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Stability of Enterocin AS-48 in Fruit and Vegetable Juices

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ABSTRACT

Enterocin AS-48 is a candidate bacteriocin for food biopreservation. Before addressing application of AS-48 to vegetable-based foods, the interaction between AS-48 and vegetable food components and the stability of AS-48 were studied. Enterocin AS-48 had variable interactions with fruit and vegetable juices, with complete, partial, or negligible loss of activity. For some juices, loss of activity was ameliorated by increasing the bacteriocin concentration, diluting the juice, or applying a heat pretreatment. In juices obtained from cabbage, cauliflower, lettuce, green beans, celery, and avocado, AS-48 was very stable for the first 24 to 48 h of storage under refrigeration, and decay of activity was markedly influenced by storage temperature. In fresh-made fruit juices (orange, apple, grapefruit, pear, pineapple, and kiwi) and juice mixtures, AS-48 was very stable for at least 15 days at 4°C, and bacteriocin activity was still detectable after 30 days of storage. Gradual and variable loss of activity occurred in juices stored at 15 and 28°C; inactivation was faster at higher temperatures. In commercial fruit juices (orange, apple, peach, and pineapple) stored at 4°C, the bacteriocin was completely stable for up to 120 days, and over 60% of initial activity was still present in juices stored at 15°C for the same period. Commercial fruit juices stored at 28°C for 120 days retained between 31.5% (apple) and 67.71% (peach) of their initial bacteriocin activity. Solutions of AS-48 in sterile distilled water were stable (120 days at 4 to 28°C). Limited loss of activity was observed after mixing AS-48 with some food-grade dyes and thickening agents. Enterocin AS-48 added to lettuce juice incubated at 15°C reduced viable counts of *Listeria monocytogenes* CECT 4032 and *Bacillus cereus* LWL1 to below detection limits and markedly reduced viable counts of *Staphylococcus aureus* CECT 976.

The demands of consumers for ready-to-eat minimally processed fresh foods and drinks has prompted the food processing industry to develop new preservation strategies that can efficiently lower the risk of food spoilage and the transmission of foodborne pathogens. The use of natural antimicrobial substances for food preservation is an alternative to other more drastic preservation methods, which have a limited range of applications especially in vegetable foods and drinks *(9).* The use of bacteriocins as natural preservatives has been studied extensively for foods of meat and dairy origin, but much less work has been done for vegetable-based foods and drinks. The activity of bacteriocins in foods may be limited by several factors such as inactivation, binding to food components, and poor distribution in the food matrix, but most of the data available come from assays carried out in dairy and meat products *(1, 14).* Therefore, further studies are necessary to understand the activity and stability of bacteriocins in vegetable-based foods before carrying out application studies to control selected spoilage or pathogenic bacteria that occur in these foods.

The purpose of this work was to evaluate the stability of enterocin AS-48 in several types of vegetable-based foods as a preliminary step to in-depth studies of food application. Enterocin AS-48 is a broad-spectrum cyclic peptide produced by *Enterococcus faecalis* S-48 *(13)* and its mutant strain A-48-32 *(21).* The genetic determinants and peptide structure of this bacteriocin have been described, as has its mode of action and antimicrobial activity against foodborne pathogenic bacteria such as *Bacillus cereus, Listeria monocytogenes, Staphylococcus aureus,* and *Salmonella* Choleraesuis *(20).* The available data on this bacteriocin have provided a sound basis for the study of its use in foods, and preliminary studies carried out in dairy foods have already proven the ability of AS-48 to control *Bacillus cereus (23).* The results presented here may help to establish the conditions for optimal use of this bacteriocin in vegetable-based foods and fruit juices.

MATERIALS AND METHODS

Strains and cultivation conditions. *E. faecalis* strain A-48-32 *(21)* was used for bacteriocin production. *E. faecalis* strain S-47 from our collection was used for standard determination of bacteriocin activity. *L. monocytogenes* CECT 4032 and *S. aureus* CECT 976 were supplied by the Spanish Type Culture Collection (CECT, Valencia, Spain). *B. cereus* LWL1 was kindly supplied by Dr F. M. van Leusden (Microbiological Laboratory for Health Protection, National Institute for Public Health and the Environment, Bilthoven, The Netherlands). Bacterial strains were cultivated routinely on brain heart infusion broth (Scharlab, Barcelona, Spain) at 37°C and stored at 4°C.

Bacteriocin production and assay. Partially purified bacteriocin concentrates were obtained from cultured broths of pro-

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TABLE 1. *Interaction of enterocin AS-48 with fruit and vegetable juices*[a]

Fruit or vegetable juice	pH	Residual activity (%)[b]
Control (distilled water)		100.00 ± 1.0
Chard	6.61	0
Celery	5.73	85.71 ± 3.5
Broccoli	6.60	0
Leek	5.39	50.05 ± 3.8
Cabbage	6.20	100.02 ± 0.5
Cauliflower	6.60	18.57 ± 7.5
Endive	5.98	34.28 ± 3.0
Lettuce	5.96	40.05 ± 3.5
Spinach	6.41	40.05 ± 1.5
Broad bean	6.50	0
Green bean	5.86	57.14 ± 3.0
Asparagus	5.84	18.57 ± 4.5
Mushroom	6.41	0
Potato	6.15	0
Carrot	6.36	0
Tender onion	5.82	0
Avocado	6.33	57.14 ± 2.5
Artichoke	6.24	0
Caper	4.10	0
Eggplant	5.26	40.02 ± 5.0
Pumpkin	5.57	34.28 ± 1.5
Tomato	4.30	0
Cucumber	5.44	0
Red pepper	4.81	0
Kiwi	3.16	85.71 ± 2.5
Pineapple	3.39	67.14 ± 7.5
Grapefruit	4.07	85.71 ± 5.0
Orange	3.79	79.18 ± 6.5
Apple	3.55	100 ± 1.0
Pear	4.32	100 ± 0.5
Peach	4.02	100 ± 0.5

[a] Bacteriocin concentrate was diluted 10-fold in juices and tested for residual activity against *E. faecalis* S-47. The percentage of residual bacteriocin activity was calculated with respect to a control sample diluted in distilled water.

[b] Values are the mean ± SD of triplicate samples.

ducer strains processed by cation exchange chromatography as described by Abriouel et al. *(3)*. Bacteriocin preparations were filtered through low-protein-binding filters (Millex GV, Millipore Corp., Belford, Mass.) before use. Samples were serially diluted and tested for bacteriocin activity by the agar-well diffusion method *(12)* in 8-mm-diameter wells with *E. faecalis* S-47 as the test strain. One 100-μl sample was placed in each well. The detection limit of the assay was one arbitrary unit (AU), defined as the highest dilution that produced a visible zone of inhibition (9 mm diameter). A titration curve was obtained after plotting the zones of inhibition obtained versus the reciprocal of the dilution.

Preparation of fruit and vegetable juices. Fruits and vegetables were purchased at local stores, and fresh juices were prepared with a Citroplus (Moulinex, Slough, Berkshire, UK) and a Frutti Pro (Moulinex) fruit juice extractor, respectively, under aseptic conditions. Fruit juice mixtures were obtained by mixing equal portions (vol/vol) of the freshly made juices. Commercial orange juice (Kasfruit, Vitoria, Spain; pH 4.12), apple juice (Don Simón, Murcia, Spain; pH 3.92), peach juice (Kasfruit; pH 4.02), and pineapple juice (Kasfruit; pH 3.68) were also used.

Determination of bacteriocin interaction and stability in fruit and vegetable juices. To test bacteriocin interactions with fruit and vegetable juices, partially purified bacteriocin was diluted in sterile distilled water (control) or in juice and mixed for 1 min at room temperature before being tested for residual activity.

Bacteriocin stability in fruit and vegetable juices was determined as follows. Juice samples containing a titratable bacteriocin concentration of 70 AU/ml were distributed in sterile Eppendorf test tubes (1-ml sample per tube) and stored at temperatures of 4 and 15°C in a refrigerated incubation chamber and at 28°C. After specified incubation periods, samples were tested for residual bacteriocin activity. The percentage of residual activity was calculated by comparison with control activity (AS-48 diluted in sterile distilled water) at time 0 of incubation. All experiments were carried out in triplicate. Fruit and vegetable juices without added bacteriocin were tested as negative controls.

Effect of thermal pretreatment of vegetable juices on bacteriocin activity. Vegetable juices were heated by immersion in a water bath (Memmert, Schwabach, Germany) at 72°C for 10 min or at 98°C for 5 min and then cooled under running tap water before addition of AS-48. Immediately after addition of the bacteriocin, residual bacteriocin activity was determined.

Determination of protease activity in fruit and vegetable juices. Proteolytic activity was determined using casein as the substrate. A 0.5-ml aliquot of 10 mg/ml casein (Sigma, Madrid, Spain) in 50 mM sodium phosphate buffer, pH 7.2 (PanReac, Barcelona, Spain) was mixed with 0.1 ml of juice and incubated at 45°C for 2 h. The reaction was stopped by addition of 0.5 ml of 20% (wt/vol) trichloroacetic acid (Sigma). The unreacted precipitated substrate was removed by centrifugation at 12,000 × *g* for 10 min at room temperature, and the absorbance of the supernatant was read at 280 nm. An arbitrary enzyme unit (U) was defined as the amount of enzyme that produces an increase of 0.01 absorbance unit (1 cm light-path) per minute under the assay conditions.

Interaction of AS-48 with food-grade dyes and thickening agents. Aqueous solutions of different dyes and thickening agents (DOMCA S.A., Granada, Spain) were mixed with AS-48 (70 AU/ml final concentration) and then tested for bacteriocin activity. Dye solutions without added bacteriocin were tested as negative controls.

Antimicrobial activity of AS-48 against selected bacterial pathogens in vegetable juice. Overnight cultures of *B. cereus* LWL1, *L. monocytogenes* CECT 4032, and *S. aureus* CECT 976 were used separately to inoculate (0.02%, vol/vol) freshly prepared lettuce juice (pH 5.96). After addition of AS-48 (70 AU/ml final concentration), the samples were incubated at 15°C. At specified incubation times, aliquots of controls (without AS-48) and bacteriocin-treated samples were serially diluted and plated in triplicate on *B. cereus* selective agar (Scharlab), staphylococcal Vogel Jonhson agar (Scharlab), and PALCAM agar with *Listeria* supplement (Merck, Madrid, Spain). The average viable counts obtained after 48 h of incubation at 37°C were used to calculate the log CFU per milliliter of sample.

Data analysis. All experiments were carried out in triplicate, and the means ± standard deviation (SD) were determined. Regression curves were determined with the Excel program (Microsoft Corp., Redmond, Wash.).

TABLE 2. *Influence of bacteriocin and juice concentration on detectable activity of enterocin AS-48 in fruit and vegetable juices*[a]

Fruit or vegetable juice	Residual activity (%)[b]					
	Undiluted extract (plus 140 AU/ml AS-48)	Juice dilution (plus 70 AU AS-48):				
		1:2	1:3	1:4	1:5	1:6
Chard	0	0	10.83 ± 2.5	20.00 ± 4.5	20.00 ± 4.8	33.33 ± 4.5
Artichoke	0	10.83 ± 0.8	16.66 ± 3.8	20.00 ± 4.5	26.66 ± 6.0	26.66 ± 5.5
Caper	0	0	10.83 ± 4.3	16.66 ± 2.5	20.00 ± 4.8	33.33 ± 4.5
Broccoli	16.66 ± 4.5					
Tender onion	10.83 ± 2.3					
Mushroom	0	0	0	0	0	0
Broad bean	26.66 ± 4.0					
Potato	0	0	20.00 ± 5.5	33.33 ± 2.8	33.33 ± 7.5	33.33 ± 4.8
Cucumber	9.16 ± 3.5					
Red pepper	10.83 ± 1.0					
Tomato	0	20.00 ± 4.5	20.00 ± 2.5	26.66 ± 3.8	33.33 ± 4.5	33.33 ± 5.6
Carrot	0	0	0	0	0	0

[a] Bacteriocin concentrate was diluted in juices and tested for residual activity against *E. faecalis* S-47. The percentage of residual bacteriocin activity was calculated with respect to a control sample sample diluted in distilled water.
[b] Values are the mean ± SD of triplicate samples.

RESULTS

Interaction of enterocin AS-48 with fruit and vegetable juices. To determine whether bacteriocin activity could be influenced by interaction with food components, a bacteriocin concentrate was diluted 10-fold in fruit juices, and the activity of these dilutions was compared with an equivalent dilution made in distilled water.

Bacteriocin dilutions made in cabbage juice had activities similar to those of control dilutions made in distilled water (Table 1). Bacteriocin activity in samples diluted in celery juice was also high (85.71% of control activity). Dilutions made in pumpkin, eggplant, lettuce, spinach, leek, green bean, and avocado juices had lower activities (from 40.05 to 57.14% of control activity), and dilutions made in endive and asparagus juices had the lowest activities. In other vegetable juices tested (leek, broccoli, broad bean, mushroom, potato, carrot, tender onion, artichoke, caper, tomato, cucumber, and red pepper), bacteriocin activity was always below the detection limit of the assay. None of the vegetable juices without added bacteriocin had any antimicrobial activity against the test strain used in the assays (data not shown).

Bacteriocin dilutions in freshly made apple, peach, and pear juices had activities similar to those of dilutions in distilled water (Table 1). Activity of AS-48 diluted in orange, pineapple, grapefruit, and kiwi juice was somewhat lower, but always above 65% of the control activity. None of the fruit juices without added bacteriocin had any antimicrobial activity against the test strain.

Influence of bacteriocin concentration and juice concentration on detectable activity. Juices for which no bacteriocin activity had been detected in previous assays were further investigated by using a higher bacteriocin concentration and by using a lower juice concentration (Table 2).

After addition of a twofold-higher bacteriocin concentration (140 AU/ml), some bacteriocin activity (9.16 to 26.66%) was detected in broccoli, tender onion, broad bean, cucumber, and red pepper juices, but no activity was detected in chard, artichoke, caper, potato, tomato, or carrot juices.

When AS-48 was added to juices previously diluted in distilled water, some bacteriocin activity was detected for most juices tested, except mushroom and carrot juices. In most cases, bacteriocin activity increased as more highly diluted juice was used.

Influence of heat pretreatments on detectable bacteriocin activity. To determine whether the rapid loss of activity of AS-48 in some juices could be due to enzymatic inactivation, juices were heated at temperatures of 72 or 98°C before bacteriocin addition, and the residual bacteriocin activity was then tested. For chard, caper, potato, and tomato juices heated at 72°C, variable bacteriocin activity (21.66 to 78.33%) was detected (Table 3). Activity detected in juices heated at 72 or 98°C was similar except for juice obtained from capers (with a higher activity in juice preheated at 98°C) and from carrots (for which bacteriocin activity was detected only when juice had been preheated at 98°C). In artichoke and mushroom juices, no bacteriocin activity was detected regardless of the heat pretreatment applied.

Protease activity in juices was tested before and after juices were heated at 98°C for 5 min. All juices listed in Table 3 tested positive on the protease assay (data not shown). After application of heat treatment, proteolytic activity disappeared for chard, artichoke, potato, and carrot juices, but only partial inactivation was detected in artichoke, caper, mushroom, and tomato juices (data not shown).

Stability of enterocin AS-48 in vegetable juices. To determine the influence of incubation conditions on bacte-

TABLE 3. *Influence of heat pretreatment on detectable activity of enterocin AS-48 in fruit and vegetable juices*[a]

Fruit or vegetable juice	Residual activity (%) in juice heated at[b]:	
	72°C (10 min)	98°C (5 min)
Chard	67.14 ± 5.5	60.45 ± 7.2
Artichoke	0	0
Caper	21.66 ± 6.8	53.33 ± 4.5
Mushroom	0	0
Potato	78.33 ± 3.0	73.33 ± 6.5
Carrot	0	53.33 ± 5.8
Tomato	46.66 ± 4.2	40.05 ± 6.5

[a] Bacteriocin concentrate was diluted 10-fold in heat-treated juices after being cooled at room temperature and then tested for residual activity against *E. faecalis* S-47. The percentage of residual bacteriocin activity was calculated with respect to a control sample diluted in distilled water.

[b] Values are the mean ± SD of triplicate samples.

riocin stability, freshly made vegetable juices with added enterocin AS-48 were stored at 4 and 15°C for various periods of time. At 4°C, no loss of bacteriocin activity was detected during the first 24 h of incubation for all juices tested (Fig. 1). During prolonged incubation at 4°C, detectable activity in juices from cabbage, lettuce, and green beans decreased gradually (Fig. 1A, 1C, and 1D). In juices from cauliflower, celery, and avocado, a large drop of activity was observed at 72 h, but then the level of activity remained stable for up to 15 days of storage (Fig. 1B, 1E, and 1F). More than 20% of the initial activity was detected after 15 days of storage in all juices tested.

Loss of bacteriocin activity occurred much more rapidly in juices stored at 15°C, especially lettuce and avocado juices for which no bacteriocin activity was detected after 7 days of incubation (Fig. 1C and 1F). For the rest of the juices tested, variable bacteriocin activity was detected after 7 days of storage, but no activity was detected after 15 days.

Stability of enterocin AS-48 in fruit juices. Stability of enterocin AS-48 was tested in freshly made fruit juices

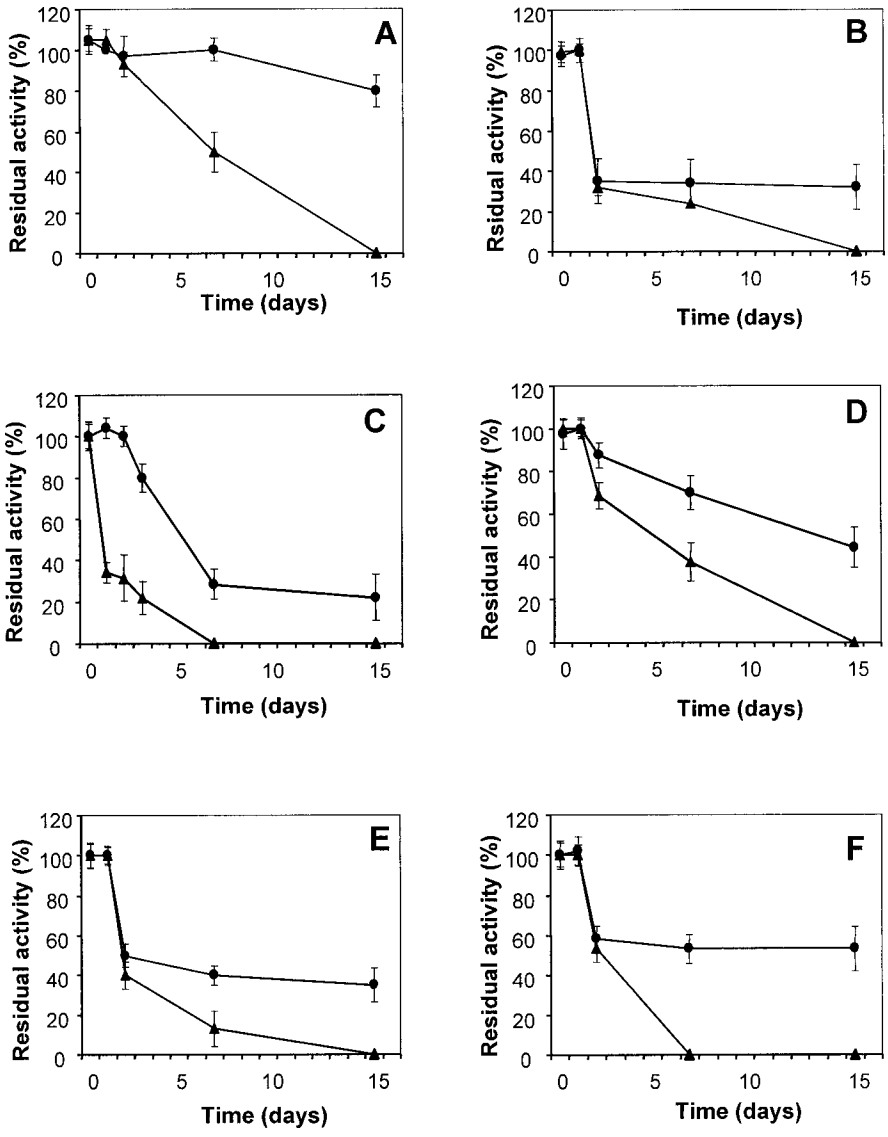

FIGURE 1. *Stability of enterocin AS-48 in vegetable juices stored at 4°C (●) or 15°C (▲). Juices were made from cabbage (A), cauliflower (B), lettuce (C), green beans (D), celery (E), and avocado (F). All residual enterocin AS-48 bacteriocin activities were measured against* E. faecalis *S-47.*

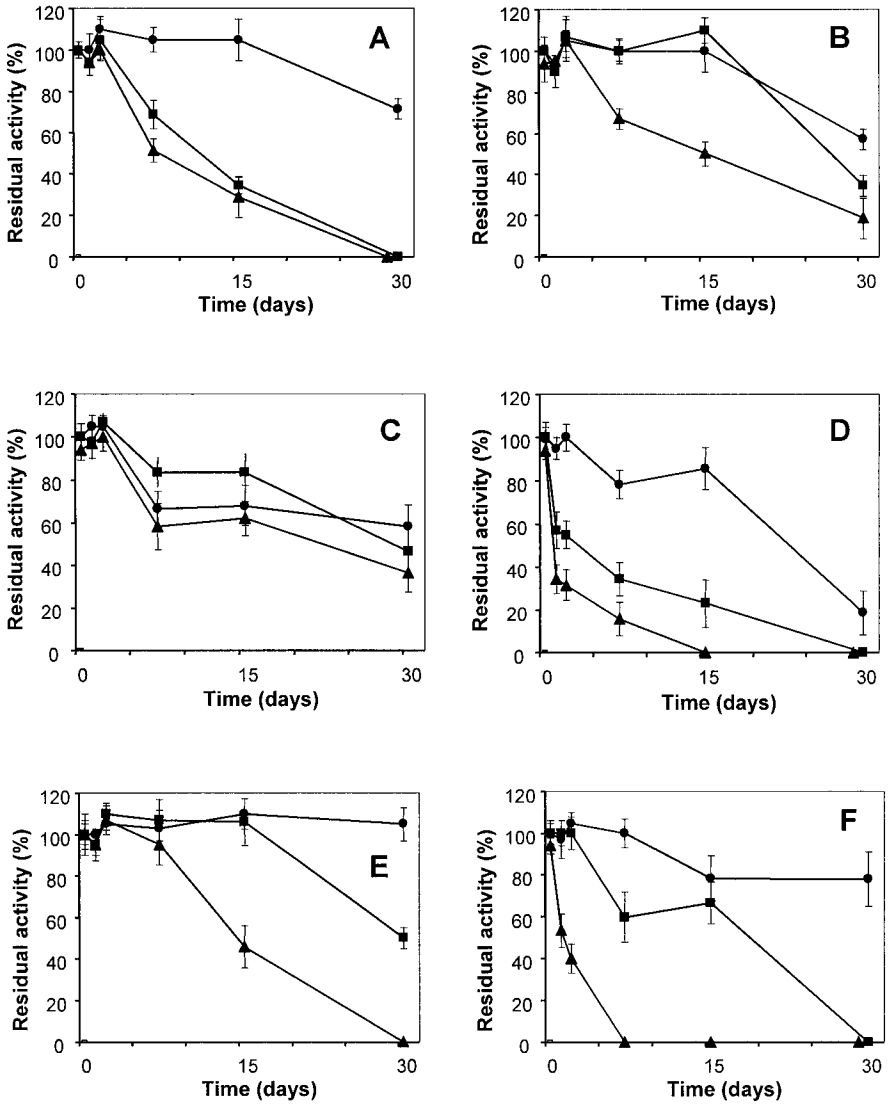

FIGURE 2. *Stability of enterocin AS-48 in freshly made fruit juices stored at 4°C (●), 15°C (■), or 28°C (▲). Juices were made from oranges (A), apples (B), grapefruits (C), pears (D), pineapples (E), and kiwis (F). All residual enterocin AS-48 bacteriocin activities were measured against* E. faecalis *S-47.*

stored at 4, 15, and 28°C. In natural orange juice, the detectable bacteriocin activity remained stable during the first 15 days of storage at 4°C, and some loss of activity was detected only after 30 days (Fig. 2A). At 15 or 28°C, activity remained stable during the first 3 days of incubation but then decreased gradually below detectable levels after 30 days of storage. Bacteriocin activity in apple juice remained stable during the first 15 days of storage at 4 or 15°C, followed by some loss of activity at 30 days, which was more pronounced in samples stored at 15°C (Fig. 2B). Loss of bacteriocin activity was more pronounced in apple juice stored at 28°C, but more than 20% of initial activity was still detected after 30 days. In grapefruit juice, bacteriocin activity remained stable during the first 3 days of storage for all temperatures tested (Fig. 2C). After prolonged incubation, variable loss of activity occurred, but differences among samples from different incubation temperatures were not as great as those among sample of various fruit juices, and all samples retained over 40% of initial activity. For pear juice stored at 4°C, bacteriocin activity remained above 80% during the first 15 days of storage, although it decreased afterward (Fig. 2D). Loss of bacteriocin activity occurred more rapidly in samples stored at 15 or 28°C, and complete inactivation was detected at 30 and 15 days, respectively. Bacteriocin diluted in pineapple juice was completely stable for up to 30 days at 4°C, for up to 15 days at 15°C, and for 7 days at 28°C (Fig. 2E). For the two higher temperatures, partial or total loss of activity was detected after 30 days of storage. In kiwi juice, bacteriocin activity remained very high for up to 30 days at 4°C and for up to 15 days at 15°C (Fig. 2F). However, rapid inactivation was observed in samples stored at 28°C.

Stability of enterocin AS-48 was also tested in mixtures of freshly made fruit juices. For mixtures of peach and grapefruit juices, bacteriocin activity remained stable for up to 15 days regardless of storage temperature (Fig. 3A). After 30 days of storage, bacteriocin activity was reduced to 34 to 40% of initial activity at temperatures of 15 and 28°C, but less reduction was observed in samples stored at 4°C. For mixtures of pear and apple juices, bacteriocin activity remained stable during the first 3 days of storage at temperatures of 4 and 15°C, with a gradual loss of activity during prolonged incubation (Fig. 3B). Bacteriocin inactivation occurred more rapidly in juice mixtures stored at 28°C. For mixtures containing orange, pineapple, grapefruit, peach, pear, and kiwi juices bacteriocin activity

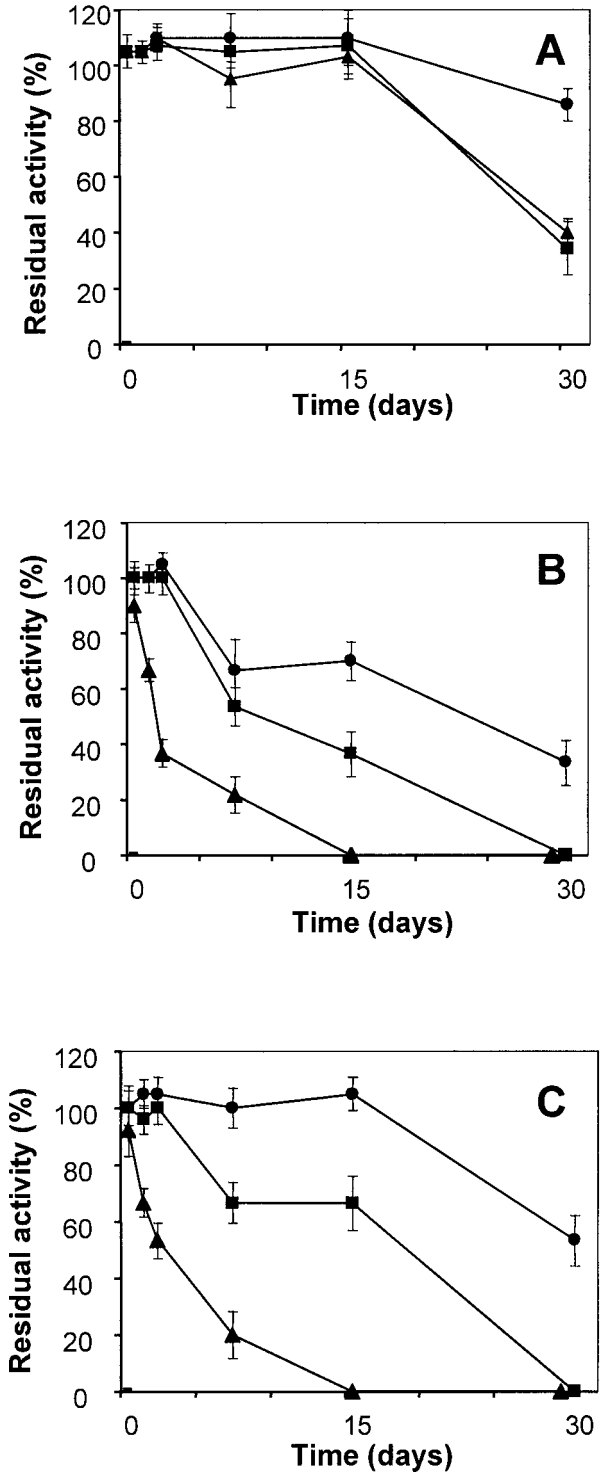

FIGURE 3. *Stability of enterocin AS-48 in mixtures of freshly made fruit juices stored at 4°C (●), 15°C (■), or 28°C (▲). Juice mixtures were made from peaches and grapefruits (A), pears and apples (B), and oranges, pineapples, grapefruits, peaches, pears, and kiwis (C). All residual enterocin AS-48 bacteriocin activities were measured against* E. faecalis *S-47.*

remained stable in samples stored at 4°C for up to 15 days and remained very high (above 60% of initial activity) for the same period in samples stored at 15°C (Fig. 3C). However, rapid inactivation was observed in samples stored at 28°C.

Stability of AS-48 was also tested in four commercial fruit juices (orange, apple, peach, and pineapple) during long-term storage under different conditions (Fig. 4). As a rule, bacteriocin activity was markedly more stable in commercial fruit juices than in freshly made juices. In all four juices tested, bacteriocin activity remained completely stable in samples stored at 4°C for up to 120 days. Loss of activity at incubation temperatures of 15 and 28°C was also less pronounced. For example, orange, peach, and pineapple juices stored at 28°C for 120 days retained over 50% of initial bacteriocin activity (Fig. 4A, 4C, and 4D). Similar results were obtained for apple juice stored at 15°C, but in this case the degree of inactivation during storage at 28°C was higher, as indicated by the larger negative value obtained for the slope of the regression line (Fig. 4B). As expected, bacteriocin activity in distilled water remained stable during the entire test period, regardless of storage temperature (Fig. 4E).

Bacteriocin stability also was tested in commercial juices frozen at −20°C. In all four juices tested, the bacteriocin remained stable for the entire storage period of 3 months (data not shown).

Interaction with food-grade dyes and thickening agents. Bacteriocin activity was tested in aqueous solutions of food-grade dyes and thickening agents (Table 4). Bacteriocin activity was very high in the presence of most of the dyes tested, with more than 85% of control activity. Some inactivation was detected when AS-48 was mixed with solutions containing sunset yellow, erythrosine, or tartrazine, but in all cases activity was always above 65% of control. However, no loss of activity was detected when these dyes were used at twofold-lower concentrations (data not shown). The thickening agents tested did not interfere with bacteriocin activity, which was always above 85% of control activity. None of the dyes or thickening agents had any antimicrobial activity against *E. faecalis* S-47 when tested in the absence of AS-48.

Antimicrobial effects of AS-48 in juice. The antimicrobial activity of AS-48 was tested against *B. cereus* LWL1, *L. monocytogenes* CECT 4032, and *S. aureus* CECT 976 inoculated into lettuce juice and incubated at 15°C. Counts of viable *B. cereus* LWL1 and *L. monocytogenes* CECT 4032 were reduced below detection limits (10 CFU/ml) after 24 h of incubation, whereas those for untreated controls increased markedly (Fig. 5A and 5B). The effect of AS-48 against *S. aureus* CECT 976 was more limited; counts of viable cells increased during the first 24 h of incubation (Fig. 5C). However, variable reductions from 2.0 to 2.8 log units were detected during prolonged incubation of samples, whereas counts for the untreated control increased markedly.

DISCUSSION

In various studies, fruits and vegetables have acted as vehicles for transmission of pathogenic bacteria *(7, 11, 25),* and these products have been implicated in foodborne outbreaks *(11, 18, 25–28).* Many different sources may contribute to contamination of vegetable foods and juices with

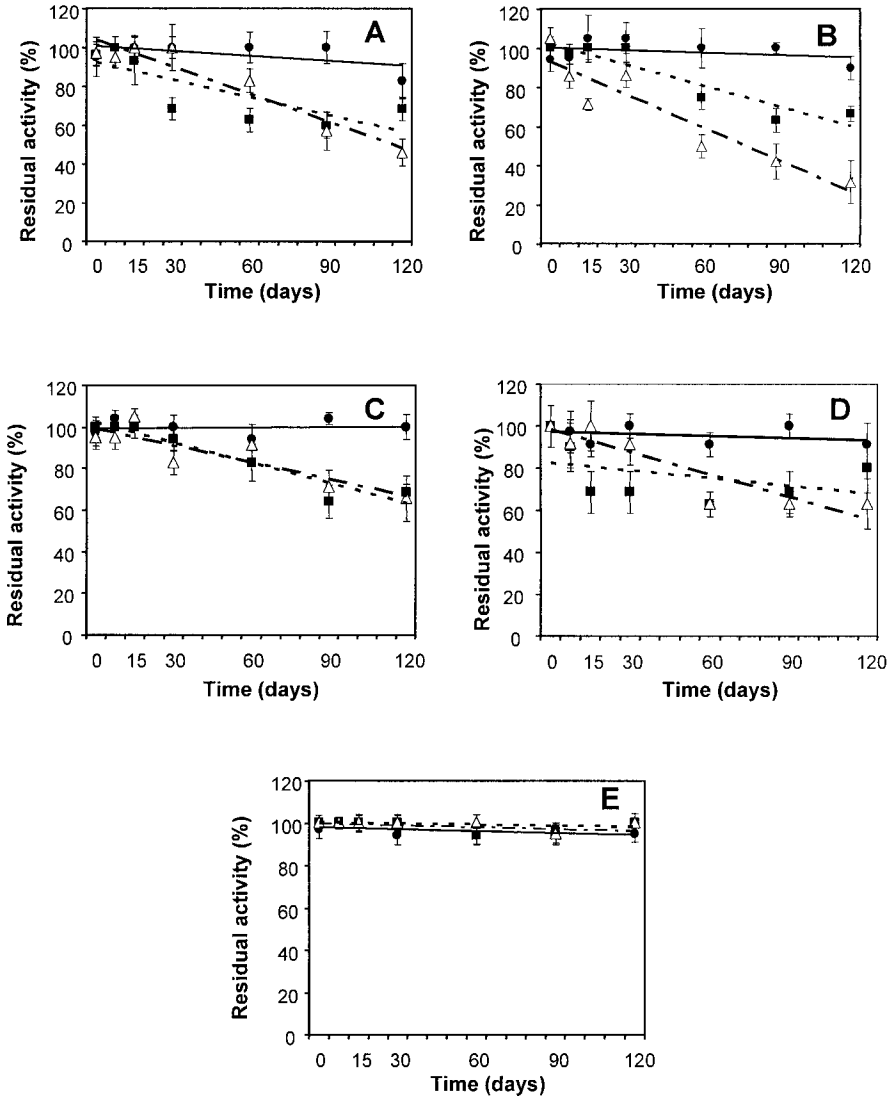

FIGURE 4. *Stability of enterocin AS-48 in commercial fruit juices stored at 4°C (●–●), 15°C (■·····■), or 28°C (△——△). The slope values (*m*) of regression lines are given. (A) Orange:* $m_{4°C} = -0.6264$; $m_{15°C} = -2.2309$; $m_{28°C} = -3.4911$; *(B) apple:* $m_{4°C} = -0.3053$; $m_{15°C} = -2.6009$; $m_{28°C} = -4.1496$; *(C) peach:* $m_{4°C} = 0.0476$; $m_{15°C} = -2.4454$; $m_{28°C} = -2.0331$; *(D) pineapple:* $m_{4°C} = -0.2296$; $m_{15°C} = -0.9063$; $m_{28°C} = -2.6844$; *(E) distilled water (control):* $m_{4°C} = -0.2115$; $m_{15°C} = -0.2096$; $m_{28°C} = -0.1326$. *All residual enterocin AS-48 bacteriocin activities were measured against* E. faecalis *S-47.*

pathogenic bacteria, which may survive and multiply and/or invade vegetable tissues. Food processing operations (such as chopping, handling, and refrigeration storage) may facilitate proliferation of pathogenic bacteria *(7).* The currently available treatments used to reduce the microbial load and/or eliminate pathogenic bacteria in vegetable-based foods are not always satisfactory because they may modify the organoleptic properties, may be unacceptable to consumers (e.g., irradiation), or may be ineffective against some bacterial forms (e.g., endospores). Therefore, it is necessary to investigate alternative or complementary treatments.

Although the use of bacteriocins for preservation of vegetable-based foods and juices has not been well studied, this group of antimicrobial substances may find application in this category of food products, especially for preservation of certain value-added vegetable foods. Enterocin AS-48 is an attractive candidate for biopreservation because of its broad antimicrobial spectrum of activity against such foodborne pathogens as *L. monocytogenes*, *B. cereus*, and *S. aureus (2, 5, 22).* Although the activity of AS-48 in dairy and meat products has been confirmed *(4, 23)*, activity in vegetable-based foods has not been reported. Activity and stability of AS-48 was evaluated in a variety of fruit and vegetable juices as a preliminary step in the investigation of the antimicrobial activity of this bacteriocin against selected spoilage and pathogenic bacteria occurring in these types of foods.

The results obtained in this study indicate that the activity of enterocin AS-48 in fruit and vegetable juices depends largely on the type of vegetable or fruit. The activity of bacteriocins in the foods has been determined in only a few studies, and activity was reported as considerably reduced or not detectable after some days of storage *(15, 19, 24).* Bacteriocin activity and stability in foods may be influenced by several factors *(14)*, including the pH of the food *(9, 24)*, the food composition (especially the presence of substances to which bacteriocins can bind such as fat *(6, 8, 10, 17)* or proteins *(1, 14, 24)*), and proteolytic inactivation *(1, 24).* Enterocin AS-48 is a pH-stable protease-sensitive cationic peptide of amphypathic nature *(16, 20).* Therefore, interaction with food components may occur both through positively charged residues and through hydrophobic regions of the molecule. In vegetable juices, activity of AS-48 does not seem to be influenced by the food pH; loss of activity was detected both in acidic substrates

TABLE 4. *Interaction of enterocin AS-48 with food-grade dyes and thickening agents*[a]

Test material	Residual activity (%)[b]
Dye (mg/liter)	
Control (distilled water)	100.00 ± 0.5
Annato (35)	100.00 ± 1.5
Chlorophyl (10)	85.71 ± 2.5
Beet red (200)	85.71 ± 0.7
Erythrosine (40)	71.42 ± 4.5
Erythrosine (20)	100.22 ± 1.5
Cochineal red (10)	85.71 ± 3.5
Ponceau red (40)	92.85 ± 2.5
Sunset yellow (40)	67.42 ± 4.5
Sunset yellow (20)	100.05 ± 2.4
Tartrazine (15)	71.42 ± 5.2
Tartrazine (7.5)	100.00 ± 1.2
Thickening agent (%)	
K-carragenate (0.2)	85.71 ± 4.5
Arabic gum (0.2)	110.02 ± 4.5
Xantan gum (0.2)	100.03 ± 2.1
Cornstarch (1.0)	100.02 ± 2.4

[a] Bacteriocin concentrate was diluted 10-fold in dye solutions and tested for residual activity against *E. faecalis* S-47. The percentage of residual bacteriocin activity was calculated with respect to a control sample diluted in distilled water.
[b] Values are the mean ± SD of triplicate samples.

(e.g., tomato) and in substrates with pH close to neutrality (e.g., chard and celery). Vegetable foods used in this study are not fat rich, and therefore loss of activity should be attributed to interaction with other food components or to enzymatic inactivation. Application of heat treatments to vegetable extracts ameliorated the loss of bacteriocin activity in most cases, which suggests that enzymatic inactivation may be responsible for loss of activity detected in some vegetable extracts. Protease assays confirmed the presence of proteolytic activity.

The second issue addressed in this work concerns the stability of enterocin AS-48 in fruit and vegetable juices during incubation for prolonged periods at different temperatures. Higher stability would presumably allow longer protection against spoilage or pathogenic bacteria. Results indicate that stability of AS-48 is clearly dependent on the type of food. Bacteriocin inactivation occurred much more rapidly in vegetable juices than in fruit juices. This finding should be taken into consideration for specific bacteriocin applications, especially when protection during the entire shelf life of a product is required, whereas application of bacteriocin treatment to eliminate or significantly reduce the population of selected pathogenic or spoilage bacteria may be satisfactory in other cases.

The food incubation temperature was also a key factor for bacteriocin stability; inactivation always occurred more rapidly at higher temperatures. For example, bacteriocin activity remained relatively high in most of the vegetable juices stored at 4°C for at least 24 to 72 h, whereas decay of activity occurred much more rapidly in samples stored at

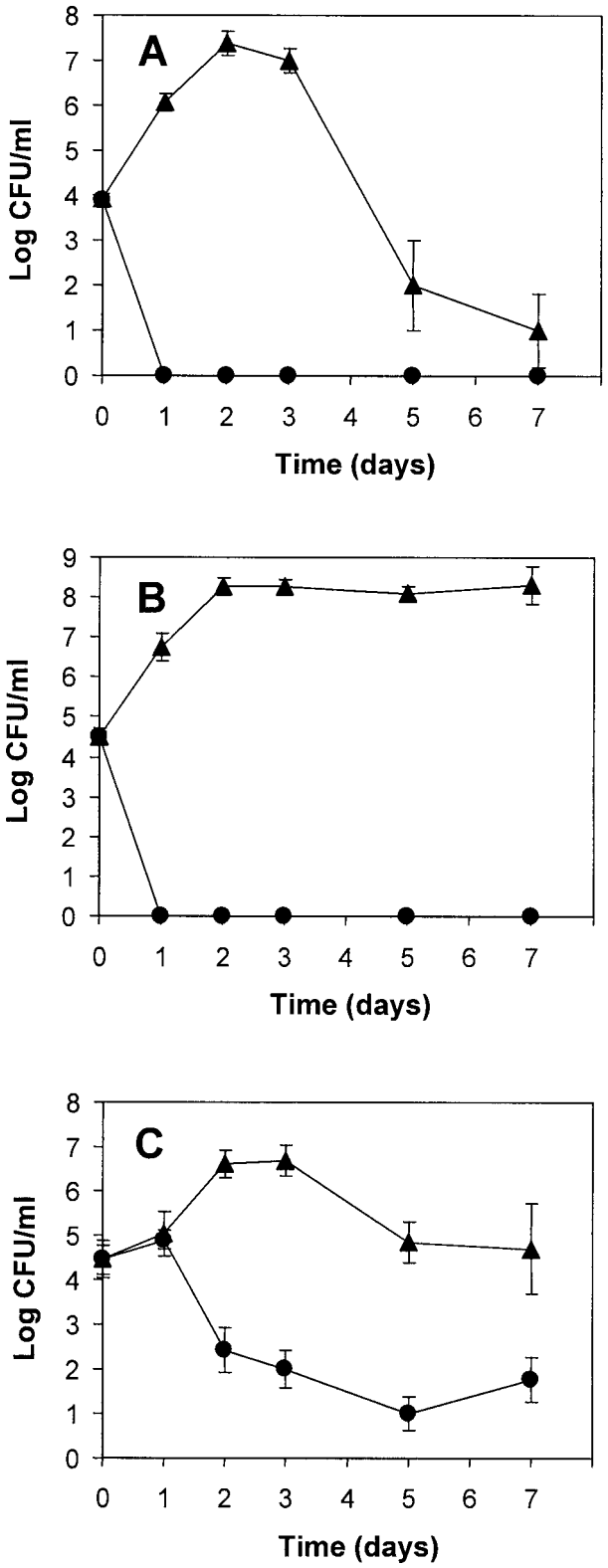

FIGURE 5. *Antimicrobial activity of enterocin AS-48 (70 AU/ml) in lettuce juice against* Bacillus cereus *LWL1 (A),* Listeria monocytogenes *CECT 4032 (B), and* Staphylococcus aureus *CECT 976 (C). Samples were incubated at 15°C, and the concentrations of viable cells in control cultures (▲) and in bacteriocin-treated cultures (●) were determined at desired incubation points by plating on selective media.*

15°C. A similar effect was observed in freshly made fruit juices. However, temperature-dependent inactivation was less pronounced in commercial fruit juices, probably because the bacteriocin inactivating agents (endogenous enzymes or contaminating bacteria) probably were destroyed during the industrial manufacturing processes. The prolonged stability of AS-48 in commercial fruit juices suggests that added bacteriocin could provide protection during the entire shelf life of juices.

A third issue addressed here was whether added bacteriocin could control proliferation of potentially pathogenic bacteria in vegetable foods. Lettuce juice was chosen as a model food system. Both *L. monocytogenes* and the psychrotrophic and toxigenic *B. cereus* strain LWL1 were rapidly inactivated after bacteriocin addition, whereas high cell counts were obtained in the untreated controls. The assay was carried out at 15°C, a temperature abuse condition that may be reached easily during many processing and handling operations. Control of *S. aureus* was more limited, although ca. 3-log reductions in counts of viable cells were achieved. *S. aureus* also has a limited capacity for growth under the incubation conditions tested. Results of antimicrobial activity tests carried out in lettuce juice indicate that in spite of the apparent loss of detectable bacteriocin activity in this substrate there were still enough AS-48 molecules available to kill the target bacteria. These results are promising for future applications of this bacteriocin as a preservative for vegetable-based foods.

The results presented in this study also indicate that the use of enterocin AS-48 is compatible with addition of food-grade dyes and thickening agents. AS-48 could be used as a bioprotectant in a broad range of vegetable-based foods in combination with other additives. Further studies on the antimicrobial activity of AS-48 against selected pathogenic and spoilage bacteria in foods will determine the usefulness of this antimicrobial peptide as a bioprotectant in vegetable-based foods and drinks.

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