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Inhibition of toxicogenic *Bacillus cereus* in rice-based foods by enterocin AS-48

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Abstract

The antimicrobial effect of the broad-spectrum bacteriocin enterocin AS-48 against the toxicogenic psychrotrophic strain *Bacillus cereus* LWL1 has been investigated in a model food system consisting of boiled rice and in a commercial infant rice-based gruel dissolved in whole milk stored at temperatures of 37 °C, 15 °C and 6 °C. In food samples supplemented with enterocin AS-48 (in a concentration range of 20–35 μg/ml), viable cell counts decreased rapidly over incubation time, depending on the bacteriocin concentration, the temperature of incubation and the food sample. Enterotoxin production at 37 °C was also inhibited. Heat sensitivity of endospores increased markedly in food samples supplemented with enterocin AS-48: inactivation of endospores was achieved by heating for 1 min at 90 °C in boiled rice or at 95 °C in rice-based gruel. Activity of enterocin AS-48 in rice gruel was potentiated by sodium lactate in a concentration-dependent way.

Keywords: Bacteriocin; Enterocin AS-48; *Bacillus cereus*; Rice-based foods; Biopreservation

1. Introduction

Bacillus cereus is a food-poisoning bacterium that may cause two types of gastrointestinal disorders: the emetic syndrome, caused by ingestion of a preformed toxin in the food, and the diarrhoeal syndrome, caused by a different toxin that can be formed in the food but also in the small intestine (Granum and Lund, 1997; Granum, 2001). Due to its ubiquitous distribution in nature, *B. cereus* occurs frequently in a wide range of food raw materials. Rice-based products and farinaceous foods such as pasta and noodles are frequently contaminated and involved in *B. cereus* poisoning (Kramer and Gilbert, 1989). Levels of *B. cereus* greater than 10^3 cfu/g have been found in both cooked and uncooked rice and in cereal products all over the world (Gilbert et al., 1974; Mortimer and McCann, 1974; Raevuorie et al., 1976; Schiemann, 1978; Shinagwa et al., 1979; Bryan et al., 1981; Holmes et al., 1981; Lee et al., 1995; Rusul and Yaacob, 1995; te Giffel et al., 1997; Nichols et al., 1999; Little et al., 2002; Sarrías et al., 2002). Other foods including meats, milk, sauces and desserts are also frequently implicated in *B. cereus* food poisoning (Notermans et al., 1997). Dried milk products are frequently contaminated with *B. cereus* spores. In foods containing both dairy and cereal ingredients, such as cereal-based infant foods, the risk of *B. cereus* poisoning may even be higher (Becker et al., 1994), and contaminated weaning foods are frequently responsible for episodes of diarrhoea in children (Motarjemi et al., 1993; Motarjemi and Nout, 1996). *B. cereus* spores survive pasteurization processes with decimal reduction times at 100 °C of 2.2–5.4 min and, in addition, vegetative cells from some strains can grow down to 4–5 °C (Dufrenne et al., 1995; Choma et al., 2000). Under specific conditions, mild heat treatments might even activate rather than inactivate dormant spores, thereby increasing the risk of

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pathogen outgrowth and food poisoning (Kim and Foegeding, 1990).

The demands of consumers for mildly processed foods with a limited refrigerated shelf-life have promoted research to improve processing technologies that may lower the risk of *B. cereus* poisoning. Natural antimicrobial substances, such as bacteriocins, are being investigated for food preservation and to replace chemical preservatives (Cleveland et al., 2001; Devlieghere et al., 2004), although nisin is currently the only bacteriocin widely used (Thomas et al., 2000). Some bacteriocins, such as nisin or lacticin 3147, prevent spore outgrowth and enterotoxin production by *B. cereus* (Jaquette and Beuchat, 1998; Morgan et al., 2001). Enterocin AS-48 is a broad-spectrum cyclic peptide produced by *Enterococcus faecalis* (Gálvez et al., 1986, 1989a; Martínez-Bueno et al., 1998; González et al., 2000), with a bactericidal mode of action (Gálvez et al., 1991) against several foodborne pathogenic bacteria (Gálvez et al., 1989b; Abriouel et al., 1998; Mendoza et al., 1999). The antimicrobial activity of enterocin AS-48 against *B. cereus* has been established in conventional culture media under laboratory conditions (Abriouel et al., 2002) as well as in cheese inoculated with a bacteriocinogenic strain (Muñoz et al., 2004). In this work, we describe the antimicrobial activity of enterocin AS-48 against a toxicogenic psychrotrophic strain of *B. cereus* in a model food system consisting of boiled rice and in a rice-based infant formula dissolved in whole milk.

2. Materials and methods

2.1. Bacterial strains and cultivation conditions

The psychrotrophic and enterotoxigenic strain *B. cereus* LWL1 (Dufrenne et al., 1995) was kindly supplied by Dr. F.M. van Leusden (Microbiological Lab. for Health Protection, Natl. Inst. Publ. Health and Environ., The Netherlands). The strain *E. faecalis* A-48-32 (a cured mutant from strain *E. faecalis* S-48 producing solely enterocin AS-48; Gálvez et al., 1985; Martínez-Bueno et al., 1990) was used to produce enterocin AS-48. The cured mutant strain *E. faecalis* B-48-47 (Martínez-Bueno et al., 1990) was used as negative control. *E. faecalis* S-47 (Gálvez et al., 1985) was used as test strain to determine bacteriocin activity. Bacilli and enterococci were cultivated in Brain Heart Infusion broth (BHI; Scharlab, Barcelona, Spain), at 37 °C. Tryptic Soy Agar (TSA, Scharlab) was used as plating medium for viable cell count. Enterococci and *B. cereus* were routinely stored at 4 °C in BHI-agar slants. Strains were maintained as frozen stocks at −80 °C in 40% glycerol.

2.2. Preparation of endospore suspensions

Spore crops were obtained according to Beuchat et al. (1997). Cultures grown in BHI broth for 24 h were surface spread on a solid sporulation medium consisting of nutrient agar (NA, Oxoid, Madrid) supplemented with 0.05 g/l of $MnSO_4$ (NAMS agar) and incubated for 4 days at 37 °C to obtain at least 90–95% spores. Spores were collected with a sterile cotton swab and resuspended in sterile distilled water (3 ml per plate). The pool of spores collected from the different plates was 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 (6–7 log units/ml, as determined by plating on TSA) and stored in Eppendorf tubes at −20 °C until use. Spores were activated to germinate by heat (80 °C for 10 min), followed by 1-h incubation on ice. The number of spores was determined by serially diluting the heat-shocked spore suspensions in sterile saline solution and plating by triplicate on TSA. Plates were incubated for 48 h at 37 °C and the grown colonies were counted.

2.3. Preparation of bacteriocin extracts and determination of bacteriocin activity

Enterocin AS-48 was obtained from cultured broths of the producer strain *E. faecalis* A-48-32 after concentration by cation exchange chromatography as described by Abriouel et al. (2003). The partially purified bacteriocin samples obtained by this process contained enterocin AS-48 as the sole antibacterial substance, with an estimated purity of 48% (Abriouel et al., 2003). A cultured broth of the non-bacteriocinogenic mutant strain *E. faecalis* B-48-47 concentrated by an identical procedure was added (8.0%, vol/vol) as negative control. Bacteriocin concentrates and negative control concentrates were filtrated 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. A specific activity value of 3.5 AU/μg protein was previously determined for purified enterocin AS-48 (Abriouel et al., 2003).

2.4. Effect of enterocin AS-48 on vegetative cells and endospores of B. cereus in boiled rice and in rice gruel

Vegetative cells from exponential-phase cultures of *B. cereus* LWL1 (incubated for 8 h at 37 °C in BHI broth) as well as endospore suspensions prepared as described above were inoculated into the desired rice-based preparation. Boiled rice was prepared from round grain white rice (SOS Cuétara S.A., Madrid). Rice (200 g) was boiled in water (250 ml) for 30 min, and the resulting paste was diluted 1:5 with distilled water under stirring for 10 min to obtain a homogeneous slurry (pH 8.79). Rice gruel was prepared from a commercial rice-based infant formula (rice cream; Nestlé España, S.A., Esplugas del Llobregat, Barcelona) by dissolving 25 g of the commercial powder in 200 ml of whole milk (Puleva SA., Granada, Spain) at 70 °C under stirring (pH 6.71). Food samples (10 ml each, in duplicate) were pre-cooled at desired incubation temperatures for 1 h before inoculation with *B. cereus* and addition of bacteriocin. Enterocin AS-48 was

added at various final concentrations to boiled rice inoculated with vegetative cells (20 μg/ml) or endospores (25 μg/ml), as well as to the rice gruel inoculated with vegetative cells (20, and 30 μg/ml) or with endospores (35 μg/ml). Following inoculation and/or bacteriocin addition, duplicate samples were thoroughly mixed and distributed in sterile capped test tubes and incubated at temperatures of 6 °C, 15 °C (in a refrigerated incubation chamber; Memmert, Schwabach, Germany) or 37 °C. At desired intervals of incubation (4 and 8 h, and 1, 2, 3, 7, 9 and 14 days), food samples were serially diluted in sterile saline solution and plated in triplicate on TSA. Plates were incubated at 37 °C for 48 h and the average number of colonies was used to calculate the initial concentration of viable cells, expressed as log units (the $\log_{10}$ colony forming units, cfu) per ml.

2.5. Determination of enterotoxin production

Food samples were removed at desired intervals of incubation, centrifuged in a microcentrifuge, filtrated through 0.22-μm pore size Millex GV filters (Millipore Corp.) and tested for reciprocal toxin titre using *B. cereus* enterotoxin reverse passive latex agglutination toxin detection kit (BCET-RPLA; Oxoid) according to the manufacturer's instructions. Enterotoxin titre was expressed in units (U) as the reciprocal of the last dilution that caused a visible agglutination.

2.6. Effect of enterocin AS-48 on endospore heat resistance

Boiled rice as well as rice gruel were inoculated in triplicate with LWL1 endospore suspensions (not activated to germinate). Aliquots (1 ml) of each inoculated food were placed in sterile glass tubes (12 × 80 mm, Corning Glass Works, Medfield, MA) with or without enterocin AS-48 (16 μg/ml, final concentration) and immersed in a water bath (Memmert) previously warmed at desired temperature (85, 90 or 95 °C). At 1-min intervals of incubation, samples (0.2 ml) were removed from each tube under sterile conditions and placed immediately into ice-cold sterile test tubes. Then, they were serially diluted in ice-cold sterile saline solution and plated on TSA for viable counts. Decimal reduction times (D) were calculated as the negative reciprocals of the slopes of the regression lines of the survival curves (obtained by plotting the $\log_{10}$ survivors, log S, vs. time). Decimal reduction temperatures (z) were determined as the negative reciprocals of the slopes of the regression lines for $\log_{10}$ D values vs. temperature.

2.7. Combined effect of enterocin AS-48 and sodium lactate

Vegetative cells of strain LWL1 were inoculated into rice gruel in duplicate and incubated at 37 °C with sodium lactate (Merck, Madrid) at final concentrations of 0.5%, 1.0%, 1.5% or 3.0% either alone or in combination with enterocin AS-48 (at final concentrations of 8 and 16 μg/ml). At 0, 4, 8, 24 and 48 h of incubation, samples were serially diluted and plated on TSA for viable cell counts.

2.8. Statistical analysis

The average data ± standard deviations were determined with Excel programme (Microsoft Corp., USA). Statistical analysis was performed with Statgraphics Plus version 5.1 (Statistical Graphics Corp, USA).

3. Results

3.1. Bacteriocin stability in rice-based foods

Before carrying out in situ biocontrol assays, the stability of enterocin AS-48 (15 μg/ml, final concentration) in boiled rice and in rice gruel stored at temperatures of 6 °C, 15 °C and 37 °C was tested. At desired intervals of incubation (0, 3, 7, 14, and 30 days), the residual enterocin AS-48 activity in food samples was tested by the agar well diffusion assay described under Materials and methods. In boiled rice, between 85% and 90% of initial bacteriocin activity (as measured at time zero of incubation) could still be detected within the first 14 days regardless of storage temperature (data not shown). After 30 days, activity was reduced to 71% in samples stored at 6 °C or at 45% in samples stored at 15 or 37 °C. In rice gruel dissolved in whole milk, over 90% of initial activity could still be detected regardless of storage temperature. After 30 days of storage, 90% of initial activity could still be detected in samples stored at 6 °C, but activity was reduced to 47% at 15 °C or to 40% at 37 °C (data not shown).

3.2. Inhibition of B. cereus by enterocin AS-48 in boiled rice

Boiled rice inoculated with suspensions of vegetative cells or heat-activated endospores of *B. cereus* LWL1 was supplemented with enterocin AS-48 (20 μg/ml for vegetative cells, and 25 μg/ml for endospores) and incubated at temperatures of 37 °C, 15 °C or 6 °C.

At 37 °C, vegetative cells of strain LWL1 reached high cell concentrations after 8 h of incubation and produced highest enterotoxin titre (10 U) at 24 h (Fig. 1A). After prolonged incubation, enterotoxin titre decreased. In rice samples supplemented with enterocin AS-48, the concentration of viable cells decreased by 4.7 log units during the first 8 h of incubation (Fig. 1A), followed by a slight increase of viable counts and then by a slower decrease. From day 7 of incubation on, the concentrations of viable cells remained below the detection limit (<20 cfu/ml). A negative control added of a concentrated cultured broth of the non-bacteriocinogenic mutant strain *E. faecalis* B-48-47 prepared by an identical procedure showed no signs of growth inhibition and reached identical cell counts as control cultures without any added preparation (data not shown), indicating that enterocin AS-48 was responsible for the inhibition observed in samples supplemented with the enterocin concentrate. Enterotoxin production was inhibited in samples treated with enterocin AS-48 to remarkably low titres (1 U at first day) or no detectable levels (Fig. 1A). Heat-activated endospores germi-

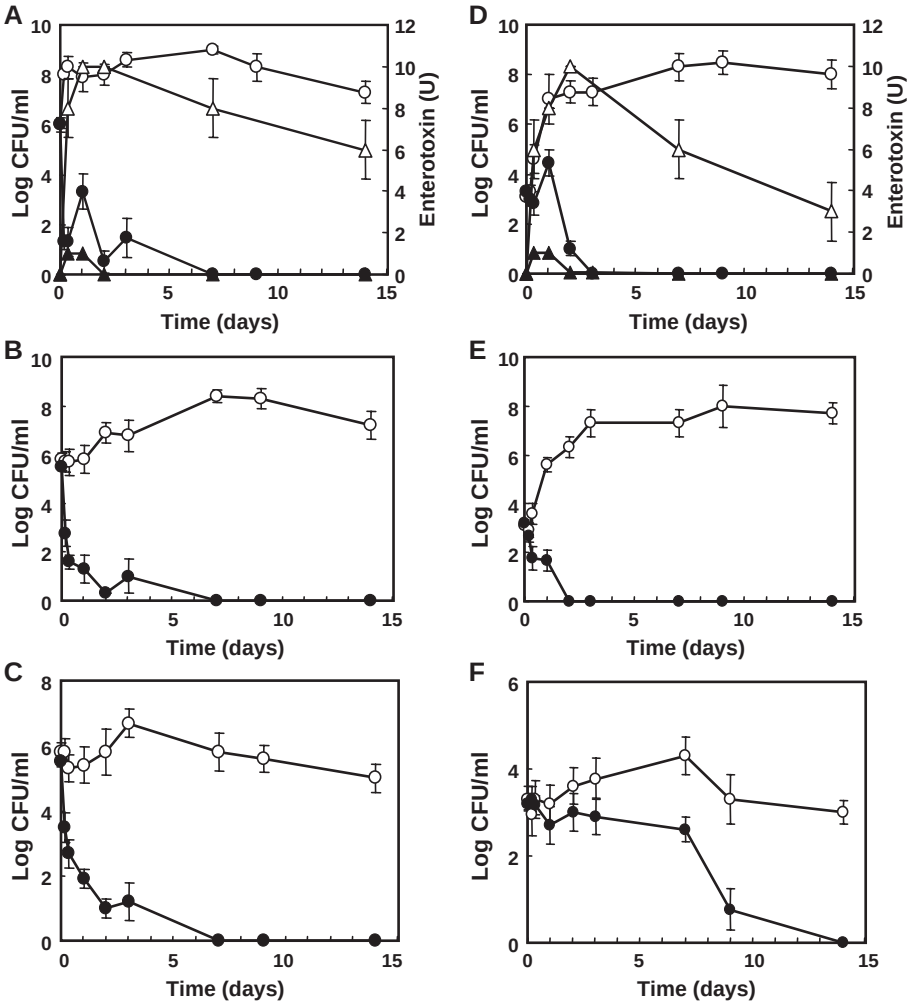

Fig. 1. Antimicrobial activity of enterocin AS-48 in boiled rice inoculated with vegetative cells or endospores of *B. cereus* LWL1 at different temperatures of incubation. (A–C) Vegetative cells incubated with 20 μg/ml of enterocin AS-48 at 37 °C (A), 15 °C (B) or 6 °C (C). (D–F) Endospores incubated with 25 μg/ml of enterocin AS-48 at 37 °C (D), 15 °C (E) or 6 °C (F). The concentration of viable cells in controls (○) and in bacteriocin-treated samples (●) was determined. In samples incubated at 37 °C, enterotoxin production was determined in controls (△) and in bacteriocin-treated samples (▲). The average data shown in graph are representative for duplicate assays. Error bars corresponding to standard deviations are shown.

nated rapidly and produced enterotoxin in the rice-based substrate at 37 °C (Fig. 1D). In samples supplemented with enterocin AS-48, growth was also inhibited early during incubation and no viable cells were detected from day 3 on. Enterotoxin titre was also remarkably reduced.

In boiled rice stored at 15 °C, the concentration of viable cells decreased markedly within the first day of incubation with enterocin AS-48, followed by a much slower decrease down to undetectable levels after day 7 (Fig. 1B). In samples inoculated with endospores, viable cell counts were reduced below detection limits after day 2 of incubation with enterocin AS-48 (Fig. 1E).

For vegetative cells incubated at 6 °C with enterocin AS-48, the concentration of viable cells decreased sharply (by 2.8 log units) during the first 8 h of incubation and then more slowly during the following days (Fig. 1C). No viable cells were detected from day 7 on. In rice samples inoculated with endospores and supplemented with enterocin AS-48, the concentration of viable cells did not increase but still remained quite high within the first 7 days (Fig. 1F). After this, viable counts decreased more rapidly, and no viable cells were detected at 14 days of incubation.

3.3. Inhibition of *B. cereus* by enterocin AS-48 in rice gruel

To test the effect of enterocin AS-48 in rice gruel dissolved in whole milk, gruels were inoculated with suspensions of vegetative cells or endospores of strain LWL1 and incubated at temperatures of 37 °C, 15 °C or 6 °C with or without added bacteriocin.

Vegetative cells of strain LWL1 grew rapidly in gruels incubated at 37 °C (Fig. 2A). Enterotoxin titres also reached highest values (10 U) at 24 h and decreased during further incubation. In gruels supplemented with enterocin AS-48 at 20 μg/ml, the concentration of viable cells oscillated with variable decreases and increases within the first 24 h of incubation, but no viable cells were detected after 48 h incubation (Fig. 2A). For a higher bacteriocin concentration (30 μg/ml), no viable

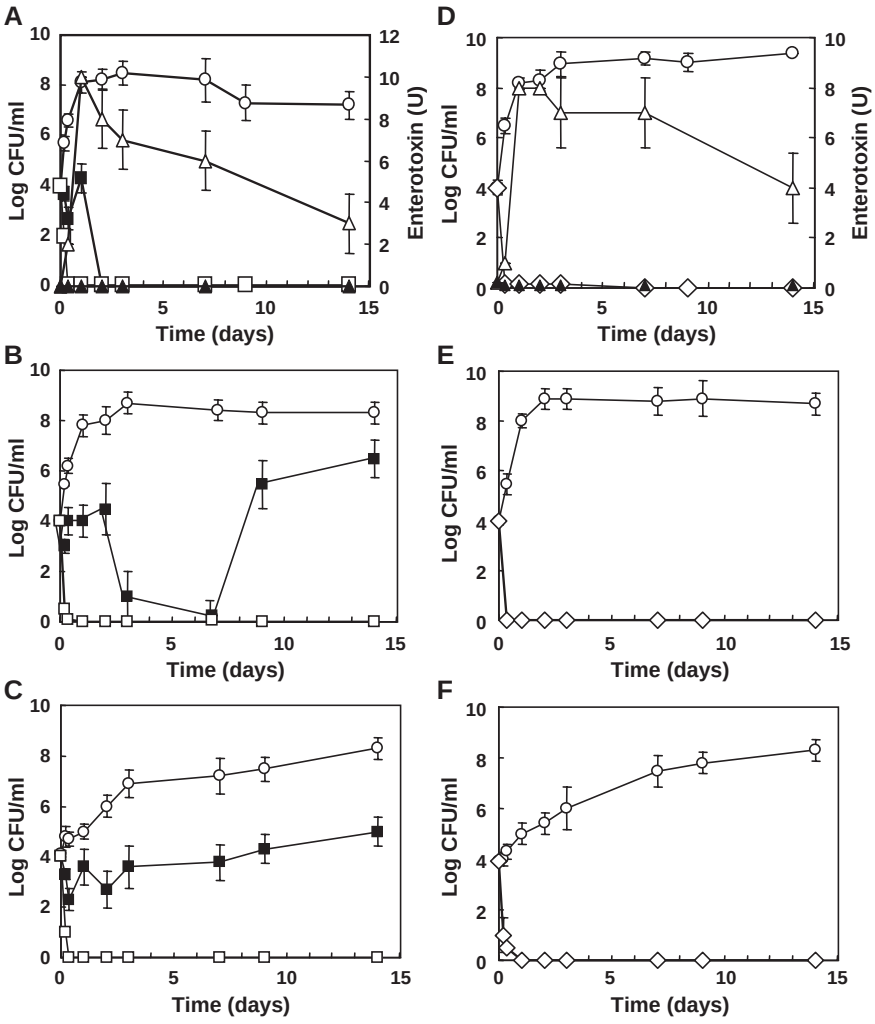

Fig. 2. Antimicrobial activity of enterocin AS-48 in rice gruel inoculated with vegetative cells or endospores of *B. cereus* LWL1 at different temperatures of incubation. (A–C) Vegetative cells incubated with 20 (■) and 30 μg/ml (□) of enterocin AS-48 at 37 °C (A), 15 °C (B) or 6 °C (C). (D–F) Endospores incubated with 35 μg/ml of enterocin AS-48 (◊) at 37 °C (D), 15 °C (E) or 6 °C (F). The concentration of viable cells in controls (○) and in bacteriocin-treated samples was determined. In samples incubated at 37 °C, enterotoxin production was determined in controls (△) and in bacteriocin-treated samples (▲). The average data shown in graph are representative for duplicate assays. Error bars corresponding to standard deviations are shown.

cells were detected after 8 h incubation and no enterotoxin production was detected either (Fig. 2A). Suspensions of endospores activated to germinate were inoculated in gruels and treated with a single bacteriocin concentration of 35 μg/ml. In controls incubated at 37 °C, dense populations of bacilli were observed after 24 h, and enterotoxin titres reached 8 U (Fig. 2D). However, in gruels supplemented with enterocin AS-48, no viable cells or enterotoxin production were detected during the whole test period (Fig. 2D).

In gruels inoculated with vegetative cells at 15 °C, a bacteriocin concentration of 20 μg/ml failed to inhibit growth of the bacilli during the tested incubation period (Fig. 2B), but no viable cells were detected after 8 h of incubation with a higher bacteriocin concentration of 30 μg/ml (Fig. 2B). In gruels inoculated with endospores at 15 °C and supplemented with 35 μg/ml of enterocin AS-48, no viable cells were detected during the whole incubation period (Fig. 2E).

At 6 °C, a bacteriocin concentration of 20 μg/ml only produced some inhibition of bacterial growth in gruels inoculated with vegetative cells, but no viable cells were detected in samples supplemented with 30 μg/ml (Fig. 2C). In gruels inoculated with endospores, addition of enterocin AS-48 (35 μg/ml) resulted in a gradual reduction of viable counts, which remained below the detection limits after 24 h (Fig. 2F).

3.4. Effect of enterocin AS-48 on endospore heat resistance

The combined effect of enterocin AS-48 (at a final concentration of 16 μg/ml) and heat was tested on intact endospores (without previous activation of germination) inoculated in boiled rice (Fig. 3A) and in rice gruel (Fig. 3B).

Thermal inactivation of endospores in boiled rice and in rice gruel showed first-order kinetics (Fig. 3A and B). The *D* values as well as the *z* values (calculated from linear regressions of $\log_{10} D$ values vs. temperature as shown in Fig. 3C) obtained for endospores in boiled rice and in rice gruel are shown in Table 1. In control samples without added bacteriocin, the *D*

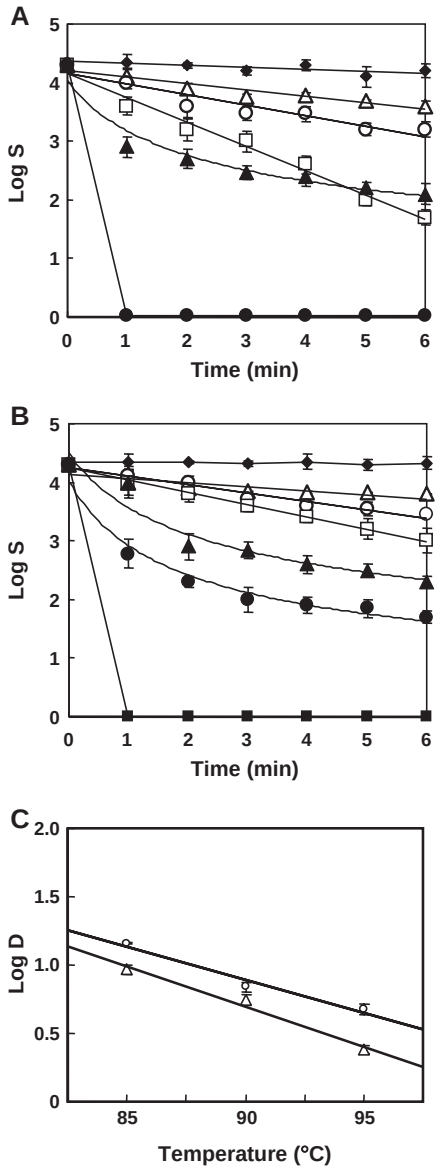

Fig. 3. Effect of enterocin AS-48 on thermal resistance of endospores of *B. cereus* LWL1 inoculated in boiled rice (A) and rice gruel (B). Endospore suspensions were incubated in triplicate at temperatures of 37 °C (♦), 85 °C (△), 90 °C (○) or 95 °C (□) for different heating times either alone (open symbols) or with enterocin AS-48 at a final concentration of 16 μg/ml (closed symbols), and the average $\log_{10}$ survivors (log *S*) was determined. Standard deviation values are represented. In (C), the linear regressions for the $\log_{10}$ of decimal reduction times (log *D*) of spores in boiled rice (△) and in rice gruel (○) are shown.

values obtained in rice gruel were always higher compared to boiled rice, and the differences were statistically significant for each of the temperatures tested (Table 1). Similarly, the *z* value obtained for rice gruel was also higher compared to boiled rice. For samples heated with enterocin AS-48, best fits were obtained for two-order potential curves rather than regression lines. In boiled rice, the combined treatment of enterocin AS-48 and heat was more efficient than heat alone. For example, the concentration of survivors was 1.5 log units lower in samples heated at 85 °C for 6 min with enterocin AS-48 compared to samples heated for the same period of time without bacteriocin (Fig. 3A). This effect was more pronounced at 90 °C, since no survivors were detected after heating in the presence of enterocin AS-48 for 1 min, which means a reduction of at least 4.3 log units. A D_{90} value of 0.23 min was calculated from the slope of the straight regression line obtained between time 0 and 1 min of treatment (Table 1). Similar results were obtained in rice gruel, with increasing reductions in the log survivors for samples heated in the presence of enterocin AS-48 (Fig. 3B). However, similar to results obtained for heat treatments alone, the sporocidal effect of the combined treatment was also less pronounced in rice gruel, and it was necessary to heat samples at 95 °C for 1 min to achieve a complete inactivation of the endospore population. The D_{95} value calculated for this treatment was 0.21 min (Table 1).

To corroborate that intact endospores were resistant to enterocin AS-48 in the absence of a heat treatment, endospore suspensions in boiled rice as well as in rice gruel were incubated at 37 °C with the same bacteriocin concentration as above (16 μg/ml). As expected, no reduction in viable cell counts was observed during the 6-min incubation period regardless of the food tested.

3.5. Combined effect of enterocin AS-48 and sodium lactate

To determine if the activity of enterocin AS-48 in rice gruel could be potentiated by sodium lactate, different concentrations of lactate were tested alone as well as in combination with bacteriocin concentrations of 8 and 16 μg/ml. Sodium lactate had no effect on the viability of vegetative cells of strain LWL1, and only some inhibition of growth was observed even at a final concentration of 3.0% (Fig. 4A). However, when used in combination with a sublethal bacteriocin concentration of 8 μg/ml, the bactericidal effect of enterocin AS-48 increased in proportion to the sodium lactate concentration tested (Fig. 4B). At sodium lactate concentrations of 1.0% or 1.5%, no viable cells were detected after 24 h of incubation. This effect was even more pronounced at 3.0% lactate, since no viable cells were detected after 4 h. For a bacteriocin concentration of 16 μg/ml, a higher bactericidal activity was detected at lower lactate concentrations, and no viable cells were detected after

Table 1
Calculated *D* and *z* values for *B. cereus* LWL1 endospores in boiled rice and in rice gruel

Treatment	Boiled rice	Rice gruel
	D values (min)	
85 °C	9.25±0.51	14.43±0.19 $P<0.0001$
90 °C	5.56±0.48	6.74±0.54 P 0.048
95 °C	2.41±0.16	4.74±0.41 P 0.00078
90 °C+enterocin AS-48	0.23[a]	
95 °C+enterocin AS-48		0.21[a]
	z values (°C)[b]	
	6.85	8.24

[a] Calculated from the slope of the straight regression line obtained for interval 0–1 min.
[b] *z* values of samples heated without enterocin AS-48.

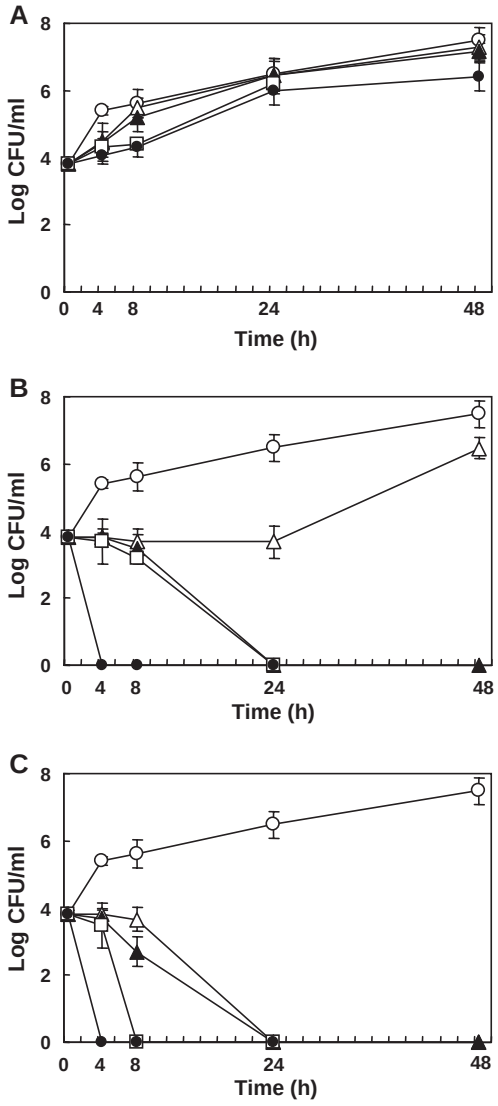

Fig. 4. Effect of sodium lactate and enterocin AS-48 on vegetative cells of *B. cereus* LWL1 inoculated in rice gruel at 37 °C. Sodium lactate was tested at final concentrations of 0.5% (△), 1.0% (▲), 1.5% (□) or 3.0% (●) either alone (A) or in combination with enterocin AS-48 at final concentrations of 8 μg/ml (B) or 16 μg/ml (C). Controls without added preservative or enterocin AS-48 (○). The average data shown in graph are representative for duplicate assays. Error bars corresponding to standard deviations are shown.

24 h of incubation with 0.5% or 1.0% lactate or after 8 h incubation with 1.5% lactate (Fig. 4C).

4. Discussion

As consumers demand foods with less intense processing treatments, fresh-tasting and ready to eat, the chances for growth of bacteria capable of overcoming the different hurdles employed in the food industry increase, and development of new or alternative technologies is necessary to satisfy the requirements for improved safety. In this context, endospore-forming bacteria such as *B. cereus* represent one of the highest risks in low-acid foods due to heat resistance of endospores, the broad temperature range in which some strains can grow, and the capacity to produce food-poisoning toxins. Rice-based foods are frequently contaminated with *B. cereus* and, hence, represent a high risk of *B. cereus* food poisoning because spores surviving cooking heat treatments may germinate and reach high cell concentrations in the food (Gilbert et al., 1974; Mortimer and McCann, 1974; Raevuorie et al., 1976; Parry and Gilbert, 1980; Johnson et al., 1984; Becker et al., 1994; Khodr et al., 1994; Ueda, 1994; Nichols et al., 1999). The need to include additional hurdles to control *B. cereus* in the food chain could be satisfied by incorporation of natural antimicrobial substances, such as bacteriocins. Since enterocin AS-48 is active against all strains of *B. cereus* tested (Abriouel et al., 2002), we tested the capacity of this bacteriocin to control the psychrotrophic and toxicogenic strain LWL1 in model food systems consisting of a boiled rice slurry and a rice gruel dissolved in whole milk.

Activity of bacteriocins in foods is greatly influenced by different factors including food composition, interaction with food components, bacteriocin stability, pH, storage temperature and others (Gänzle et al., 1999; Aasen et al., 2003). The stability of enterocin AS-48 detected in boiled rice and in rice gruel for at least 14 days regardless of storage temperature suggests that enterocin AS-48 could be used as bioprotectant during an equivalent shelf-life period. Enterocin AS-48 also showed a high stability at low temperature for a prolonged period, which could be of advantage to inhibit the slow growth of psychrotrophic strains during refrigeration storage of foods. In addition, the high solubility of enterocin AS-48 at neutral pH (being of at least 20 mg/ml at pH 7.5; Sánchez-Barrena et al., 2003) could be an advantage for preservation of low-acid foods compared to other bacteriocins with lower solubility at neutral pH such as nisin (Jaquette and Beuchat, 1998; Gänzle et al., 1999). Previous results also indicated that highest activity of enterocin AS-48 against *B. cereus* was detected in the pH interval of 6–8 (Abriouel et al., 2002). Therefore, enterocin AS-48 would be suitable to inhibit growth of this bacterium in non-acid foods such as cooked rice or rice gruel.

The capacity of enterocin AS-48 to inhibit proliferation of the psychrotrophic strain *B. cereus* LWL1 in a boiled rice slurry and in rice gruel contaminated with vegetative cells as well as with endospore suspensions and to reduce the concentration of viable cells below detection limits in a storage period of 14 days was demonstrated for bacteriocin concentrations in the range of 20–35 μg/ml. These are 3- to 5-fold higher compared to bactericidal concentrations obtained in BHI broth in previous experiments (Abriouel et al., 2002). In agreement with our results, activity of other bacteriocins in foods is also lower compared to experiments carried out in vitro using culture media (Gänzle et al., 1999), and the food type and composition can also be determinant factors for bacteriocin activity. Therefore, inhibitory concentrations need to be determined specifically for each type of food when considering application of bacteriocins.

The activity of enterocin AS-48 against vegetative cells of *B. cereus* LWL1 was influenced by incubation temperature, especially at sub-optimal bacteriocin concentrations. For example, a bacteriocin concentration of 20 μg/ml was sufficient to maintain *B. cereus* below detection limits in rice gruel stored

at 37 °C but insufficient to avoid proliferation at 15 or 6 °C. At 15 °C, regrowth of vegetative cells observed during prolonged incubation clearly compromises the safety of such food samples. Since food is often a complex and heterogeneous matrix (Fleet, 1999), interaction with food components as well as uneven bacteriocin distribution or the formation of bacteriocin concentration gradients may facilitate the generation of microenvironments where bacteria can find an increased protection against environmental factors. This could be avoided in part by adding a higher bacteriocin concentration, as shown in experiments from this work. Therefore, for food applications, it is important to use bacteriocin concentrations that are high enough to inactivate the bacterial population from the early stages of treatment and to avoid regrowth of survivors during prolonged food storage.

Both the boiled rice and the rice gruels inoculated with heat-activated endospore suspensions were also protected against *B. cereus* proliferation by enterocin AS-48. Previous results have shown that endospores of *B. cereus* gradually become sensitive to enterocin AS-48 during the germination and outgrowth while intact spores are resistant (Abriouel et al., 2002). For this reason, slightly higher bacteriocin concentrations were used for food samples inoculated with endospore suspensions. Since endospores represent one of the main risks for food contamination with *B. cereus*, it is important to use bacteriocin concentrations high enough to avoid proliferation of sporulated *B. cereus* in foods. The lower effect of enterocin AS-48 in spore-contaminated boiled rice samples stored at 6 °C was probably due to the longer time required for spore germination. Therefore, the conditions unfavourable to endospore germination in food should also decrease killing by enterocin AS-48 and vice versa.

Due to the high heat resistance of endospores (Choma et al., 2000) and the increasing demand of consumers for mildly processed foods, considerable research has been devoted to improve mild thermal processing techniques and the development of alternative procedures such as hydrostatic pressure treatments (Knorr, 2002; Lado and Yousef, 2002). Most of these techniques do not, however, efficiently inactivate spores of *B. cereus*, and technical and economical constraints limit the pressure that can be reasonably used for food applications. Although intact endospores also usually resistant to antimicrobial substances such as bacteriocins, combined treatments of nisin on germinating spores of *B. cereus* have been described (Pol et al., 2001). While intact endospores of *B. cereus* are resistant to enterocin AS-48 (Abriouel et al., 2002), results from this work indicate that heat resistance of endospores decreases markedly in the presence of enterocin AS-48 depending on the heat temperature. Furthermore, heat inactivation was more pronounced in boiled rice than in rice gruel dissolved in whole milk, suggesting an influence of the food type/composition on endospore inactivation. Accordingly, optimal conditions for endospore inactivation should be determined for each type of food. The fact that short heat treatments applied in the presence of relatively low bacteriocin concentration reduce viable counts of endospore suspensions below detection limits suggests that heat and enterocin AS-48 act synergistically against endospores. These are promising results, suggesting that application of heat treatments in the presence of enterocin AS-48 may be an efficient method to inactivate *B. cereus* endospores in foods. In previous works, nisin treatment has also demonstrated to increase the heat sensitivity of *Bacillus stearothermophilus* and *Bacillus licheniformis* endospores in food systems (Beard et al., 1999; Wandling et al., 1999), indicating the potential industrial application of such combined treatments.

In previous work (Abriouel et al., 2002), we showed that the activity of enterocin AS-48 against vegetative cells of *B. cereus* in BHI broth was potentiated by sodium lactate. Experiments carried out in rice gruel also indicate that the activity of enterocin AS-48 is potentiated by sodium lactate depending both on the lactate and the bacteriocin concentration used. Accordingly, control of *B. cereus* in foods containing lactate could be achieved with much lower concentrations of enterocin AS-48, decreasing the costs of bacteriocin addition. The application of combined treatments including organic acids or their salts has also been described for other bacteriocins. For example, Buncic et al. (1995) found that the sensitivity of *Listeria monocytogenes* to nisin (400 IU/ml) increased in combination with lactate (40 g/l) and the chelating agent sodium polyphosphate (5 g/l) in buffered saline (pH 5.5), and further reports have confirmed the increased antibacterial activity of nisin in combination with sodium lactate in several food systems (Scannell et al., 1997; Nykanen et al., 2000; Long and Phillips, 2003; Ukuku and Fett, 2004).

The efficacy of enterocin AS-48 against *B. cereus* inoculated in cheese has also been demonstrated in a previous work (Muñoz et al., 2004) by inoculation with an enterococcal strain that produced the bacteriocin in situ. Although the method of choice for application of enterocin AS-48 (be it in situ production or addition of a bacteriocin concentrate) will largely depend on the type of food, the results of previous works and the data presented in this work support the use of enterocin AS-48 for the preservation of foods with a higher risk for transmission of foodborne pathogens such as *B. cereus* and the development of combined treatments consisting of bacteriocin and other physico-chemical barriers to enhance the final antimicrobial effect. The use of enterocin AS-48 could also be helpful for application of mild treatments without compromising the safety of foods.

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