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# Inactivation of *Geobacillus stearothermophilus* in canned food and coconut milk samples by addition of enterocin AS-48

Pilar Martínez Viedma [a], Hikmate Abriouel [a], Nabil Ben Omar [a], Rosario Lucas López [a], Eva Valdivia [b], Antonio Gálvez [a,*]

[a] *Área de Microbiología, Departamento de Ciencias de la Salud, Facultad de Ciencias Experimentales, Universidad de Jaén, 23071 Jaén, Spain*
[b] *Department of Microbiology, University of de Granada, 18071 Granada, Spain*

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The cyclic bacteriocin enterocin AS-48 was tested on a cocktail of two *Geobacillus stearothermophilus* strains in canned food samples (corn and peas), and in coconut milk. AS-48 (7 μg/g) reduced viable cell counts below detection levels in samples from canned corn and peas stored at 45 °C for 30 days. In coconut milk, bacterial inactivation by AS-48 (1.75 μg/ml) was even faster. In all canned food and drink samples inoculated with intact *G. stearothermophilus* endospores, bacteriocin addition (1.75 μg per g or ml of food sample) rapidly reduced viable cell counts below detection levels and avoided regrowth during storage. After a short-time bacteriocin treatment of endospores, trypsin addition markedly increased *G. stearothermophilus* survival, supporting the effect of residual bacteriocin on the observed loss of viability for endospores. Results from this study support the potential of enterocin AS-48 as a biopreservative against *G. stearothermophilus*.

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## 1. Introduction

Spoilage of canned vegetables is usually caused by thermophilic spore-forming bacteria (Brackett, 2001). Flat sour spoilage will likely occur if canned products contaminated with thermophilic spores are stored at temperatures above 43 °C. Thermophilic bacteria were initially reported to be the prime cause of spoilage of canned corn (Denny, 1981). *Bacillus stearothermophilus* was identified as responsible for spoilage of canned asparagus (Lin et al., 1968). *Geobacillus stearothermophilus* (formerly *B. stearothermophilus*) is a Gram-positive, non-pathogenic, spore-forming bacterium that thrives in high temperature environments. It typically survives canning and sterilization procedures of food products. Spores of *G. stearothermophilus* are extremely heat resistant, up to 20 times more resistant than *Clostridium botulinum* spores (Lin et al., 1968; Feeherry et al., 1987; Lopez et al., 1997; Watanabe et al., 2003; Iciek et al., 2008). *G. stearothermophilus* spores can and do survive thermal processing in a commercially sterile product (Cameron and Esty, 1926; Denny, 1981; Brackett, 2001), and may cause spoilage problems especially where foods must be stored at elevated temperatures for a long time (e.g., canned food vending machines or military operations in tropical climates). Growth of *G. stearothermophilus* spores results in flat sour spoilage because acid is produced but with little or no gas generated (Brackett, 2001). Low-acid foods such as meat and marine products, milk, vegetables, meat and vegetable mixtures (such as soups) can be spoiled by *G. stearothermophilus* under improper storage conditions (Ayres et al., 1980). Furthermore, the capacity of this bacterium to adhere to stainless steel and grow in biofilms appears to be a likely cause of contamination of manufactured dairy products (Flint et al., 2001).

Feeherry et al. (1987) noted that many food products cannot withstand the heat treatment needed to inactivate thermophilic spores (to achieve at least a 6D reduction). Therefore, other measures such as the control of contamination of ingredients by thermophilic organisms, rapid cooling below 43 °C after thermal processing, and controlled storage are required to prevent spoilage. Since bacterial endospores are also resistant to a variety of other treatments such as irradiation, high hydrostatic pressure, pulsed electric fields, and chemicals, alternative methods for inactivation of endospore-formers should be of great value to the food processing industry. Among the proposed methods to avoid the problems of bacterial food spoilage is the application of bacteriocins, as part of hurdle technology (Cleveland et al., 2001; Devlieghere et al., 2004; Gálvez et al., 2007). So far, nisin is the only

* Corresponding author. Present address: Área de Microbiología, Departamento de Ciencias de la Salud, Facultad de Ciencias Experimentales, Edif. B3, Universidad de Jaén, Campus Las Lagunillas s/n, 23071 Jaén, Spain. Tel.: +34 953 212160; fax: +34 953 212943.
*E-mail address:* agalvez@ujaen.es (A. Gálvez).

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bacteriocin reported to inhibit thermophilic bacteria in canned vegetable foods (Thomas et al., 2000). Enterocin AS-48 is a broad-spectrum cyclic antimicrobial peptide (reviewed by Maqueda et al., 2004) which is active against several food spoilage and pathogenic bacteria (Muñoz et al., 2004; Ananou et al., 2005; Cobo Molinos et al., 2005; Grande et al., 2005, 2006a,b, 2007a,b; Lucas et al., 2006; Muñoz et al., 2007). This bacteriocin was very active against *B. coagulans* in canned foods (Lucas et al., 2006), *B. licheniformis* in apple cider (Grande et al., 2006a), *B. cereus* in rice-based foods (Grande et al., 2006b), and *Alicyclobacillus acidoterrestris* in fruit juices (Grande et al., 2005). Treatment with enterocin AS-48 decreased the heat resistance of bacterial endospores for *B. coagulans*, *B. licheniformis* and *B. cereus* (Grande et al., 2006a,b; Lucas et al., 2006), while endospores of *A. acidoterrestris* became inactivated by bacteriocin treatment at room temperature (Grande et al., 2005). These results would suggest that the bacteriocin may also be effective against other endospore-formers in food systems. With these premises, the purpose of the present study was to determine the efficacy of enterocin AS-48 against *G. stearothermophilus* vegetative cells and endospores in different types of canned fruit juice and vegetable foods during storage under temperature abuse conditions.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

*G. stearothermophilus* CECT 43 (type strain) from deteriorated canned food, and *G. stearothermophilus* CECT 49 from canned food with flat sour spoilage were supplied by the Colección Española de Cultivos Tipo (CECT, Burjasot, Valencia, Spain). *Enterococcus faecalis* A-48-32 (Martinez-Bueno et al., 1990) was used to produce enterocin AS-48. *E. faecalis* S-47 was used as an indicator strain for determination of bacteriocin activity. Geobacilli were cultivated routinely on tryptic soy broth (TSB, Scharlab, Barcelona) or tryptic soy agar (TSA, Scharlab) at 45 °C. Enterococci were grown on Brain Heart Infusion broth (BHI, Scharlab) at 37 °C.

### 2.2. Bacteriocin preparation

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). Bacteriocin concentrates were desalted through 2000 mw cut-off dialysis tubing (Sigma) and 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 (Abriouel et al., 2003). 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 reported specific activity value of 3.5 AU/μg protein (Abriouel et al., 2003).

### 2.3. Preparation of inocula and endospore suspensions

*G. stearothermophilus* exponential phase vegetative cells were grown in TSB at 45 °C for 48 h before they were used for inoculation of food and drink samples. In order to obtain endospore preparations, *G. stearothermophilus* cultures were grown in TSA for approximately 10–15 days at 45 °C. The level of sporulation was monitored by staining the spores with a solution of 5% malachite green and then with 0.5% safranin followed by examination under light microscopy. Once sporulation had reached at least 95%, the plates were flooded with sterile distilled water and the growth was scraped off with a sterile swab in order to collect the spores. Spores were washed three times in distilled water and separated by centrifugation (13,000 ×g, for 5 min), and then stored in distilled water at −20 °C until use. The spore concentration was determined by serial dilution and viable cell count on TSA.

### 2.4. Food and drink samples

Canned corn (containing corn grains, water and salt; pH 6.37; Bonduelle Iberica, Alcobendas, Spain), canned peas (containing peas, water, and salt; pH 6.19; Bonduelle Iberica) and coconut milk (containing 33% coconut extract and 67% water; pH 6.37; TIGER TIGER Marketing, Nottingham, UK) were bought from local supermarkets. Cans were opened under aseptic conditions and the content of cans was used for bacteriocin tests.

### 2.5. Bacteriocin treatments

Food and beverage samples (5 g, or 5 ml) in duplicate were inoculated with a cocktail of vegetative cells or endospore suspensions ($3.0–3.5 \times 10^6$ CFU/ml) from strains CECT 43 and 49 prepared as described above, and then supplemented with enterocin AS-48 at desired final concentrations (1.75–7 μg per g or ml of sample). Untreated controls and bacteriocin-treated samples were placed in an incubation chamber with a controlled temperature of 45 °C (Memmert, Schwabach, Germany). At desired intervals, peas and corn samples were homogenised with a Stomacher 80 (Biomaster, Seward, UK) at maximum speed for 30 s. Drink samples were homogenised by vortexing. Samples were serially diluted with sterile saline solution and then plated in triplicate on TSA agar plates and incubated at 45 °C for 5 days. The average number of colonies was used to calculate the viable cell concentrations. These were expressed in $\log_{10}$ colony-forming units (CFU) per gram or ml of solid or liquid food sample, respectively. The detection limit was 10 CFU.

### 2.6. Trypsin rescue of bacteriocin-treated endospores

Since intact endospores treated with the bacteriocin did not produce viable counts, a treatment with trypsin (which inactivates AS-48) was applied in order to determine whether the bacteriocin adsorbed on the spores would be responsible for inactivation during the germination and outgrowth phases, or if intact spores could be inactivated directly by the bacteriocin before germination. Intact endospores of *G. stearothermophilus* CECT 43 were inoculated in TSB broth with or without enterocin AS-48 (0.75 μg/ml) and incubated at 30 °C. After 5 min incubation, the samples were supplemented with trypsin (Sigma, Madrid) (1 mg/ml in 0.1 M phosphate buffer) to a final concentration of 100 μg/ml, or with phosphate buffer alone. After 20 min of further incubation at 30 °C (with or without trypsin), spore suspensions were serially diluted in sterile saline solution and plated for viable counts.

### 2.7. Statistical analysis

The average data from duplicate trials ± standard deviations were determined with Excel programme (Microsoft Corp., USA). In order to determine the statistical significance of the data, a *t*-test was performed at the 95% confidence interval with Statgraphics Plus version 5.1 (Statistical Graphics Corp, USA). The significance of combined treatments was determined by comparison of data from the same incubation time.

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