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Control of *Alicyclobacillus acidoterrestris* in fruit juices by enterocin AS-48

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Abstract

Alicyclobacillus acidoterrestris is a spoilage-causing bacterium in fruit juices. Control of this bacterium by enterocin AS-48 from *Enterococcus faecalis* A-48-32 is described. Enterocin AS-48 was active against one *A. acidocaldarius* and three strains of *A. acidoterrestris* tested. In natural orange and apple juices incubated at 37 °C, vegetative cells of *A. acidoterrestris* DSMZ 2498 were inactivated by enterocin AS-48 (2.5 μg/ml) and no growth was observed in 14 days. In commercial fruit juices added of AS-48 (2.5 μg/ml) and inoculated with vegetative cells or with endospores of strain DSMZ 2498, no viable cells were detected during 90 days of incubation at temperatures of 37 °C, 15 °C or 4 °C, except for apple, peach and grapefruit juices inoculated with vegetative cells and incubated at 37 °C which were protected efficiently for up to 60 days. Remarkably, in all commercial fruit juices tested, no viable cells were detected as early as 15 min after incubation with the bacteriocin. Endospores incubated for a very short time (1 min) with increasing bacteriocin concentrations were inactivated by 2.5 μg/ml AS-48. Electron microscopy examination of vegetative cells and endospores treated with enterocin AS-48 revealed substantial cell damage and bacterial lysis as well as disorganization of endospore structure.

Keywords: Bacteriocin; AS-48; *Alicyclobacillus*; Fruit juice; Spoilage; Biopreservation

1. Introduction

In 1984, Cerny et al. reported a new type of bacterium in aseptically packaged apple juice, subsequently named *Bacillus acidoterrestris* by Deinhard et al. (1987). Later, Wisotzkey et al. (1992) proposed the new genus *Alicyclobacillus* to include this bacterium.

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Alicyclobacillus is a thermoacidophilic gram-positive to gram-variable, rod-shaped, motile, aerobic bacterium that usually contains ω-alicyclic fatty acids as the major membrane lipid component (Yamazaki et al., 1996; Walls and Chuyate, 1998). Microorganisms of the genus *Alicyclobacillus* have a pH range for growth between 2.0 and 7.0 and are able to grow in a temperature range of 20 to 70 °C (Walls and Chuyate, 1998). *Alicyclobacillus* spores have been shown to resist acidic environments and high temperatures applied in hot fill processes used for fruits and vegetable juices, rendering this bacterium as a potential cause of spoilage (Murakami et al., 1998; Pontius et al., 1998; Splittstoesser et al., 1994, 1998; Walls and Chuyate, 1998; Eiroa et al., 1999; Silva and Gibbs, 2001). *Alicyclobacillus* has been detected in a wide range of fruit juices and products as well as processing facilities, where it enters most probably on fruit surfaces contaminated from soil during production and harvesting (McIntyre et al., 1995; Wisse and Parish, 1998; Eiroa et al., 1999). Acidic juices, and especially apple juice, are more prone to spoilage caused by this bacterium, but heat-activated spores have also been shown to grow on tomato, apple, orange, pineapple, grapefruit and white grape juices (Pettipher et al., 1997).

The use of bacteriocins for food preservation has been a matter of extensive work in recent years (Giraffa, 1995; Holzapfel et al., 1995, Hugas, 1998; Ennahar et al., 1999; Cleveland et al., 2001; O'Sullivan et al., 2002), but application of bacteriocins in preservation of fruit juices has seldom been studied, nisin being the only exception. In 1999, Komitopoulou et al. reported inhibition of spore outgrowth by nisin in apple, grapefruit and orange juice, and Yamazaki et al. (2000) also reported similar effects in acidic drinks inoculated with *A. acidoterrestris* spores. Enterocin AS-48 is a broad-spectrum cyclic peptide that has been extensively characterized as far as chemical composition (Gálvez et al., 1986, 1989a), molecular structure (González et al., 2000), genetic determinants (Martínez-Bueno et al., 1998), mode of action (Gálvez et al., 1991) and antimicrobial activity against foodborne pathogenic bacteria (Gálvez et al., 1989b; Abriouel et al., 1998, 2002; Mendoza et al., 1999). This bacteriocin may be considered as an alternative to nisin for food biopreservation. This work describes the antimicrobial activity of enterocin AS-48 against vegetative cells and endospores of *Alicyclobacillus acidoterrestris* in fruit juices under different storage conditions.

2. Materials and methods

2.1. Bacterial strains and cultivation conditions

Alicyclobacillus acidocaldarius CECT 4328, *A. acidoterrestris* LMG 16906, *A. acidoterrestris* DSMZ 2498 and *A. acidoterrestris* DSMZ 3922 were provided by the Colección Española de Cultivos Tipo (CECT), the Laboratory voor Microbiologie Universiteit Gent (LMG) and the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), respectively. Strain DSMZ 2498 (from spoiled juice) was selected to study the antimicrobial effect of enterocin AS-48 in fruit juices. Strains were grown at 37 °C in the *Alicyclobacillus acidocaldarius* medium (AAM) described by Yamazaki et al. (2000), composed of yeast extract (1.0 g), $(NH_4)_2SO_4$ (0.2 g), $MgSO_4 \cdot 7H_20$ (0.5 g), $CaCl_2 \cdot 2H_2O$ (0.25 g), KH_2PO_4 (1.0 g) and glucose (1.0 g) per liter of distilled water (pH 5.3). YPDA composed of potato dextrose agar (PDA; Scharlab, Barcelona, Spain) supplemented with agar (5.0 g) and yeast extract (2.0 g) per liter (pH 5.8) was used as solid growth medium.

The bacteriocin-producer strain *Enterococcus faecalis* A-48-32 is described elsewhere (Martínez-Bueno et al., 1990). *Enterococcus faecalis* S-47 (Gálvez et al., 1985) was used as test strain to determine bacteriocin activity. Enterococci were cultivated in brain heart infusion broth (BHI; Scharlab, Barcelona, Spain) at 37 °C. Enterococci and *Alicyclobacillus* were routinely stored at 4 °C on BHI-agar and YPDA slants, respectively. Strains were maintained as frozen stocks at −80 °C in 40% glycerol.

2.2. Preparation of spore suspensions

Cultures grown for 48 h at 37 °C in AAM broth were spread onto YPDA in Petri dishes and incubated at 37 °C for 8 days. Sporulation was confirmed by microscopy following staining with malachite green. After reaching more than 90% of sporulation, spores were collected with a sterile swab and resuspended in sterile distilled water (2 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 (10^6–10^7 spores/ml, as determined by plating on YPDA) and stored in Eppendorf tubes at −20 °C until use.

2.3. Preparation of bacteriocin extracts

Enterocin AS-48 concentrates were prepared from cultured broths of the producer strain *E. faecalis* A-48-32 in CMG-medium followed 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) under aseptic conditions and tested for bacteriocin activity by the agar well diffusion method (5 μg AS-48/well) using sterile stainless steel cylinders of 8 mm (outer) diameter (Gálvez et al., 1986). Samples were incubated at 37 °C for 48 h until the zones of inhibited growth were clearly visible.

2.4. Determination of the spectrum of inhibition of enterocin AS-48 against Alicyclobacillus

Bacteriocin concentrates were tested against the different strains of *Alicyclobacillus* by the agar well diffusion method described above, by using YPDA as the solid medium.

2.5. Effect of enterocin AS-48 on exponential-phase cultures

Bacteriocin concentrates were added to 24-h exponential-phase cultures of *A. acidoterrestris* growing in AAM medium at 37 °C. At desired intervals of incubation, aliquots of cultures were serially-diluted in sterile saline solution and plated in triplicate on YPDA. Plates were incubated at 37 °C for 3 days and the average number of colonies was used to calculate the concentration of viable cells, expressed as colony forming units (CFU) per ml.

2.6. Effect of enterocin AS-48 on vegetative cells and endospores of A. acidoterrestris in fruit juices

Vegetative cells from exponential-phase cultures of *A. acidoterrestris* growing in AAM and endospore suspensions respectively were inoculated into the desired fruit juices in duplicate. Fresh orange and apple juices (pH 3.86 and 3.55, respectively) were made from Valencia oranges and golden delicious apples by using a Moulinex Citroplus (Moulinex, Berkshire, UK) and a Moulinex Frutti Pro (Moulinex) fruit juice extractor respectively, under aseptic conditions. Commercial orange juice (Kasfruit, Vitoria, Spain; pH 4.12), apple juice (Don Simón, Murcia, Spain; pH 3.92), pineapple juice (Kasfruit; pH 3.68), peach juice (Kasfruit; pH 4.02), and grapefruit juice (Don Simón; pH 3.58) were purchased in local stores. Two 200-ml tetrabricks of each fruit juice were used for each experiment. Fruit juices were incubated for 1 h at the desired test temperature before they were inoculated with vegetative cells from an exponential-phase culture in AAM or with spore suspensions in distilled water. Then, enterocin AS-48 was added to the juices at the desired final concentration. Juice mixtures were distributed in sterile capped test tubes and incubated at temperatures of 4 °C, 15 °C or 37 °C for different periods of time. Viable counts were determined as described above.

2.7. Electron microscopy

Exponential-phase cells as well as endospore suspensions of *A. acidoterrestris* DSMZ 2498 strain (ca. 10^8 CFU/ml) in AAM broth were treated with AS-48 at 37 °C. Due to the higher cell concentrations required for microscopic analyses, a higher bacteriocin concentration (15 μg/ml) was also used. At desired incubation times, samples (1.5 ml) were collected in Eppendorf tubes by centrifugation (12,000×*g* for 10 min) and then incubated at 4 °C for 2 h in a fixation solution [2.0 vol.%/vol.% glutaraldehyde (Merck, Madrid, Spain) and 1.0 vol.%/vol.% formaldehyde (Merck) in 0.1 M sodium cacodylate buffer, pH 7.4; Merck] followed by three washes (5 min each) in 0.1 M cacodylate buffer, pH 7.4 (Merck). Fixed samples were prepared for electron microscopy examination at the Technical Services of the University of Granada: samples were embedded into EMBed 812® resin (Electron Microscopy Science, Hartfield, PA), sectioned and mounted on copper grids and stained with 2.0 vol.%/vol.% uranyl acetate (Merck) and 2.0 vol.%/vol.% lead citrate (Merck) before they were viewed under a Carl Zeiss EM10C transmission electron microscope (Carl Zeiss, Jena, Germany) operating at 80 kV.

2.8. Statistical analysis

Statistical analyses were performed using the SPSS-PC 11.0 software (SPSS, Chicago, IL, USA). Data relating to microbiological counts along the incubation period of juices were subjected to ANOVA. The presence of enterocin AS-48 was used as factor with two categories: juices added with enterocin AS-48 and juices without AS-48.

3. Results

3.1. Spectrum of inhibition

Alicyclobacillus acidocaldarius CECT 4328 as well as the three strains of *A. acidoterrestris* were sensitive to AS-48 when tested by an agar well diffusion assay (results not shown). Most strains showed a similar sensitivity to AS-48.

3.2. Effect of AS-48 on exponential-phase cells in a laboratory substrate

Vegetative cells from *A. acidoterrestris* LMG 16906 and DSMZ 2498 strains were incubated in AAM at 37 °C with bacteriocin concentrations of 1.25 and 2.5 μg/ml (Fig. 1). For the lowest bacteriocin concentration tested, viable cell counts decreased rapidly within the first 1 to 3 days of incubation. However, the remaining viable cells were able to multiply during the following incubation period. For a bacteriocin concentration of 2.5 μg/ml, no viable cells of either strain were detected after 24 h of incubation. For the remaining incubation period (up to 14 days), the concentrations of viable cells remained below the detection limit of 20 CFU/ml.

3.3. Effect of AS-48 on A. acidoterrestris DSMZ 2498 in fresh-made fruit juices

Vegetative cells from an exponential-phase culture of strain DSMZ 2498 in AAM were inoculated onto fresh-made orange and apple juices and incubated at 37 °C, using a bacteriocin concentration of 2.5 μg/ml (Fig. 2). In orange juice, viable cell counts increased by 4.08 $\log_{10}$ units within the first 7 days of incubation. In orange juice containing enterocin AS-48, no viable cells were detected during the whole incubation period, from 8 h after bacteriocin addition up to day 15 of incubation. In apple juice, viable cell counts of control samples increased by 4.37 $\log_{10}$ units after 10 days of incubation. As in orange juice, no viable cells were detected for bacteriocin-treated apple juice during the whole incubation period.

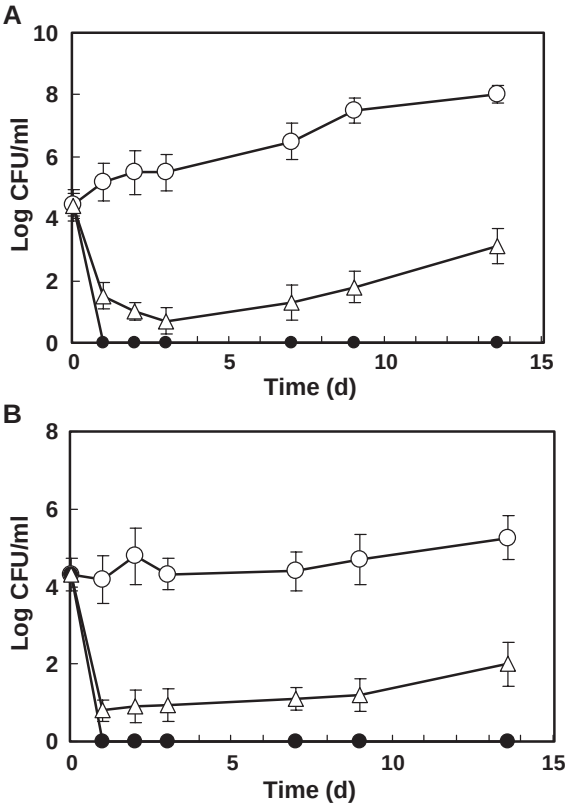

Fig. 1. Effect of enterocin AS-48 on exponential-phase vegetative cells of *Alicyclobacillus acidoterrestris* LMG 16906 (A) and *A. acidoterrestris* DSMZ 2498 (B) growing in AAM broth at 37 °C. Controls (○). Cultures added AS-48 at a concentration of 1.25 (Δ) and 2.5 μg/ml (●). Data represent the average of two assays ± standard deviation.

3.4. Effect of enterocin AS-48 on A. acidoterrestris DSMZ 2498 in commercial fruit juices

Five commercial fruit juices (orange, apple, pineapple, peach and grapefruit juice) were inoculated with vegetative cells or with endospores and stored at 37 °C, 15 °C or 4 °C with enterocin AS-48 (2.5 μg/

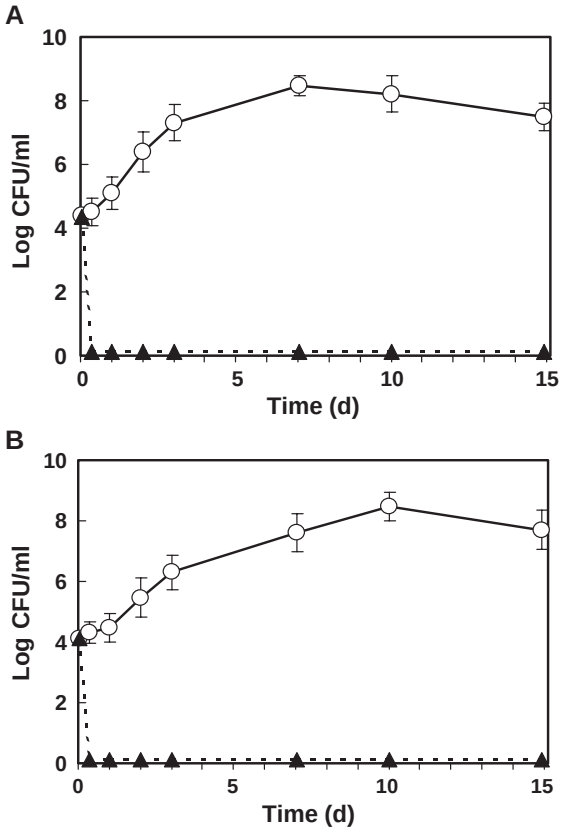

Fig. 2. Effect of enterocin AS-48 on vegetative cells of *Alicyclobacillus acidoterrestris* DSMZ 2498 at 37 °C inoculated in natural orange juice, pH 3.86 (A) and apple juice, pH 3.55 (B). Controls (○). Samples added AS-48 at a concentration of 2.5 μg/ml (▲). Data represent the average of two assays ± standard deviation.

ml) added. The following observations were made (data not shown).

For fruit juices inoculated with vegetative cells and incubated at 37 °C without bacteriocin, the concentrations of viable cells increased to a variable degree within the first 15 to 30 days of incubation. Highest counts were obtained for peach juice, followed by orange, apple and grapefruit juices while the lowest counts were obtained in pineapple juice. In fruit juices added of enterocin AS-48, no viable cells were detected after 15 min of incubation at 37 °C. Viable cell counts remained below the detection limits in orange and pineapple juices for up to 90 days of incubation, and for up to 60 days in apple, peach and grapefruit juices. In the last three cases, the concentrations of viable cells after 90 days of incubation were still significantly lower ($P<0.001$) compared with the controls.

For fruit juices inoculated with endospores and incubated at 37 °C without bacteriocin, variable increases in the concentrations of viable cells were also detected depending on the fruit juice being tested. Highest counts were obtained in peach, apple and grapefruit juices and lowest counts were obtained in pineapple juice, for which no growth was observed. In fruit juices inoculated with endospore suspensions, addition of enterocin AS-48 reduced the concentrations of viable counts below the detection limits within the first 15 min of incubation in all cases. Furthermore, no viable cells were detected in any of the bacteriocin-treated juices for an incubation period of up to 90 days.

For fruit juices inoculated with either vegetative cells or endospores and incubated at 15 °C, no bacterial growth was observed in any case, but viable counts remained quite high (at least over 4.30 $\log_{10}$ CFU/ml) during the whole incubation period of 90 days. By contrast, in fruit juices treated with bacteriocin AS-48, no viable cells were detected after 15 min of incubation, regardless of whether they were inoculated with vegetative cells or with endospores. Furthermore, no viable cells were detected during the whole 90 days of incubation period.

Viable cell counts from control fruit juices inoculated with either vegetative cells or with endospores and stored at 4 °C for 90 days decreased slowly during the incubation period, but always remained at least above 3.69 $\log_{10}$ CFU/ml at day 90. As in the previous cases, in fruit juices (inoculated with vegetative cells or with endospores) containing enterocin AS-48, viable cell counts decreased below the detection limits 15 min after bacteriocin addition and remained at this level for the whole incubation period.

3.5. Influence of bacteriocin concentration and incubation time on the viability of endospores from A. acidoterrestris DSMZ 2498

The results described above indicated that endospores of *A. acidoterrestris* DSMZ 2498 were inactivated after a short time of incubation with enterocin AS-48. For a more precise determination

of this effect, endospore suspensions of *A. acidoterrestris* DSMZ 2498 were inoculated in AAM and also in commercial orange juice and incubated at 37 °C with increasing bacteriocin concentrations for one and 60 min. Both in AAM and in orange juice, viable cell counts obtained after 1 min of incubation decreased in proportion to the bacteriocin concentration added (Fig. 3). In both cases, a bacteriocin concentration of 2.5 μg/ml reduced the viable cell counts below the detection limit. Furthermore, viable counts obtained after 60 min of incubation of endospores inoculated in AAM did not differ significantly ($P=0.154$) from counts obtained after 1 min of incubation. In the case of orange juice, viable counts obtained after 60 min of incubation were also below the detection limit for a bacteriocin concentration of 1.25 μg/ml.

3.6. Ultrastructural changes of vegetative cells and endospores of A. acidoterrestris DSMZ 2498 treated with enterocin AS-48

Electron microscopy examination of vegetative cells of *A. acidoterrestris* DSMZ 2498 treated with enterocin AS-48 revealed severe structural changes compared to untreated control cells (Fig. 4). Short after bacteriocin addition, cell wall damage (Fig. 4B) and loss of cytoplasmic content were observed.

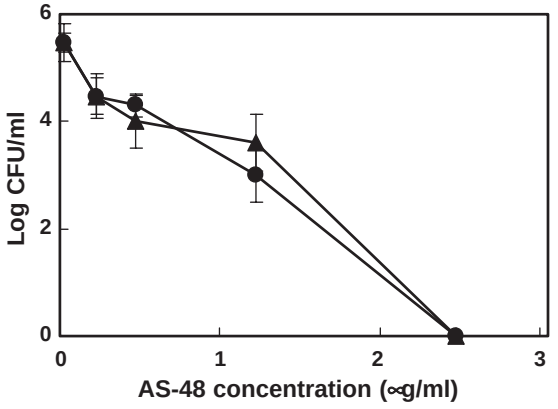

Fig. 3. Relationship between the added bacteriocin concentration and viable endospore concentrations of *Alicyclobacillus acidoterrestris* DSMZ 2498 after one min of incubation in AAM broth (▲) and in orange juice (●). Data represent the average of two assays ± standard deviation.

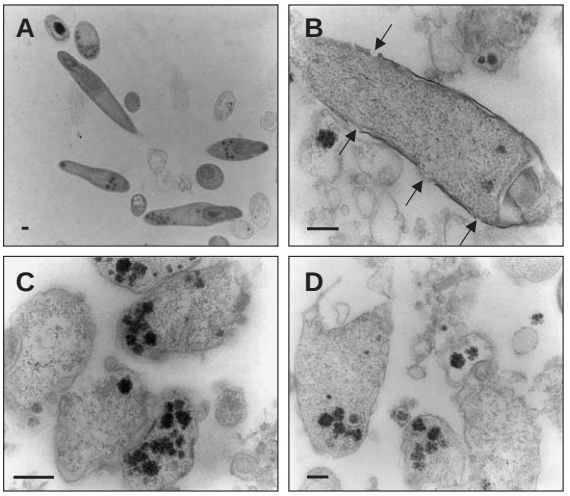

Fig. 4. Electron microscopy examination of *Alicyclobacillus acidoterrestris* DSMZ 2498 vegetative cells treated with enterocin AS-48. Cell suspensions (ca. 1.0×10^8 CFU/ml) in AAM broth at 37 °C were examined at time zero without bacteriocin (A) or at different times with enterocin AS-48 (15 μg/ml): 60 min (B), 7 h (C) or 24 h (D). Bar=0.2 μm. Localized cell wall degradation is indicated by the arrow.

Cell disorganization was more pronounced after 7 h of incubation, yielding abundant cell debris (Fig. 4C). Bacteriocin-treated samples also contained small membrane vesicles, some of which seemed to protrude from the cells and were seen also after prolonged incubation (Fig. 4D).

Endospores of *A. acidoterrestris* DSMZ 2498 showed densely stained spore protoplasts surrounded by a less dense spore cortex and a thick and multilayered spore coat (Fig. 5A). During the course of germination, the cortex became less dense and larger in size, and the endospore coats became more diffuse and partially degraded. After 24 h of incubation, control preparations contained mainly vegetative cells (Fig. 5B). After 15 min of incubation with enterocin AS-48, some endospores showed a protoplast surrounded by a cortex less densely stained and also of larger size, and some also showed localized and partial degradation of the spore coats (Fig. 5C). After 8 h of incubation with the bacteriocin, spore protoplasts showed an irregular shape and did not stain homogeneously (Fig. 5D). At that time, degraded endospores were also found (Fig. 5E). After 24 h of incubation, some endospores still retained intact spore coats and dense protoplasts (results not shown), while

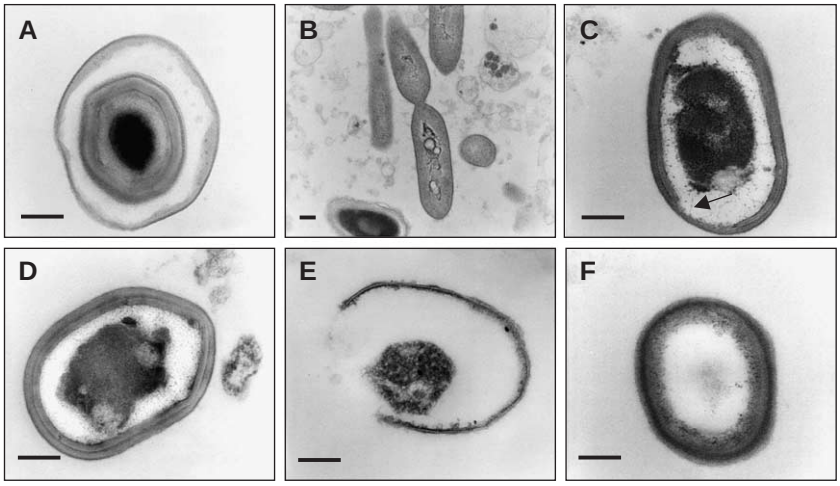

Fig. 5. Electron microscopy examination of *Alicyclobacillus acidoterrestris* DSMZ 2498 endospores treated with enterocin AS-48 (15 μg/ml). Endospore suspensions (ca. 1.0×10^8 CFU/ml) in AAM broth at 37 °C without bacteriocin were examined at time zero (A) and 24 h of incubation (B). Endospore suspensions added of AS-48 (15 μg/ml) were observed after 15 min (C), 8 h (D, E) or 24 h of incubation (F). Bar = 0.2 μm. Localized degradation of spore coat is indicated by the arrow.

others were surrounded by more diffuse layers and showed an ill-defined protoplast of very low density (Fig. 5F).

4. Discussion

The globalisation of the food market and modern food-processing technologies create new ecological niches for microbes. One example can be found in *Alicyclobacillus acidoterrestris*, an endospore-forming bacterium frequently found in soil. Thanks to its capacity to grow in a broad pH range of 2.0 to 7.0 (Walls and Chuyate, 1998), this bacterium can easily multiply in acidic fruit juices. Endospores from this bacterium are also capable of germinating and outgrowing under high acidic conditions in orange juice (Pettipher et al., 1997) and also in apple, orange and grapefruit juices with pH values of 3.42 to 3.90 (Komitopoulou et al., 1999). Vegetative cells of *A. acidoterrestris* are highly thermotolerant, requiring more intense heat treatments for inactivation than any other microbial cells that may be present in juices like, for example, lactic acid bacteria or yeasts. In addition, they also produce endospores of a strong heat resistance, with reported D values exceeding 60 min at 85 °C and close to 8 min at temperatures of 95–97 °C (Pontius et al., 1998; Eiroa et al., 1999). Since the hot fill processes applied in manufacture of most commercial juices usually holds the products for about 2 min at temperatures between 88 and 96 °C (McIntyre et al., 1995), spores of this bacterium have become a potential spoilage concern. Fruit juices and fruit juice-containing drinks that are most susceptible to this bacterium are either fresh (not heat-treated) or pasteurised (but not UHT-treated) and stored unpreserved at ambient temperature (Pettipher et al., 1997). Therefore, alternative methods to control juice spoilage by this bacterium are required, especially if less intense treatments are to be applied in order to satisfy consumers demands for better preserved and fresh-tasting juices and drinks.

Nisin is the only bacteriocin that has been tested so far for preservation of fruit juices and drinks against spoilage caused by *Alicyclobacillus* (Komitopoulou et al., 1999; Yamazaki et al., 2000). The antimicrobial spectrum and the physico-chemical properties of enterocin AS-48 suggest that this bacteriocin may also be used for biopreservation. This is the first report on the activity of enterocin AS-48 against *Alicyclobacillus* and also on its application in fruit juices. The results shown in this work indicate that strains of *Alicyclobacillus* may be inhibited by AS-48 both in a laboratory growth medium as well as in home-made and in commer-

cial fruit juices of several types. Furthermore, a bacteriocin concentration as low as 2.5 μg/ml appears to be enough to maintain fruit juices tested free of detectable *A. acidoterrestris* viable cells for prolonged periods of time under temperature conditions that permit growth and spoilage by this bacterium. This bacteriocin concentration falls in the range of the nisin concentrations reported to inhibit *A. acidoterrestris* in orange juice, grapefruit juice and apple juice (Komitopoulou et al., 1999) as well as in orange and fruit-mixed drinks (Yamazaki et al., 2000). Conceivably, incorporation of enterocin AS-48 into commercial fruit juices could be useful in order to apply less intense heat treatments without increasing the risk for spoilage caused by *A. acidoterrestris*. It should be remarked that inactivation of *A. acidoterrestris* by enterocin AS-48 was also detected under temperature conditions (like, for example, 15 °C or 4 °C) below the reported temperature growth range of this bacterium. Presumably, addition of AS-48 to vegetable juices right after extraction could provide an extra protection in situations where there is a risk for temperature abuse (like, for example, during large-scale long distance transportation and handling of refrigerated fruit juices prior to manufacture of the final commercial products).

Enterocin AS-48 was also very active against endospores of *A. acidoterrestris*, which were inactivated in all fruit juices and under all incubation conditions tested. Furthermore, a very short time of incubation (even with bacteriocin concentration as low as 2.5 μg/ml) was enough to cause endospore inactivation, suggesting a rapid adsorption of bacteriocin molecules. The bacteriocin nisin has also been shown to inactivate endospores of *A. acidoterrestris* (Komitopoulou et al., 1999; Yamazaki et al., 2000). For the food processing industry, endospores represent the most difficult life form to inactivate, since not only show a higher resistance to heat treatments but also to food treatments like ultra-high pressure or pulsed electric fields of high intensity (Knorr, 1995; Butz and Tauscher, 2002). Since *A. acidoterrestris* endospores require intense heat treatments for inactivation (Pontius et al., 1998; Eiroa et al., 1999), application of enterocin AS-48 may represent a technological advantage to currently used inactivation processes.

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