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Efficacy of Enterocin AS-48 against Bacilli in Ready-to-Eat Vegetable Soups and Purees

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ABSTRACT

The broad-spectrum bacteriocin enterocin AS-48 was tested for biopreservation of ready-to-eat vegetable foods (soups and purees) against aerobic mesophilic endospore-forming bacteria. By adding AS-48 (10 μg/ml), *Bacillus cereus* LWL1 was completely inhibited in all six vegetable products tested (natural vegetable cream, asparagus cream, traditional soup, homemade-style traditional soup, vegetable soup, and vichyssoise) for up to 30 days at 6, 15, and 22°C. A collection of strains isolated from spoiled purees showed slightly higher resistance to AS-48 in the order *Paenibacillus* sp. > *Bacillus macroides* > *B. cereus,* although they were also completely inhibited in natural vegetable cream by AS-48 at 10 μg/ml. However, cocktails of five or eight strains composed of *B. cereus* (three strains), *B. macroides* (two strains), and *Paenibacillus* sp., *Paenibacillus polymyxa,* and *Paenibacillus amylolyticus* showed higher bacteriocin resistance with AS-48 of up to 50 μg/ml required for complete inactivation in natural vegetable cream stored at 22°C. Repetitive extragenic palindromic sequence-based PCR (REP-PCR) analysis showed that paenibacilli (along with some *B. cereus*) was the predominant survivor in the cocktails after bacteriocin treatment. To increase the effectiveness of enterocin AS-48, the bacteriocin was tested (at 20 μg/ml) against the eight-strain cocktail in natural vegetable cream in combination with other antimicrobials. The combination of AS-48 and nisin had a slight but significant additive effect. Bactericidal activity was greatly enhanced by phenolic compounds (carvacrol, eugenol, geraniol, and hydrocinnamic acid), achieving a rapid and complete inactivation of bacilli in the tested puree at 22°C.

Endospore-forming bacteria are often considered food safety hazards because of their wide distribution in nature, frequency in raw materials, and higher resistance of the endospores to treatments applied during food processing. *Bacillus cereus* is the most important cause of food poisoning within the genus *(43). B. cereus* produces one emetic and at least three diarrheic toxins *(11, 18, 21, 25, 43),* and it may cause two types of gastrointestinal disorders: the emetic syndrome, caused by the ingestion of a preformed toxin in the food, and the diarrheal syndrome, caused by a different toxin that can be formed in the food but also in the small intestine *(18, 19). B. cereus* spores survive pasteurization processes. Decimal reduction times at 90°C of 2.2 to 9.2 min have been reported *(9).* In addition, vegetative cells from some strains can grow at 4 to 5°C *(6, 9).* Strains of *Bacillus subtilis, Bacillus pumilus,* and *Bacillus licheniformis* have also been linked to incidents of foodborne illness *(23, 32, 41).* Enterotoxin production has been demonstrated for *Bacillus* sp. strains *B. licheniformis, B. megaterium, B. firmus,* and *B. simplex (41, 43, 44),* as well as for *B. subtilis, B. mojavensis, B. pumilus,* and *B. fusiformis (12),* and in some cases, the enterotoxins produced by *Bacillus* spp. other than *B. cereus* are believed to be transcribed from genes that are similar to those of *B. cereus* enterotoxins *(33, 39, 40).* In view of the high molecular polymorphism of *B. cereus* enterotoxin genes *(10),* the genetic background and toxicogenic potential of other species related to *B. cereus* need to be reevaluated. Furthermore, endospore-forming bacteria are of concern in food spoilage, especially in cooked, chilled foods. In commercial purees, several species of *Bacillus* and its relatives have been identified and shown to cause food spoilage during abuse temperature storage *(5, 20).*

One of the current approaches under study to control endospore-forming bacteria in foods is based on the use of natural antimicrobial substances such as bacteriocins *(7, 8).* The bacteriocin enterocin AS-48 from *Enterococcus faecalis* is a broad-spectrum cyclic peptide that has been characterized in depth as far as its composition and structure, mode of action, and genetic determinants (reviewed by Maqueda et al. *(26)*). Enterocin AS-48 is active against different species of bacilli *(14),* and its inhibitory effects against *B. cereus* in broth, milk, cheese, lettuce juice, and rice-based foods, as well as *Bacillus coagulans* in canned fruit and vegetable foods, have been described previously *(1, 15, 17, 24, 29).* However, because the efficacy of bacteriocins greatly depends on the target bacteria and the food substrate, further research was carried out in the present study to evaluate the effectiveness of enterocin AS-48 on different aerobic mesophilic endospore formers (including

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different strains of *B. cereus, Bacillus macroides,* and *Paenibacillus*) in ready-to-eat vegetable soups and purees and to assess the complexity of the bacterial population on bacteriocin sensitivity. The combined action of enterocin AS-48 with other natural antimicrobials such as nisin and phenolic compounds was also tested as a way to enhance inhibition of a mixture of bacilli in the food system under study.

MATERIALS AND METHODS

Bacterial strains and cultivation conditions. *E. faecalis* A-48–32 *(27)* was used for bacteriocin production. *B. cereus* LWL1, LWL3, and LWL10 were kindly supplied by Dr. F. M. van Leusden (Microbiological Lab. for Health Protection, National Institute of Public Health and Environment, The Netherlands). *B. macroides* P53-2, *B. macroides* P51-5, *Paenibacillus* sp. P51-3, *Paenibacillus amylolyticus* P52-3, and *Paenibacillus polymyxa* P12-5 from spoiled vegetable purees *(20)* were kindly supplied by Dr. M. H. Guinebretiere (INRA, Avignon, France). Bacterial strains were cultivated routinely on brain heart infusion broth (Scharlab, Barcelona, Spain) or tryptic soy agar (TSA; Scharlab) at 30°C and stored at 4°C.

Food samples. Commercial vegetable foods were purchased at local supermarkets: traditional soup (pH 5.85; Don Simon, Murcia, Spain), homemade-style traditional soup (pH 5.84; Don Simon), vegetable soup (pH 5.9; Carrefour), natural vegetable cream (pH 6.13; Don Simon), asparagus cream (pH 6.17; Don Simon), and vichyssoise (pH 6.24; Gallina Blanca, Barcelona, Spain). The first three contained different mixtures of vegetables as main ingredients plus olive oil. The natural vegetable cream as well as asparagus cream contained milk cream in addition to olive oil and vegetables, while the vichyssoise contained vegetables plus whole milk, vegetable oil, and butter. Vegetable foods were commercially sterilized and ready-to-eat, and they were sold at ambient temperature in 1-liter packs. Food packages were cleaned with a sterile swab dipped in 70% ethanol and opened under sterile conditions right before use. Once opened, they were kept at 4°C for no longer than 7 days.

Bacteriocin preparation. Partially purified bacteriocin concentrates were obtained from cultured broths of the producer strain, followed by cation exchange chromatography as described by Abriouel et al. *(2)* and filtration and titration for bacteriocin activity as described elsewhere *(15).*

Bacteriocin treatments. Bacterial strains were grown overnight in brain heart infusion broth at 30°C. Overnight cultures (containing mostly vegetative cells) were diluted in a sterile saline solution and added to the vegetable soups being tested to achieve an initial cell concentration of 10^3 to 10^5 CFU/ml. Bacilli were tested individually or as a cocktail of five strains (composed of *B. cereus* and *B. macroides* strains) or eight strains (which, in addition, included *Paenibacillus* strains). Before inoculation, foods were incubated for 1 h at the desired temperature. The artificially contaminated foods were supplemented with enterocin AS-48 to the desired final concentration, mixed thoroughly, distributed in hermetically sealed sterile plastic test tubes (5 ml per tube), and then incubated at the desired temperatures. At specific times during incubation, duplicate samples were removed for each treatment and serially diluted in ice-cold sterile saline solution. Serial dilutions were plated in triplicate on TSA plates and incubated at 30°C for 48 h. The average viable cell counts were expressed as the log CFU per milliliter of sample.

REP-PCR typing. Bacterial colonies representative of mixed cultures (taken as the square-root number of colonies grown on TSA plates) were purified, and total bacterial DNA was extracted by the method of Pitcher et al. *(35),* with an additional step involving RNase at the end of the procedure. Repetitive extragenic palindromic sequence-based PCR (REP-PCR) was carried out with primers REP 1R-Dt and REP 2-Dt *(20)* in a final volume of 25 μl containing 45 pmol of each primer, 200 μM (each) deoxynucleoside triphosphates (Amersham Biosciences, Barcelona, Spain), 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.35 U of *Taq* DNA polymerase (Amersham). Amplifications were performed in a PCR thermocycler (Mastercycler Personal, Eppendorf, Madrid, Spain) with the following program: 95°C for 3 min and then 30 cycles at 90°C for 30 s, at 40°C for 1 min, and at 72°C for 1 min with a ramping of 6 min between 40 and 72°C, followed by a final extension of 8 min at 72°C. PCR products (15 μl) were separated on 1.5% agarose gel containing 1× Tris-borate-EDTA buffer *(42).* A mix of 100- and 500-bp DNA ladder (BioRad, Madrid, Spain) was used as the size standard marker. The REP-PCR profiles were visualized after staining with ethidium bromide (1 mg/ml).

Effect of enterocin AS-48 in combination with nisin and phenolic compounds. A cocktail containing all eight strains of bacilli under study was inoculated on natural vegetable cream. Nisaplin powder (Danisco España, Barcelona, Spain) was added to achieve a 1.5% (wt/vol) final concentration. Phenolic compounds (Fluka, Barcelona, Spain) were added to the food (1.0%, vol/vol) directly from commercial solutions at the final concentrations indicated in parentheses: carvacrol (63.1 mM), eugenol (64.4 mM), and geraniol (56.5 mM). Hydrocinnamic acid (20 mM) was added from freshly made 1 M solutions dissolved in propylene glycol (Fluka). The different antimicrobials were tested alone or in combination with enterocin AS-48 at a subinhibitory concentration (20 μg/ml) for the cocktail of strains being tested. Viable cell counts were determined after a 48-h incubation of samples at 30°C.

Statistical analyses. The average data of duplicate experiments ± standard deviations were determined by an Excel program (Microsoft Corp., Redmond, Wash.). To determine the statistical significance of data, a *t* test was performed at the 95% confidence interval with Statgraphics Plus version 5.1 (Statistical Graphics Corp., Princeton, N.J.).

RESULTS

Effect of enterocin AS-48 on *B. cereus* LWL1 in different types of vegetable foods. *B. cereus* vegetative cells were inoculated in the six different ready-to-eat vegetable foods under study supplemented with enterocin AS-48 (10 μg/ml) and stored at 6, 15, and 22°C. After 24 h of storage with AS-48 at 10 μg/ml, no viable bacilli were detected in any of the six tested vegetable foods after 24 h, regardless of the storage temperature (data not shown).

Effect of enterocin AS-48 on the different strains tested individually. The different strains under study were inoculated separately in natural vegetable cream supplemented with bacteriocin concentrations of 5 and 10 μg/ml (Fig. 1). Survival was determined at different intervals of incubation at 22°C. All strains were rapidly inactivated by a bacteriocin concentration of 10 μg/ml and remained below detectable levels for the entire incubation period (Fig. 1A through 1C). However, differences in strain sensitivities

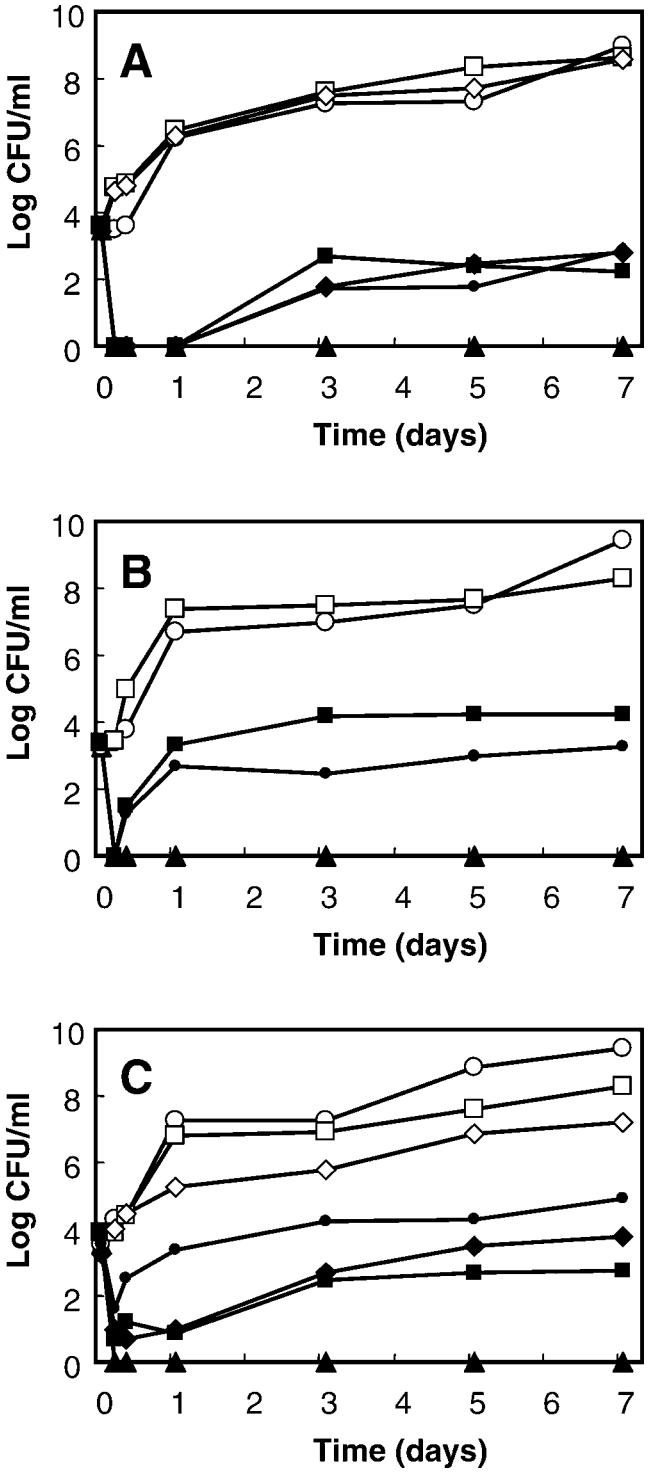

FIGURE 1. *Sensitivity of individual strains to enterocin AS-48 in natural vegetable cream at 22°C. (A)* B. cereus *LWL1 (○), LWL3 (◇), and LWL10 (□). (B)* B. macroides *P51-5 (□) and P53-2 (○). (C)* P. polymyxa *P12-5 (○),* P. amylolyticus *P52-3 (□), and* Paenibacillus *sp. P51-3 (◇). Open symbols: controls. Closed symbols: samples supplemented with AS-48 at 5 μg/ml. Cultures treated with AS-48 at 10 μg/ml (▲).*

were observed at a lower bacteriocin concentration (5 μg/ml), depending mainly on the species and genus being tested. For *B. cereus,* viable counts remained below detectable levels from 4 to 24 h of incubation for the three strains tested (LWL1, LWL3, and LWL10), followed by regrowth of the population to ca. 2 to 3 log units per ml after day 3 (Fig. 1A). Strains of *B. macroides* showed slightly lower sensitivity, with regrowth observed after 8 h of incubation (Fig. 1B). Lowest sensitivity was obtained for strains of *Paenibacillus,* where AS-48 (5 μg/ml) never reduced viable counts below the detection limit (Fig. 1C). Among these, *Paenibacillus* sp. P51-3 was the most resistant strain, as indicated by the lower reduction of viable cell counts during early incubation and faster regrowth of survivors (Fig. 1C). No viable bacteria were detected in the natural vegetable cream without added bacilli used as a negative control (data not shown).

Effect of enterocin AS-48 on mixed populations of endospore formers at different storage temperatures. In natural vegetable cream stored at 6°C, a bacteriocin concentration of 12.5 μg/ml reduced viable cell counts below detectable levels by day 7, both for the mixed population of five (*B. cereus* and *B. macroides* strains) as well as eight strains (*B. cereus, B. macroides,* and *Paenibacillus* strains), although regrowth of cultures was observed during prolonged incubation in both cases (Fig. 2A and 2D). For AS-48 (25 μg/ml), the reduction in viable counts was permanent during the entire 30-day storage period (Fig. 2A and 2D).

For samples stored at 15°C, growth in the controls was faster than in samples containing bacteriocin. For the cocktail of five strains, a bacteriocin concentration of 25 μg/ml reduced viable cell counts below detectable levels until the end of the storage period, but it failed to prevent regrowth in samples containing the eight-strain cocktail (Fig. 2B and 2E). The lowest inhibitory effects were obtained at 22°C, in which AS-48 at 25 μg/ml failed to inhibit proliferation of bacilli for both cocktails of strains (Fig. 2C and 2F). Nevertheless, a higher bacteriocin concentration (50 μg/ml) rapidly reduced the concentration of bacilli below detection levels and prevented regrowth during 30 days of storage (Fig. 2C and 2F).

The identity of survivors after bacteriocin treatment was investigated by REP-PCR with primers previously described by Guinebretiere et al. *(20).* The three strains of *B. cereus* tested showed highly similar band patterns but were clearly different from the remaining strains (Fig. 3A). *B. macroides* P51-5 and P52-3 showed clearly distinctive patterns. *P. polymyxa* P12-5 also showed a distinctive pattern, but *Paenibacillus* sp. P51-3 and *P. amylolyticus* P52-3 showed more variable patterns after repeated DNA extraction and amplification, presumably due to variations in extraction. The colonies isolated from plates corresponding to bacteriocin-treated samples stored at 6°C showed REP-PCR profiles corresponding to *B. cereus, P. polymyxa,* and *P. amylolyticus* strains (Fig. 3B). However, after prolonged incubation, the surviving populations became more homogeneous, with typical small colonies on agar plates showing REP-PCR profiles corresponding to *P. polymyxa* P12-5 as well as *Paenibacillus* sp. P51-3 (Fig. 3C). Similarly, paenibacilli were the predominant survivors in samples stored at 15 as well as 22°C during prolonged incubation, along with some colonies exhibiting REP-PCR patterns typical of *B. cereus* (Fig. 3D).

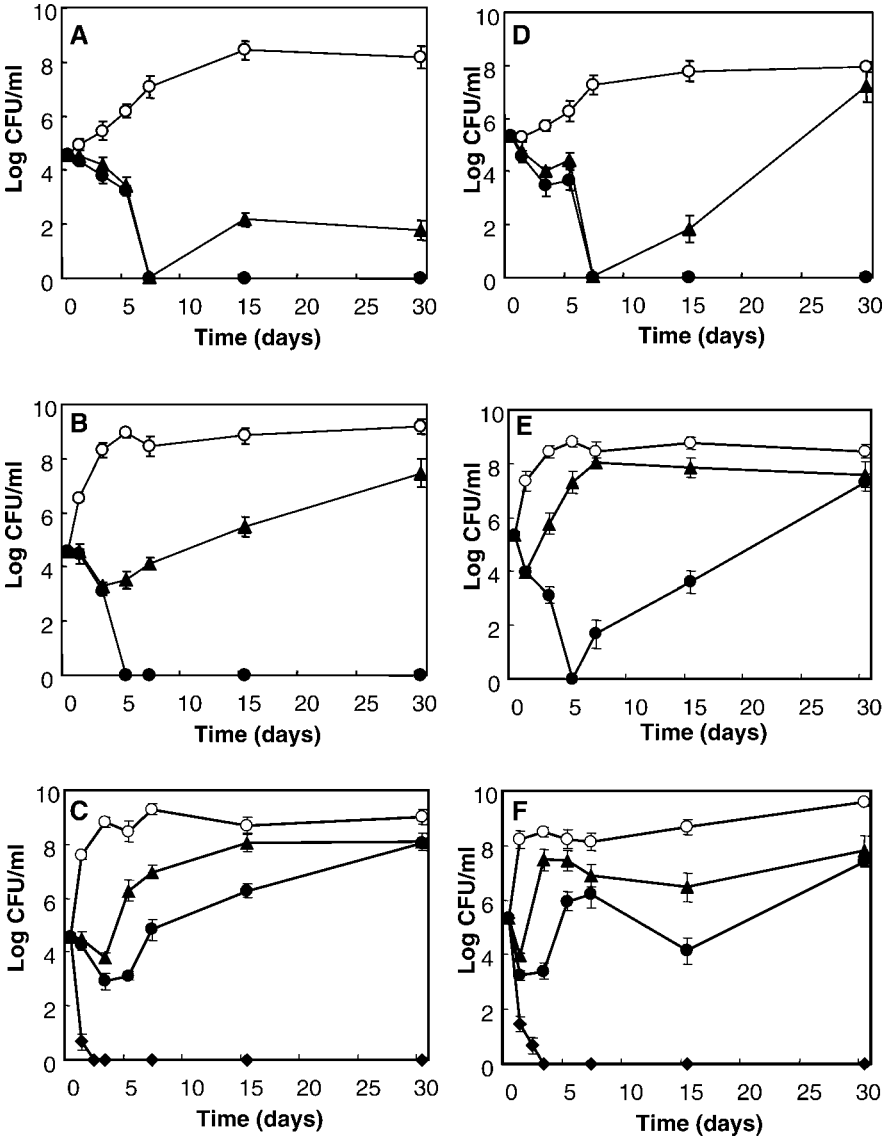

FIGURE 2. *Effect of enterocin AS-48 at final concentrations of 12.5 (▲), 25 (●), or 50 (♦) μg/ml on a mixture of* Bacillus cereus *LWL1,* B. cereus *LWL3,* B. cereus *LWL10,* B. macroides *P53-2, and* B. macroides *P51-5 (A, B, C) or* B. cereus *LWL1,* B. cereus *LWL3,* B. cereus *LWL10,* B. macroides *P53-2,* B. macroides *P51-5,* Paenobacillus *sp. P51-3,* P. amylolyticus *P52-3, and* P. polymyxa *P12-5 (D, E, F) inoculated on natural vegetable cream and stored at temperatures of 6°C (A, D), 15°C (B, E), or 22°C (C, F). Controls (○). The average data of duplicate experiments ± standard deviations (error bars) are shown.*

Combined effect of AS-48 with other antimicrobial substances. The cocktail of eight strains was tested for sensitivity to enterocin AS-48 (at a subinhibitory concentration of 20 μg/ml) in combination with other antimicrobial substances in natural vegetable cream stored at 22°C. Although the addition of 1.5% nisaplin reduced the viable counts of bacilli below detection levels at day 1 of incubation, a progressive regrowth of cultures was observed after prolonged incubation (Fig. 4A). No recovery was observed for a higher nisaplin concentration of 2.0% (data not shown). For the combination of AS-48 (20 μg/ml) and nisaplin (1.5%), viable counts were significantly lower ($P < 0.05$) at days 3, 5, and 7 compared with samples treated with nisaplin alone (Fig. 4A), suggesting an additive effect. However, in spite of the additive effect, there was no complete inhibition of bacilli.

Carvacrol, eugenol, and hydrocinnamic acid had only slight inhibitory effects on growth of the bacilli cocktail during the first 3 days of incubation, whereas geraniol had a higher inhibitory effect (Fig. 4B). After prolonged incubation (14 days) with phenolic compounds, however, viable cell counts decreased below detection levels in all cases until the end of incubation period. The inhibitory effect of enterocin AS-48 was potentiated by phenolic compounds in all cases. This effect was clearly visible within the first 3 days of incubation, in which no viable cells were detected for any of the combinations of bacteriocin and phenolic compounds, as shown in Figure 4B.

DISCUSSION

Endospore-forming bacteria are one of the major threats in the food industry because of their wide distribution in soil and plant materials, the resistance of endospores to most of the food processing treatments, and the implication of endospore formers in food spoilage and poisoning. In the present study, the effect of enterocin AS-48 against individual strains as well as a cocktail of bacilli was tested in ready-to-eat vegetable foods. In agreement with a previous report on rice-based foods *(15), B. cereus* was completely inhibited in all types of vegetables tested for up to 30 days, even at a much lower bacteriocin concentration of 10 μg/ml. Results from the present study also indicate that AS-48 is inhibitory to other bacilli not tested previously, such as strains of *B. macroides* as well as *Paeniba-*

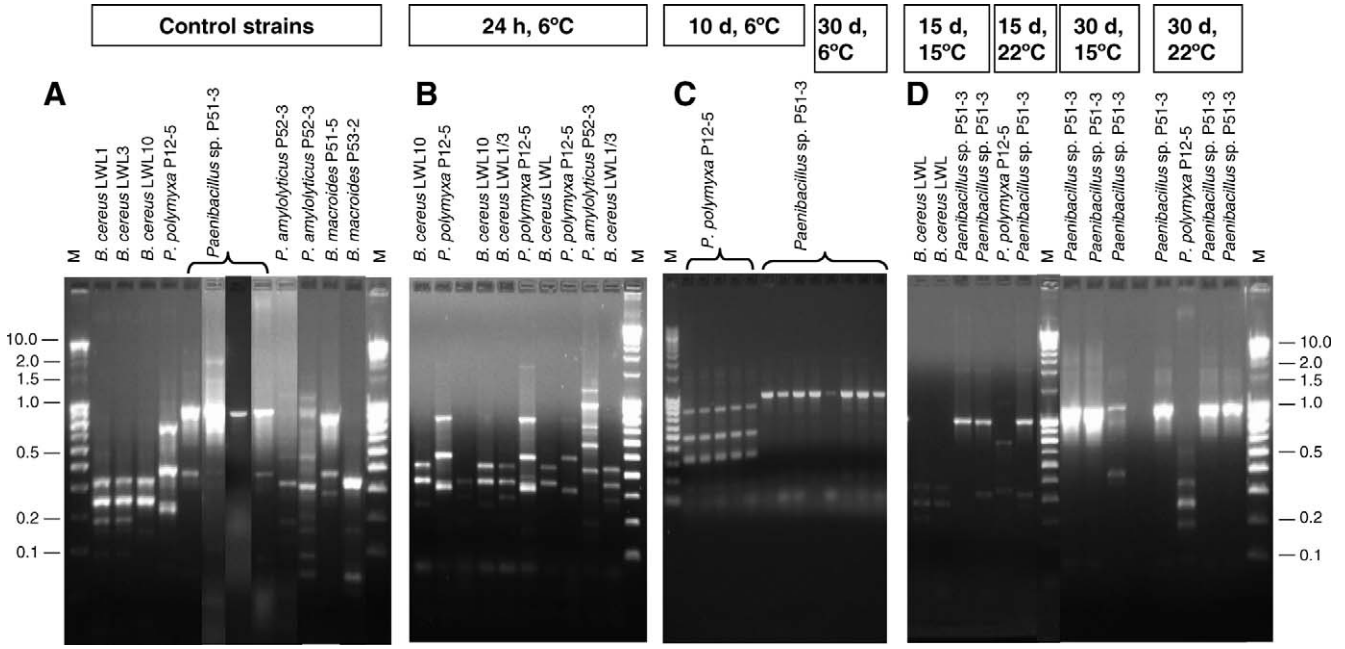

FIGURE 3. *REP-PCR profiles obtained for control strains used as a cocktail in natural vegetable cream (A) and survivors after bacteriocin treatments for samples stored at 6°C (B, C) as well as 15 and 22°C (D). M, mix of 100-bp and 1-kb molecular marker (Bioline).*

cillus isolated from zucchini purees, although paenibacilli showed higher bacteriocin resistance.

Because vegetable purees may contain a mixture of different bacteria *(20)* and there were no previous reports on the activity of AS-48 on mixed populations of bacilli, our interest was to determine the effects of this bacteriocin on a cocktail that included toxigenic *B. cereus* strains as well as other strains isolated from spoiled purees (*B. macroides* and *Paenibacillus* strains). As substrate, we chose a commercial natural vegetable soup containing spinach, carrot, green beans, peas, broccoli, leek, potato, onion, and garlic. Comparatively, the cocktail of strains showed a much higher resistance to the added bacteriocin than the strains tested individually, especially at 22°C, and bacteriocin resistance seemed to increase as more strains were included in the cocktail and the inoculum was higher, requiring up to 50 μg/ml for an effective and rapid inactivation. These results are of great relevance for application of enterocin AS-48 in foods and suggest that bacteriocin concentrations high enough to provide the necessary safety margins should be applied where different strains or species of the target bacterium coexist.

It is also interesting to know which of the target bacteria in the mixed population show an increased resistance. To address this question, we carried out a comparative study of REP-PCR profiles obtained for individual colonies isolated from viable cell count plates under different incubation conditions. The results obtained clearly indicated that paenibacilli were the predominant bacteria surviving bacteriocin treatment, in agreement with their higher resistance shown in preliminary experiments. However, some colonies of *B. cereus* were also isolated, indicating a risk for enterotoxin production. Analysis of REP-PCR profiles has been used previously to compare the capacity for proliferation of spoilage bacilli in vegetable foods under different storage temperatures *(20),* but, to our knowledge, this is the first example of application of this method to study survival during a bacteriocin treatment.

To increase the effectiveness of enterocin AS-48 against the cocktail of bacilli, the bacteriocin was tested in combination with other antimicrobials. The activity of bacteriocins can be potentiated by combination with other antimicrobial substances or treatments, as in the hurdle technology concept, to decrease the amount of inhibitory agents being added or the intensity of treatments. As an example, two or more bacteriocins can be used in combination to increase their antimicrobial activity and spectrum of inhibition and to avoid regrowth of survivors as well as to provide a barrier to the emergence of bacteriocin-resistant cells. Previous reports indicated that the simultaneous use of nisin with pediocin AcH *(22)* or with leucocin F10 *(31)* as well as lactacin B or lactacin F with nisin or pediocin AcH and lactacin 481–pediocin AcH *(28)* provides greater antibacterial activity than each bacteriocin separately. The combination of nisin and curvaticin 13 also induced a greater inhibitory effect against *L. monocytogenes* than each bacteriocin individually *(3).* The present study is the first report on the combination of enterocin AS-48 with nisin. However, the results obtained suggest that both bacteriocins have a slightly additive effect when used in combination, at least against the cocktail of bacilli tested.

One group of candidate antimicrobial substances for food preservation are the phenolic compounds, which are naturally found in many different essential oils *(4).* Phenolic compounds can be used to enhance bacteriocin activity. Nisin has been reported to act synergistically with carvacrol or thymol against *B. cereus (33, 37).* Combinations of nisin with carvacrol, eugenol, or thymol resulted in syn-

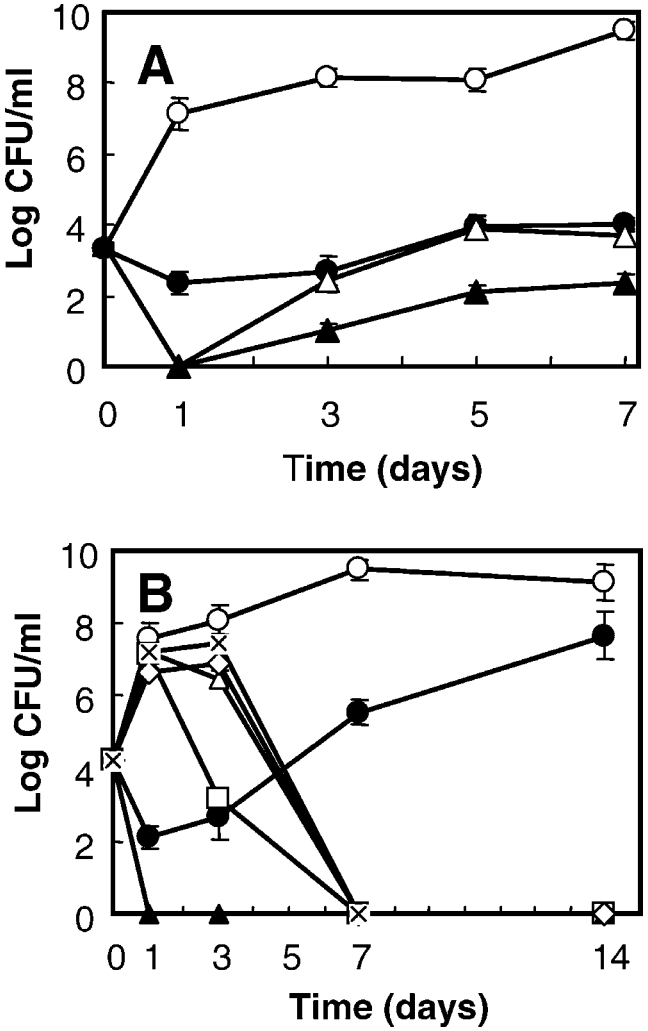

FIGURE 4. *Effect of enterocin AS-48 (20 μg/ml) on a cocktail of eight strains in natural vegetable cream at 22°C in combination with other antimicrobial substances. (A) Effect of 1.5% nisaplin (Δ) or nisaplin plus AS-48 (▲). (B) Effect of the phenolic compounds carvacrol (△), eugenol (×), geraniol (□), and hydrocinnamic acid (◇) alone. Each of the phenolic compounds in combination with AS-48 caused complete inactivation of bacilli (▲). Control cultures without additives (○). Effect of enterocin AS-48 alone (●). The average data of duplicate experiments ± standard deviations (error bars) are shown.*

ergistic action against *B. subtilis,* whereas nisin and cinnamic acid had only an additive effect *(30).* The antimicrobial activity of enterocin AS-48 against *Staphylococcus aureus* cells in vegetable sauces was potentiated significantly in combination with carvacrol, geraniol, eugenol, terpineol, caffeic acid, *p*-coumaric acid, citral, and hydrocinnamic acid *(16).* In the present study, enterocin AS-48 activity against a cocktail of bacilli was greatly potentiated by all phenolic compounds tested, rapidly reducing the number of viable cells in the food. This suggests that enterocin AS-48 and phenolic compounds can have a complementary mode of action. It has been reported that carvacrol interacts with the *B. cereus* cell membrane, where it dissolves in the phospholipid bilayer and is assumed to align between the fatty acid chains *(46).* This distortion of the physical structure would cause expansion and destabilization of the membrane, increasing membrane fluidity, which in turn would increase passive permeability *(45).* It has also been suggested that synergy between nisin and carvacrol lies in the enhanced dissipation of the membrane potential and a reduction in the pH gradient and intracellular ATP *(36).* A similar mechanism could also account for the observed potentiation of enterocin AS-48 activity, because this bacteriocin also dissipates the bacterial membrane potential *(13).* The combination of enterocin AS-48 and selected phenolic compounds could be very useful for food biopreservation under certain conditions, especially when the bacteriocin shows a lower efficacy due to the complexity of the food microbiota and the food storage temperature and when a high amount of added bacteriocin is required to effectively inhibit the target bacteria. Although phenolic compounds may impart undesirable flavor to the food, this may depend greatly on the type of food and the amount of compound added *(4).* As an example, treatment of fresh fruit with 1 mM carvacrol or cinnamic acid was found to delay spoilage without causing adverse organoleptic changes *(38).* For precise food applications, the concentrations of enterocin AS-48 and selected phenolic compounds could be adjusted to maximize their antibacterial activity at a concentration that does not produce undesirable changes in flavor or aroma.

In conclusion, results from the present study clearly indicate that the effectiveness of enterocin AS-48 depends greatly on the complexity of the food microbiota and the environmental conditions. Also, when a lower effectiveness requires the addition of a higher bacteriocin concentration, this can be compensated for by combinations with other selected antimicrobial substances.

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