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ORIGINAL ARTICLE

Inhibition of *Bacillus licheniformis* LMG 19409 from ropy cider by enterocin AS-48

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Abstract

Aims: To determine the activity of enterocin AS-48 against ropy-forming *Bacillus licheniformis* from cider.
Methods and Results: Enterocin AS-48 was tested on *B. licheniformis* LMG 19409 from ropy cider in MRS-G broth, fresh-made apple juice and in two commercial apple ciders (A and B). *Bacillus licheniformis* was rapidly inactivated in MRS-G by 0·5 μg ml^{-1}AS-48 and in fresh-made apple juice by 3 μg ml^{-1}. Concentration-dependent inactivation of this bacterium in two commercial apple ciders (A and B) stored at 4, 15 and 30°C for 15 days was also demonstrated. Counts from heat-activated endospores in cider A plus AS-48 decreased very slowly. Application of combined treatments of heat (95°C) and enterocin AS-48 reduced the time required to achieved complete inactivation of intact spores in cider A to 4 min for 6 μg ml^{-1} and to 1 min for 12 μg ml^{-1}. *D* and *z* values also decreased as the bacteriocin concentration increased.
Conclusion: Enterocin AS-48 can inhibit ropy-forming *B. licheniformis* in apple cider and increase the heat sensitivity of spores.
Significance and Impact of the Study: Results from this study support the potential use of enterocin AS-48 to control *B. licheniformis* in apple cider.

Introduction

Growth of undesirable bacteria in fruit juices during storage as well as during the fermentation processes for manufacture of beverages may cause significant economic losses to the food and beverage industry. One of the most common forms of spoilage is the formation of slime, which gives the juice or drink a ropy appearance. The strain *Bacillus licheniformis* LMG 19409 was isolated from spoiled Normandy cider (France), shown to produce exopolysaccharide and cause rope spoilage of cider (Larpin *et al.* 2002). Being an endospore-forming bacterium, spores of *B. licheniformis* may be difficult to inactivate by conventional heat treatments without compromising the organoleptic properties of the starting material and its suitability to serve as substrate for the corresponding beverage fermentation. Strains of *B. licheniformis* have also been shown to cause rope spoilage of bread (Rosenkvist and Hansen 1995; Pepe *et al.* 2003; Sorokulova *et al.* 2003). *Bacillus licheniformis* is also isolated frequently from foods, and its toxin production capacity has raised concerns about its implication in food poisoning (Salkinoja-Salonen *et al.* 1999; Taylor *et al.* 2005).

Among the alternative methods that have been suggested to avoid spoilage of food and beverages is the application of bacteriocins (Stiles 1996; Cleveland *et al.* 2001; Devlieghere *et al.* 2004). Specifically, the bacteriocin enterocin AS-48 is a broad-spectrum antimicrobial peptide produced by *Enterococcus faecalis* (Gálvez *et al.* 1986). Previous studies carried out on this bacteriocin have contributed to elucidate its molecular composition and structure as well as its mode of action and its genetic determinants (reviewed by Maqueda *et al.* 2004). More recently, satisfactory results on application of enterocin AS-48 have been reported for dairy products, meat and vegetable foods including pathogenic bacteria like *Staphylococcus aureus*, *Listeria monocytogenes* and *Bacillus cereus* (Muñoz *et al.* 2004; Ananou *et al.* 2005a,b; Grande *et al.* 2006). In fruit juices, enterocin AS-48 also showed a strong inhibitory activity against spoilage-causing *Alicyclobacillus* sp. (Grande *et al.* 2005) and also against *Escherichia coli* O157:H7 in apple juice in combination with heat or chemical preservatives (Ananou *et al.* 2005c). The purpose of this work was to test the efficacy of enterocin AS-48 against the slime-producing strain *B. licheniformis* LMG 19409 in apple cider.

Materials and methods

Bacterial strains and culture conditions

Bacillus licheniformis LMG 19409 isolated from bottled spoiled ciders produced in Normandy (Larpin *et al.* 2002) was kindly supplied by Dr Jean-Marie Laplace (Laboratoire de Microbiologie de l'Environnement, Unite' Sous Contrat INRA, Universite' de Caen, France). *Enterococcus faecalis* A-48-32 (Martínez-Bueno *et al.* 1990) was used to produce enterocin AS-48, and *E. faecalis* S-47 was used as an indicator strain for the determination of bacteriocin activity. *Bacillus licheniformis* LMG 19409 was grown on MRS broth (Scharlab, Barcelona, Spain) supplemented with 2·0% glucose, pH 6·6 (MRS-G) at 30°C. Triptic Soya Agar (TSA; Scharlab) was used as solid medium for growth. Enterococci were propagated on Brain beart–infusion broth (Scharlab) at 37°C.

Bacteriocin preparation

Cultured broths of the producer strain *E. faecalis* A-48-32 were concentrated and partially purified by cation exchange chromatography as described by Abriouel *et al.* (2003). Bacteriocin concentrates were filtered through 0·22-μm pore size, low protein-binding filters (Millex GV; Millipore Corp., Belford, MA, USA) under sterile conditions. Samples were serially diluted and tested (100 μl) for bacteriocin activity against the indicator strain *E. faecalis* S-47 by the agar well-diffusion method using stainless steel cylinders of 8 mm (outer) diameter (Gálvez *et al.* 1986). One arbitrary unit (AU) was defined as the highest dilution producing a visible (9 mm diameter) zone of inhibition. The bacteriocin concentration of samples was determined from the previously published specific activity value of 3·5 AU μg^{-1} protein (Abriouel *et al.* 2003).

Preparation of endospore suspensions

Cultures of *B. licheniformis* LMG 19409 grown in MRS-G broth for 24 h were surface spread on plates containing nutrient agar (NA; Oxoid, Madrid, Spain) supplemented with 0·05 g l^{-1} of $MnSO_4$ (NAMS agar) and incubated at 30°C to obtain at least 90–95% spores (4–5 days). Spores were resuspended in sterile distilled water (3 ml per plate). Spores were centrifuged at 5000 ***g*** for 15 min at 4°C, washed two times with sterile distilled water by repeated centrifugation and finally resuspended in sterile distilled water (7–8 log units ml^{-1}) and stored in Eppendorf tubes at −20°C until use. When necessary, spores were heated at 80°C for 10 min followed by 1-h cooling on ice in order to activate germination.

Bacteriocin treatments

Fresh-made apple juice was prepared from golden delicious apples by using a Moulinex Frutti Pro (Moulinex, Berkshire, UK) fruit juice extractor, under aseptic conditions. Apple juice was adjusted to pH 5·0 with a concentrated NaOH solution and then heated in a water bath at 100°C for 10 min and cooled with running water to room temperature. Commercial apple ciders A (Sidra Asturiana; Mayador, Villaviciosa, Oviedo) and B (Sidra Vasca; Zapiain S.A.T. Errekalde Etxea, Astigarra, Guipuzcoa) were purchased from local supermarkets. Apple ciders (pH 3·35–3·93) were supplemented with 1·0% glucose and adjusted to pH 5·0, and then heated at 100°C for 10 min and cooled to desired incubation temperature before inoculation with the contaminant strain. Liquid cultures of *B. licheniformis* LMG 19409 grown in MRS-G broth for 12 h were inoculated in duplicate onto MRS-G as well as on apple juice and commercial apple ciders A and B. Samples were supplemented with enterocin AS-48 at desired final bacteriocin concentrations and stored at incubation temperature 30°C, 15°C (by using a refrigerated incubation chamber; Memmert, Schwabach, Germany) or 4°C. At different intervals of incubation at the desired temperature, controls and bacteriocin-added samples were serially diluted in sterile saline solution plus 0·05% Tween 80 (Sigma, St Louis, MO, USA) with vigorous vortexing and plated in triplicate on TSA. Plates were incubated at 30°C for 48 h and the average number of colonies was used to calculate the initial concentration of viable cells, expressed as the $\log_{10}$ colony-forming units (CFU) per millilitre (log units). The detection limit was 10 CFU ml^{-1}. Negative controls of apple juice and commercial apple ciders were always carried out in order to corroborate that the starting food material did not contain *B. licheniformis*.

Effect of enterocin AS-48 and combined bacteriocin-heat treatments on *B. licheniformis* endospores

The effect of enterocin AS-48 on spores of *B. licheniformis* LMG 19409 was tested in commercial apple ciders A and B (both adjusted to pH 5·0, as described above). Spores were activated to germinate before they were inoculated in duplicate onto commercial apple ciders supplemented or not with enterocin AS-48 (3 and 5 μg ml^{-1}, final concentrations). At desired intervals of incubation at 4, 15 and 30°C (0, 24 and 48 h), the concentration of viable cells was determined by serial dilution and plating.

The combined effects of enterocin AS-48 and heat treatments were studied on intact spores (not activated to germinate) inoculated in triplicate onto commercial apple cider A supplemented or not with different enterocin AS-48 concentrations (3, 6 and 12 μg ml^{-1}). Aliquots (1 ml) of each inoculated cider sample were placed in sterile glass tubes (12 × 80 mm; Corning Glass Works, Medfield, MA, USA) and immersed in a water bath (Memmert) previously warmed at desired temperature (85, 90, 95 or 100°C) for different periods of time (1–6 min). Following heat treatment, samples were cooled on ice, and 0·2 ml aliquots were removed from each tube and serially diluted into ice-cold sterile saline solution and plated for viable counts. Survival curves and their corresponding regression lines were obtained by plotting the $\log_{10}$ survivors (log *S*) *vs* time. Decimal reduction times (*D*) were calculated as the negative reciprocals of the slopes of regression lines. The *z* values were calculated from linear regressions of $\log_{10}$ *D* values *vs* temperature.

Statistical analyses

The average data ± standard deviations were determined with EXCEL program (Microsoft Corp., Redmond, WA, USA). A paired *t*-test was performed at the 95% confidence interval with STATGRAPHICS PLUS version 5.1 (Statistical Graphics Corp., Rockville, MD, USA), in order to determine the statistical significance of data. The significance of combined treatments was determined by comparison of data from the same incubation time.

Results

Inhibition of *B. licheniformis* LMG 19409 by enterocin AS-48 in a culture medium and in fresh-made apple juice

Enterocin AS-48 was very active on *B. licheniformis* LMG 19409 in MRS-G broth incubated at 30°C, and its bactericidal effect was proportional to the added bacteriocin concentration (Fig. 1a). The concentration of viable cells was reduced below the detection limits after 8 h incubation with 0·5 μg ml^{-1}AS-48 or after 6 or 4 h for bacteriocin concentrations of 1·5 and 3 μg ml^{-1}.

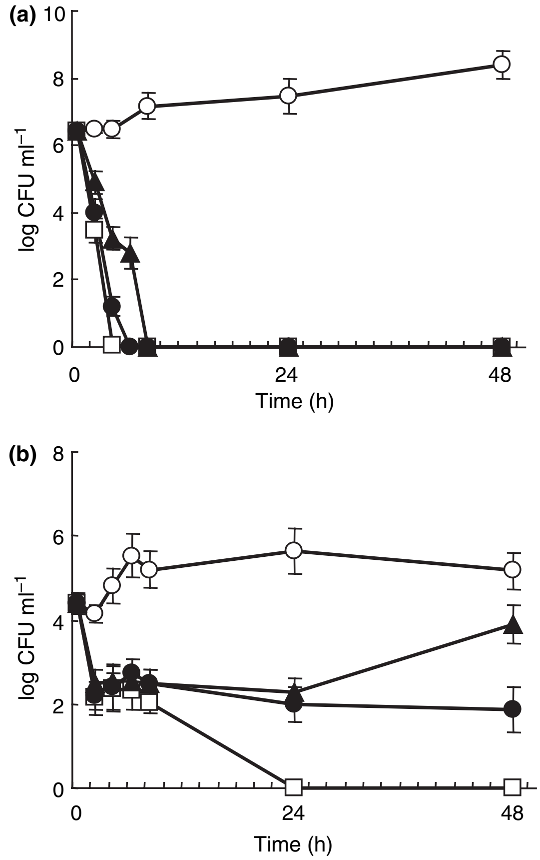

Figure 1 Effect of enterocin AS-48 on *Bacillus licheniformis* LMG 19409 inoculated in modified MRS broth (a) and in fresh-made apple juice adjusted to pH 5·0 (b). Samples were supplemented with enterocin AS-48 at final concentrations of 0·5 (▲), 1·5 (●) and 3 μg ml^{-1} (□). Controls (○). Average data from two experiments plus standard deviations (error bars) are represented.

In a fresh-made apple juice adjusted to pH 5·0, the concentration of viable cells was reduced significantly ($P < 0·05$) by approx. 1·5–2 log units after 2 h incubation with AS-48, depending on the final bacteriocin concentration (Fig. 1b). During the following incubation period, viable cell counts were significantly lower ($P < 0·05$) compared with controls for all bacteriocin concentrations tested. At 0·5 μg ml^{-1}, the concentration of viable cells increased by the end of the incubation period. However, at 3 μg ml^{-1}, no viable cells were detected after 24 h.

Effect of enterocin AS-48 against *B. licheniformis* LMG 19409 in commercial apple cider

Enterocin AS-48 was also tested on two commercial apple ciders (A and B) adjusted to pH 5·0 and supplemented with 1·0% glucose in order to stimulate growth of the contaminating bacterium. In cider A stored at 30°C, no viable cells were detected after day 2 of incubation with a bacteriocin concentration of 10 μg ml^{-1}, which corresponds to a significant reduction ($P < 0{\cdot}05$) of initial cell counts by 3·27 log units (Fig. 2a). At lower bacteriocin concentrations of 2 and 4 μg ml^{-1}, viable counts were always significantly lower ($P < 0{\cdot}05$) compared with the untreated controls, and the highest reductions (1·71 and 2·27 log units for 2 and 4 μg ml^{-1} AS-48, respectively) were detected at day 3. However, the bacilli were not completely eliminated from cider, and bacterial growth was detected at some points during further incubation. In cider B stored at 30°C, viable cell counts were also reduced below detection limits after day 2 of incubation with 10 μg ml^{-1} AS-48 (Fig. 2b). Similar to cider A, viable counts obtained in cider B at bacteriocin concentrations of 2 and 4 μg ml^{-1} were significantly lower ($P < 0{\cdot}05$) compared with the untreated controls during the whole incubation period, although bacilli were not cleared completely from cider. Highest reductions of viable counts (2·07 and 2·37 log units for 2 and 4 μg ml^{-1}, respectively) were detected at days 2 and 3 of incubation.

Enterocin AS-48 was also tested on ciders A and B stored at lower temperatures 15 and 4°C. In cider A stored at 15°C, a bacteriocin concentration of 10 μg ml^{-1} reduced the concentrations of viable bacilli below detection levels from day 1 to the end of the incubation period (Fig. 2c). This corresponds to a significant reduction ($P < 0{\cdot}05$) of initial counts by 3·27 log units. For bacteriocin concentrations of 2 and 4 μg ml^{-1}, viable counts were significantly lower ($P < 0{\cdot}05$) than the untreated control for the whole incubation period, with highest reductions of 1·93 and 2·2 log units respectively, at day 3 of incubation (Fig. 2c). However, the bacilli were not cleared from cider at these concentrations. Similar results were obtained for cider B (data not shown). In cider A stored at 4°C with 10 μg ml^{-1} AS-48, the concentration of viable bacilli was also reduced below detection limits from day 2 to the end of incubation period (Fig. 2d). As in previous cases, the lower bacteriocin concentrations tested also reduced viable counts significantly compared with the untreated controls for all samples, although the bacilli were not completely eliminated. Highest reductions of viable counts of 1·97 (for 2 μg ml^{-1} AS-48) and 2·19 log units (for 4 μg ml^{-1} AS-48) were detected at day 2 of incubation. Similar results were obtained for cider B at this temperature (data not shown).

Effect of enterocin AS-48 against *B. licheniformis* LMG 19409 endospores

Heat-activated endospores of *B. licheniformis* LMG 19409 were inoculated in commercial apple ciders A and B (pH 5·0) alone or supplemented with 3 and 5 μg ml^{-1} AS-48 and incubated at 4, 15 and 30°C. At 30°C, the concentrations of viable cells in ciders supplemented with 5 μg ml^{-1} AS-48 decreased slightly during incubation (Fig. 3). Viable counts of bacteriocin added samples were

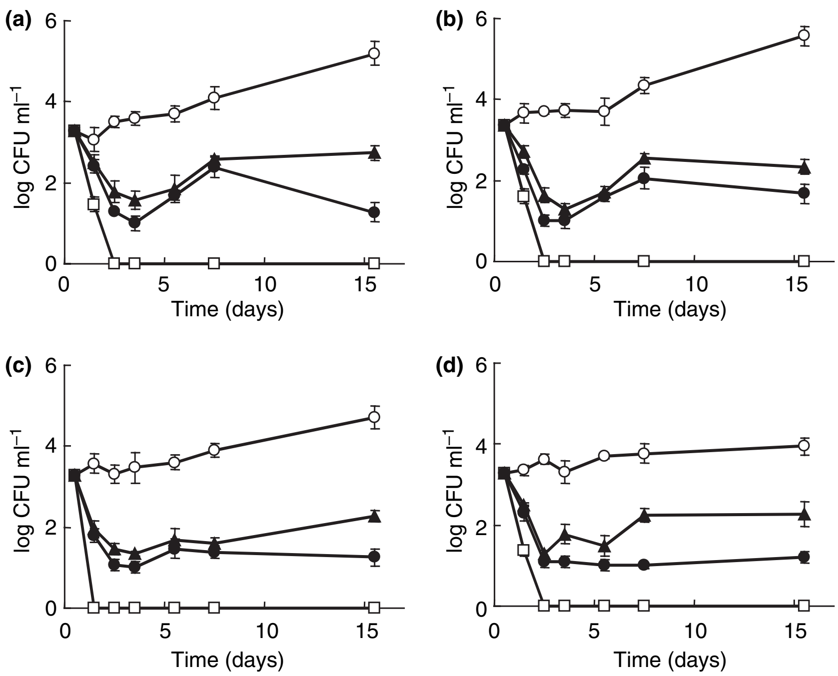

Figure 2 Effect of enterocin AS-48 against *Bacillus licheniformis* LMG 19409 in ciders A (a) and B (b) at 30°C. Cider A was also incubated at 15°C (c) and 4°C (d). Final bacteriocin concentrations were 2 (▲), 4 (●), and 10 μg ml^{-1} (□). Controls (○). Average data from two experiments plus standard deviations (error bars) are represented.

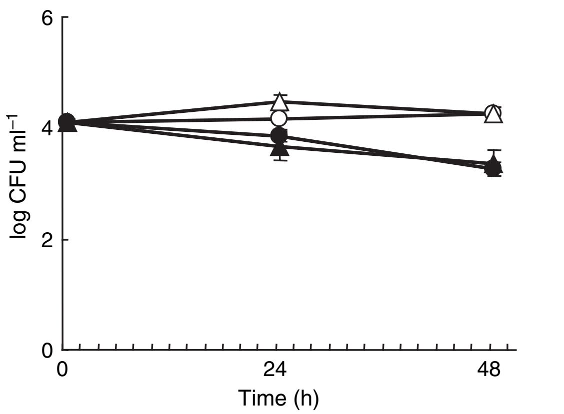

Figure 3 Effect of enterocin AS-48 on endospores from *Bacillus licheniformis* LMG 19409 inoculated in commercial apple ciders A (circles) and B (triangles) at 30°C. The final bacteriocin concentration was 5 μg ml^{-1} (closed symbols). Controls without added bacteriocin are represented by open symbols. Average data from two experiments plus standard deviations (error bars) are represented.

significantly lower ($P < 0{\cdot}05$) compared with controls without added bacteriocin at 48 h incubation for both ciders. Slight (but not statistically significant) reductions of viable counts were also detected in both ciders supplemented with 3 μg ml^{-1} AS-48 at 30°C (data not shown) as well as in ciders supplemented with 5 μg ml^{-1} AS-48 and incubated at temperatures 15 and 4°C (data not shown).

The combined effect of heat treatments and enterocin AS-48 (3, 6 and 12 μg ml^{-1}) was studied on *B. licheniformis* LMG 19409 endospores inoculated on commercial cider A (pH 5·0) without previous activation of germination. In control samples without added bacteriocin, heating at 85°C for up to 6 min had a very low effect on spore viability (Fig. 4a), as shown by the high *D* value (Table 1). As the heating temperature increased, viable cell counts decreased significantly, as well as the *D* values calculated from regression lines (Table 1). Incubation of endospore suspensions with enterocin AS-48 alone (12 μg ml^{-1}) for up to 6 min had no effect on spore viability (Fig. 4b). Application of heat treatments in combination with enterocin AS-48 had more pronounced effects on spore viability compared to heat treatments alone, and viable counts also decreased as the bacteriocin concentration and the heat temperature increased

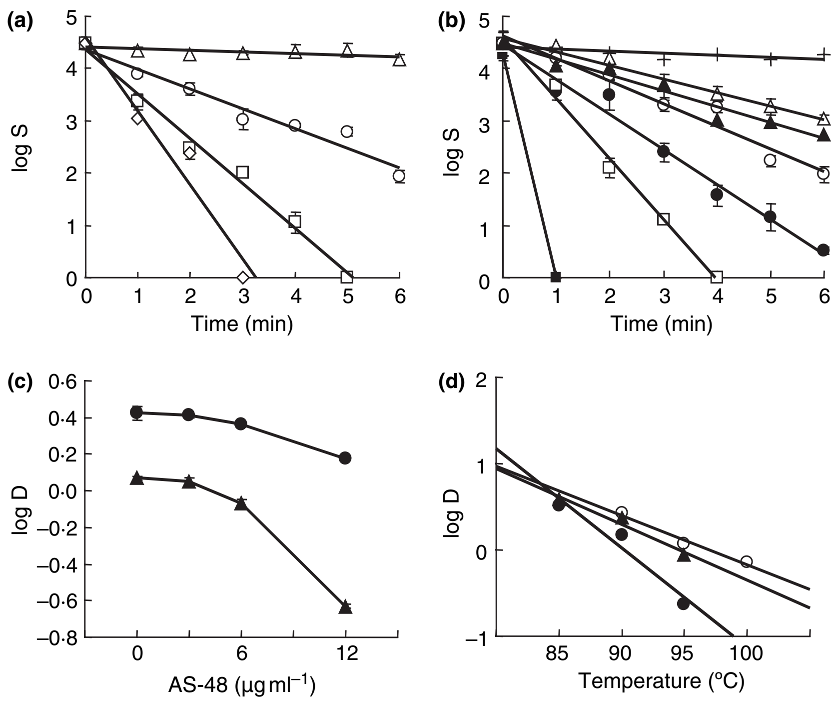

Figure 4 Effect of enterocin AS-48 on heat resistance of *Bacillus licheniformis* LMG 19409 endospores in commercial apple cider A (pH 5·0). Endospore suspensions in apple cider A without added bacteriocin (a) were heated at temperature 85°C (△), 90°C (○), 95°C (□) or 100°C (◇) for different time intervals. (b) The same heat treatments were applied to spore suspensions supplemented with enterocin AS-40 at 6 (open symbols) or 12 μg ml^{-1} (closed symbols). Spore suspensions were also incubated at 30°C with 12 μg ml^{-1} AS-48 without previous heat treatment (+). The average $\log_{10}$ survivors (log *S*) was determined, and *D* values were calculated from the reciprocal of the slopes of survival curves. The influence of bacteriocin concentration on $\log_{10}$ *D* values at temperatures 90°C (●) and 95°C (▲) is represented (c). Changes of $\log_{10}$ *D* values *vs* temperature for spore suspensions without added bacteriocin (○) or supplemented with 6 (▲) or 12 μg ml^{-1} AS-48 (●) are shown (d). Data correspond to the averages from triplicate experiments plus standard deviations (error bars).

Table 1 Calculated *D* and *z* values for *Bacillus licheniformis* LMG 19409 endospores in commercial apple cider A (pH 5·0) supplemented or not with enterocin AS-48

Heat temperature (°C)	Control	Plus AS-48 (6 μg ml^{-1})	Plus AS-48 (12 μg ml^{-1})
D values (min)			
85	32·57 ± 4·82	3·84 ± 0·13	3·33 ± 0·24
90	2·65 ± 0·34	2·32 ± 0·03	1·50 ± 0·04
95	1·17 ± 0·03	0·87 ± 0·04	0·23 ± 0·005
100	0·71 ± 0·02		
z values (°C)			
	6·99 ± 0·60	6·19 ± 0·14	3·47 ± 0·09

(Fig. 4b). At 3 μg ml^{-1} AS-48, heating at temperature 85 or 90°C had very low effect on spore viability, and it was necessary to heat samples at 95°C for 5 min or at 100°C for 2 min to achieve complete inactivation of spore suspensions (data not shown). At 6 and 12 μg ml^{-1} AS-48, the concentration of viable spores decreased in proportion to the heat treatment and the added bacteriocin concentration, achieving complete inactivation of endospores at 95°C for 4 min and 6 μg ml^{-1} AS-48 or 1 min and 12 μg ml^{-1} AS-48. *D* and *z* values also decreased as the bacteriocin concentration increased (Table 1, Fig. 4c and d).

Discussion

Consumers as well as modern food and drink manufacturing practices demand complementary methods to solve the problems associated to spoilage. Before proposing bacteriocins as alternative or complementary agents in preservation, different studies must be conducted to approximate their efficacy under practical situations. Results presented in this study indicate that enterocin AS-48 is highly active against the exopolysaccharide-producing strain *B. licheniformis* LMG 19409 when tested in a culture medium. In apple juice as well as in apple cider, although higher bacteriocin concentrations were required for effective inhibition, these were always in a low range, of 5–10 μg ml^{-1}. Moreover, inactivation of *B. licheniformis* was achieved rapidly regardless of incubation temperature when a bacteriocin concentration high enough was used. The results from the present work may be of interest for the apple cider industry, as there are scarce reports on inhibition of ropy spoilage bacteria by bacteriocins (Rosenquist and Hansen 1998; Aymerich *et al.* 2002; Katina *et al.* 2002; Messens and De Vuyst 2002; Pepe *et al.* 2003).

Enterocin AS-48 had a very low activity on endospores of *B. licheniformis* LMG 19409 inoculated on apple cider, and this effect was observed only after prolonged incubation. A previous report indicated that spores of *B. cereus* are resistant to enterocin AS-48 (Abriouel *et al.* 2002) and become gradually sensitive during the course of germination. However, a more recent work indicated that spores of *Alicyclobacillus acidoterrestris* were sensitive to enterocin AS-48 (Grande *et al.* 2005). Bacterial endospores may represent a problem in the food industry when the intensity of the heat treatments that can be applied is limited. Endospores of *B. licheniformis* may require the application of high-intensity heat treatments for inactivation. The results from the present work indicate that strain LMG 19409 shows a similar heat resistance (as measured by *D* values) compared with previous works on *B. licheniformis* (Palop *et al.* 1996). The results on the combined effect of AS-48 and heat treatments in *B. licheniformis* endospores in a commercial apple cider clearly indicate that inactivation of endospores significantly increased as the bacteriocin concentration increased. Similar results have been reported recently for the application of combined treatments of AS-48 and heat on spores of *B. cereus* inoculated in boiled rice and in a rice-based gruel (Grande *et al.* 2006). Moreover, nisin treatment has also demonstrated to increase the heat sensitivity of *B. stearothermophilus* and *B. licheniformis* endospores in food systems (Beard *et al.* 1999; Wandling *et al.* 1999). The results from the present study suggest that enterocin AS-48 may also be useful for inactivation of bacterial endospores in the food and beverage industry, allowing the application of less intense heat treatments and better preservation of the food and beverage properties.

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