acidic mango pulp. Our results indicated that bovicin HC5 was bactericidal against vegetative cells of *A. acidoterrestris*, as the viable cell number decreased in the presence of the bacteriocin (Figs 1 and 2). Growth in liquid media was completely inhibited at the lowest dose tested (40 AU ml^{-1}) and MIC determinations indicated that even lower doses (5 AU ml^{-1}) were inhibitory. Because bovicin HC5 resembles the lantibiotics (Mantovani *et al.* 2002), the effect on target bacteria could be similar to nisin. Further studies will be needed to see if bovicin HC5 also affects membrane permeability of *A. acidoterrestris* in a similar manner as seen for other Gram-positive bacteria (Mantovani and Russell 2003).

Our minimum inhibitory concentration experiments indicated that spores from *A. acidoterrestris* were more sensitive to bovicin HC5 than vegetative cells. Similar results have been reported for other spore-forming spoilage bacteria. Yamazaki *et al.* (2000) and Delves-Broughton (1990) reported that endospores from most *Clostridium*, *Bacillus* and *Alicyclobacillus* species are more sensitive to bacteriocins than vegetative cells. However, the reasons for this increased spore sensitivity to antimicrobial peptides are not well understood.

Bovicin HC5 showed sporicidal activity against spores of *A. acidoterrestris* that had been inoculated into mango pulp and phosphate buffer. Previous work indicated that nisin and enterocin AS-48 also have sporicidal activity against spores from spoilage bacteria (Delves-Broughton 1990; Grande *et al.* 2006), but the mechanism of action against endospores was not clear. It has been suggested that at least some peptides (e.g. nisin) appear to inhibit the germination process at the pre-emergent swelling stage (Delves-Broughton 1990; Mazzotta and Montville 1999).

Acidic fruits are usually processed at 95°C for *c.* 20 s (Silva and Gibbs 2001), but even these harsh conditions are insufficient to eliminate spores from thermoresistant spoilage bacteria. Our results indicated that *A. acidoterrestri* DSMZ 2498 had a *D*-value of 8.33 min in mango pulp at pH 4.0 and 95°C (Table 1), suggesting that spores could survive the processing conditions generally used in fruit juice industries. Bovicin HC5 reduced the *D*_{95°C} of *A. acidoterrestri* DSMZ 2498 spores by 95% (0.36 min), compared to controls without bacteriocin (Table 1). These results suggest that thermal processing of mango pulp could be more effective in the presence of bovicin HC5. As the effect of bovicin HC5 was sporicidal, the spores that survived the heat treatment could be inactivated by bovicin HC5 during the transition to become a vegetative cell.

We also noted that the effect of bovicin HC5 on thermal sensitivity of *A. acidoterrestri* increased at higher temperatures. Previous work indicated that bovicin HC5 is very stable even at high temperatures (121°C/20 min). Because heat can make spores more permeable to dyes and also to inhibitory substances (such as bacteriocins), the utilization of heat stable peptides could be very useful in food industries that use thermal processing. Mazzotta and Montville (1999) also described an increased nisin-sensitivity for spores of *Clostridium botulinum* as the temperature was increased. Bovicin HC5 reduced *c.* 50% the *Z*-value of spores from *A. acidoterrestri* DSMZ 2498 and it has been suggested that differences in *Z*-values are due to different mechanisms of heat inactivation (Mazzotta and Montville 1999).

Considering that nisin is the only bacteriocin used for preservation of fruit juices and the lack of studies with other bacteriocins, further experiments will be needed to characterize the sensitivity and resistance of other thermoacidophilic spoilage bacteria to bovicin HC5. Based on our results, it appears that bovicin HC5 has the potential to reduce thermal resistance of spores from *A. acidoterrestri* and to inhibit the growth of vegetative cells in acidic drinks.

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