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Treatment of Vegetable Sauces with Enterocin AS-48 Alone or in Combination with Phenolic Compounds To Inhibit Proliferation of *Staphylococcus aureus*

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ABSTRACT

The antimicrobial activity of enterocin AS-48 against *Staphylococcus aureus* was tested in vegetable sauces, alone and in combination with phenolic compounds. When added alone at 25 μg/ml, AS-48 inactivated all detectable staphylococci in napoletana and pesto sauces stored at 22°C, but it only caused limited growth inhibition when these sauces were stored at 10°C, as well as in other sauces such as carbonara and green sauce for fish. At 80 μg/ml, AS-48 eliminated all detectable staphylococci in napoletana, pesto, and green sauce for fish regardless of storage temperature, but it still had much more limited effect in carbonara sauce. Antistaphylococcal activity was potentiated significantly when AS-48 was used in combination with the phenolic compounds carvacrol, geraniol, eugenol, terpineol, caffeic acid, *p*-coumaric acid, citral, and hydrocinnamic acid. The efficacy of the combined treatments depended both on the phenolic compound and the type of sauce. In carbonara sauce stored at 22°C, the combinations of 80 μg/ml AS-48 and 20 mM hydrocinnamic acid or 126 mM carvacrol reduced viable counts of staphylococci below detection limits for up to 30 days.

Staphylococcal food poisoning is recognized among the most common causes of reported foodborne diseases *(9, 26, 29, 37, 39),* requiring hospital attention by at least 10% of the affected individuals. *Staphylococcus aureus* is found in the nostrils as well as on the skin and hair of warm-blooded animals, and up to 30 to 50% of human population are carriers. *S. aureus* is able to grow in a wide range of temperatures (7 to 48.5°C, with an optimum at 30 to 37°C) *(35),* pH (4.2 to 9.3, with an optimum of 7.0 to 7.5) *(10),* and sodium chloride concentrations (up to 15% NaCl). These characteristics enable *S. aureus* to grow in a wide variety of foods, which may become contaminated during or after processing. *S. aureus* has been isolated from several foods including meat and meat products, chicken, milk and dairy products, fermented food items, salads, vegetables, and fish products, etc. *(17, 20, 21, 36, 38).* Most strains are capable of producing one or more heat-stable enterotoxins *(8)* that are the cause of the gastrointestinal symptoms observed during intoxications *(36).*

One of the reasons that may increase the risk of staphylococcal food poisoning is the consumer demands for foods that are fresh-tasting, ready-to-eat, with an extended shelf life, less acid, and with a lower content of chemical preservatives. In this context, bacteriocins have been proposed as natural preservatives *(12, 15).* Enterocin AS-48 is a broad-spectrum cyclic peptide produced by *Enterococcus faecalis (27).* Recent studies have demonstrated the antimicrobial activity of this bacteriocin against foodborne pathogenic and spoilage bacteria both in culture media *(1, 2, 7, 30)* and in food systems *(4–6, 13, 18, 19, 31).* Enterotoxin-producing *S. aureus* strains are sensitive to this bacteriocin *(7),* and exogenously added bacteriocin as well as in situ–produced bacteriocin have been tested to control proliferation of *S. aureus* in a model meat system *(6).* Because the efficacy of bacteriocins may change significantly depending on the type of food, the purpose of the present study was to evaluate the efficacy of enterocin AS-48 in vegetable-based food systems such as vegetable sauces. This is also the first report on the potentiation of antimicrobial activity by the combined addition of enterocin AS-48 and phenolic compounds, an approach that can be useful in order to lower the added biocide concentration required to suppress *S. aureus* in foods of this category.

MATERIALS AND METHODS

Bacterial strains and cultivation conditions. The toxicogenic strain *S. aureus* CECT 976 was from the Spanish Type Culture Collection (CECT). *E. faecalis* A-48-32 *(28)* was used to produce enterocin AS-48, and *E. faecalis* S-47 was used as an indicator strain for determination of bacteriocin activity. Bacterial strains were grown on brain heart infusion broth (BHI; Scharlab, Barcelona, Spain) at 37°C and maintained routinely on BHI agar slants at 4°C.

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Bacteriocin preparation. Partially purified preparations of enterocin AS-48 were obtained from cultured broths of the producer strain *E. faecalis* A-48-32 after concentration by cation-exchange chromatography as described elsewhere *(3, 19).*

Assay of enterocin AS-48 in vegetable sauces. The different sauces used in this work were purchased at local supermarkets: napoletana sauce (pH 4.33; Buitoni, Barcelona, Spain), refrigerated napoletana sauce (pH 4.16; Buitoni), green sauce for fish (pH 5.45; Knorr, Barcelona, Spain), carbonara sauce (pH 6.03; Knorr), pesto (pH 4.77; Gallo, Barcelona, Spain), refrigerated pesto sauce (pH 4.02; Gallo), salad dressing (pH 3.82; Remia, Den Dolder, The Netherlands), Salatfix Italian sauce (pH 4.20; Hamburg, Germany), classic sauce for potatoes (pH 3.52; Kühne, Hamburg, Germany), deluxe potato sauce (pH 3.62; Calve, Bizcaia, Spain), and soy sauce (pH 4.99; Kikkoman Foods, Los Angeles, Calif.). Sauces were stored at desired temperatures of 10 and 22°C before they were inoculated (0.2%, vol/vol) with a 100-fold dilution (in sterile saline solution) of an overnight culture of *S. aureus* CECT 976 grown in BHI broth at 37°C. After this, sauces were supplemented or not with enterocin AS-48 (at final concentrations of 25 and 80 μg/ml), thoroughly mixed, and distributed in sterile capped plastic test tubes before they were stored at 10 and 22°C. Sauces without added staphylococci or bacteriocin were used as negative controls. At desired intervals, aliquots (1 ml) of the different sauces were serially diluted in sterile saline solution (0.85% NaCl) and plated in triplicate on Vogel-Johnson agar containing 0.01 g/liter of potassium tellurite (VJ agar; Scharlab). After 48 h incubation at 37°C, the average number of colonies on the plates was used to calculate the viable cell concentration of samples, expressed as CFU per milliliter. All experiments were carried out in duplicate.

Assay of enterocin AS-48 in combination with phenolic compounds. Carvacrol (31.5 mM), eugenol (32 mM), geraniol (28.25 mM), citral (27.75 mM), and terpineol (132 mM) were added to the food (either at 0.5% [vol/vol] or at 2.0% [vol/vol] for terpineol) directly from commercial solutions, yielding the final concentrations indicated in parentheses. Hydrocinnamic acid (3-phenylpropionic acid, 5 mM), caffeic acid (20 mM), ferulic acid (20 mM), chlorogenic acid (50 mM), *p*-coumaric acid (20 mM), and thymol (20 mM) were added to the food from freshly made 1-M solutions dissolved in propylene glycol (Fluka, Barcelona, Spain). The phenolic compounds were added to sauce samples inoculated with *S. aureus* CECT 976 as above, with or without enterocin AS-48 (15 μg/ml final concentration). At desired intervals of incubation at 22°C, the concentration of viable staphylococci was determined on VJ agar, as described above. Phenolic compounds were purchased from Fluka, except for citral (Sigma-Aldrich, Barcelona, Spain).

In order to determine the concentration-dependent effect of enterocin AS-48 plus hydrocinnamic acid, an overnight culture of *S. aureus* CECT 976 was diluted 100-fold in sterile saline solution and inoculated (0.2%, vol/vol) in duplicate on BHI broth supplemented with different concentrations of enterocin AS-48 (0 to 15 μg/ml) and hydrocinnamic acid (0 to 20 mM), and incubated at 22°C for 24 h. After this, the concentrations of viable cells were determined by serial dilution and plating on VJ agar.

The combined effect of enterocin AS-48 and hydrocinnamic acid as well as carvacrol was also determined on *S. aureus* inoculated in carbonara sauce during long-term storage. Sauce samples supplemented with hydrocinnamic acid (5, 10, and 20 mM final concentrations) or carvacrol (31.5, 63, or 126 mM) were supplemented or not with enterocin AS-48 (20, 50, and 80 μg/ml) before they were contaminated with *S. aureus* CET 976, as described above, and stored at 22°C. At desired intervals of incubation (0 to 30 days), duplicate samples were processed for viable cell counts on VJ agar, as described above.

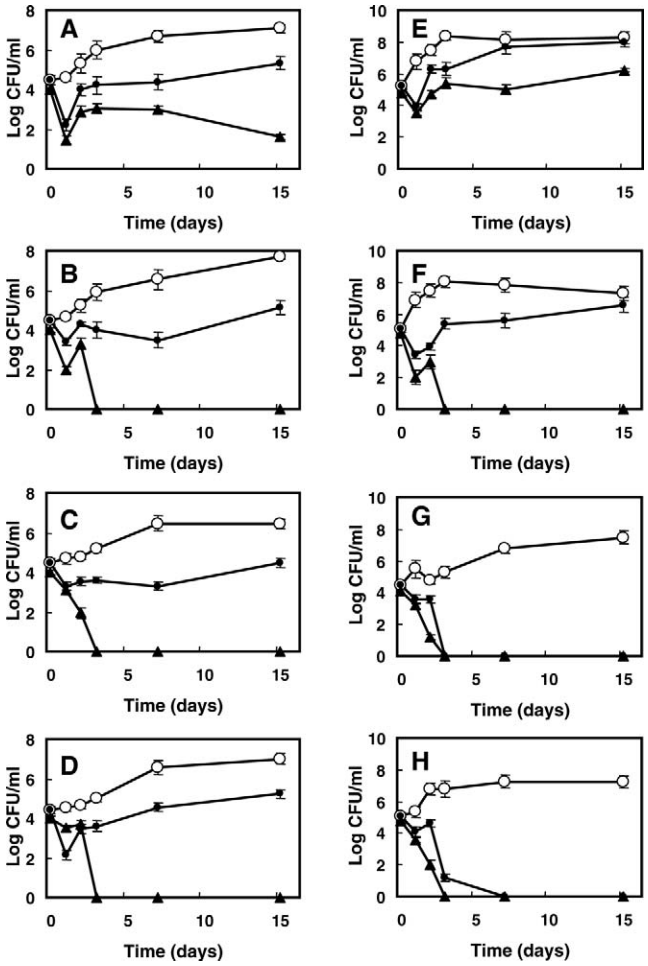

FIGURE 1. *Effect of enterocin AS-48 on* S. aureus *CECT 976 inoculated on different types of vegetable sauces: carbonara (A, E), green sauce for fish (B, F), napoletana (C, G), and pesto (D, H). Sauces were supplemented with bacteriocin concentrations of 0 (○), 25 (●) or 80 μg/ml (▲), and stored at temperatures of 10°C (A through D) or 22°C (E through H). Data represent the average of duplicate assays ± standard deviations (error bars).*

Statistical analyses. All experiments were carried out in duplicate, and the average data ± standard deviations were determined with the Excel program (Microsoft Corp., Redmond, Wash.). A *t* test was performed at the 95% confidence interval, with Statgraphics Plus, version 5.1 (Statistical Graphics Corp., Herndon, Va.), in order to determine the statistical significance of data.

RESULTS

Effect of enterocin AS-48 on *S. aureus* CECT 976 inoculated on vegetable sauces. Survival and growth of *S. aureus* CECT 976 was investigated in vegetable sauces supplemented or not with enterocin AS-48 and stored at temperatures of 10 and 22°C. Results obtained for carbonara, napoletana, pesto, and green sauce for fish are shown in Figure 1. At 10°C, a bacteriocin concentration of 25 μg/ml caused variable reductions of viable counts within the first 24 h of storage in all sauces tested, but it only caused

some inhibition of growth during the following incubation periods. Viable cell counts of bacteriocin-added samples were significantly lower ($P < 0.05$) as compared with controls in all cases (except for carbonara and green sauce for fish at day 2). A higher bacteriocin concentration (80 μg/ml) reduced viable cell counts in carbonara sauce by 2.5 log units within the first 24 h of storage (Fig. 1A). However, viable counts of 1.6 log CFU/ml were still detected after 15 days of incubation. By contrast, no viable staphylococci were detected in napoletana, pesto, or green sauce for fish after 3 days of incubation for this bacteriocin concentration (Fig. 1B through 1D).

At 22°C, a bacteriocin concentration of 25 μg/ml had a very limited effect on staphylococci in carbonara sauce, and viable counts were significantly lower ($P < 0.05$) than those of the controls only at days 1 and 3 of incubation (Fig. 1E). At 80 μg/ml, viable counts in carbonara sauce were always significantly lower ($P < 0.05$) than those of the controls. However, after the initial reduction by 1.3 log units, counts increased above 4 log units in the remaining incubation period. In green sauce for fish supplemented with 25 μg/ml AS-48, counts of staphylococci were significantly lower ($P < 0.05$) than were the controls until day 7 of storage. However, after the early decrease of 1.6 log units detected at day 1, counts continued to increase during the remaining incubation period (Fig. 1F). At 80 μg/ml, the concentrations of viable staphylococci were reduced below detection limits after day 3 of storage. In napoletana and pesto sauces, a bacteriocin concentration of 25 μg/ml reduced the concentrations of viable staphylococci below detection limits at days 3 and 7, respectively (Fig. 1G and 1H). In both cases, a higher bacteriocin concentration (80 μg/ml) only served to moderately accelerate inactivation of staphylococci. Results obtained for napoletana and pesto sauces commercialized under refrigeration were very similar to those obtained with the unrefrigerated commercial forms described above. In soy sauce supplemented with 25 μg/ml AS-48, no viable cells were detected after day 2 of storage (data not shown). However, a rapid inactivation of staphylococci was also observed in soy sauce without added bacteriocin, and no viable cells were detected after day 7 of storage (data not shown). In salad dressing, Italian sauce, classic sauce for potatoes, or deluxe potato sauce, *S. aureus* CECT 976 did not survive after 24 h of incubation (data not shown). All of them contained acetic acid as one of the declared ingredients. Therefore, these three sauces were discarded for use in AS-48 assays.

Effect of enterocin AS-48 in combination with phenolic compounds. In a preliminary experiment, phenolic compounds were tested in green sauce for fish at different concentrations in order to choose the lowest values that caused growth inhibition of staphylococci (data not shown). Phenolic compounds were then tested in combination with a subinhibitory concentration of 15 μg/ml enterocin AS-48 at 22°C. Viable cell counts of staphylococci obtained for green sauce for fish supplemented with AS-48 in combination with carvacrol, geraniol, eugenol, terpineol, caffeic acid, *p*-coumaric acid, citral, and hydrocinnamic acid were

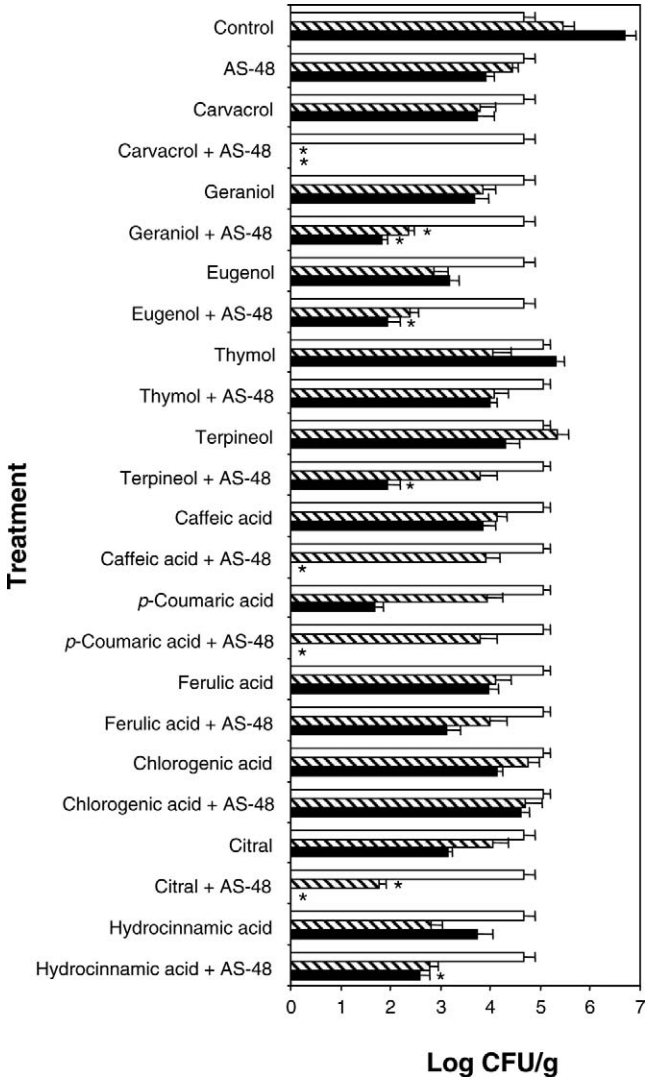

FIGURE 2. *Effect of enterocin AS-48 (15 μg/ml) on* S. aureus *CECT 976 inoculated in green sauce for fish, in combination with different phenolic compounds. Viable cell counts were determined after 8 h (dashed bars) and 24 h (solid bars) of incubation at 22°C. Controls without added preservative are represented by clear bars. Asterisks denote a statistically significant reduction of viable counts for the combined treatment compared with treatment with the phenolic compound alone. Data represent the average of duplicate assays ± standard deviations (error bars).*

significantly lower ($P < 0.05$) as compared with viable counts obtained for AS-48 or the corresponding chemical preservative separately (Fig. 2). In most cases, significant ($P < 0.05$) reductions of viable counts were obtained after 24 h of incubation, and also at 8 h for the combinations of AS-48 and carvacrol, geraniol or citral. In some of the combined treatments (carvacrol, caffeic acid, *p*-coumaric acid, or citral), the concentrations of staphylococci were reduced below detection limits.

Representatives of the chemical compounds that were active at lower concentrations were selected to corroborate their activity in three other vegetable sauces (carbonara, napoletana, and pesto). Viable counts of staphylococci were significantly lower ($P < 0.05$) as compared with controls in samples supplemented with combinations of AS-48 and

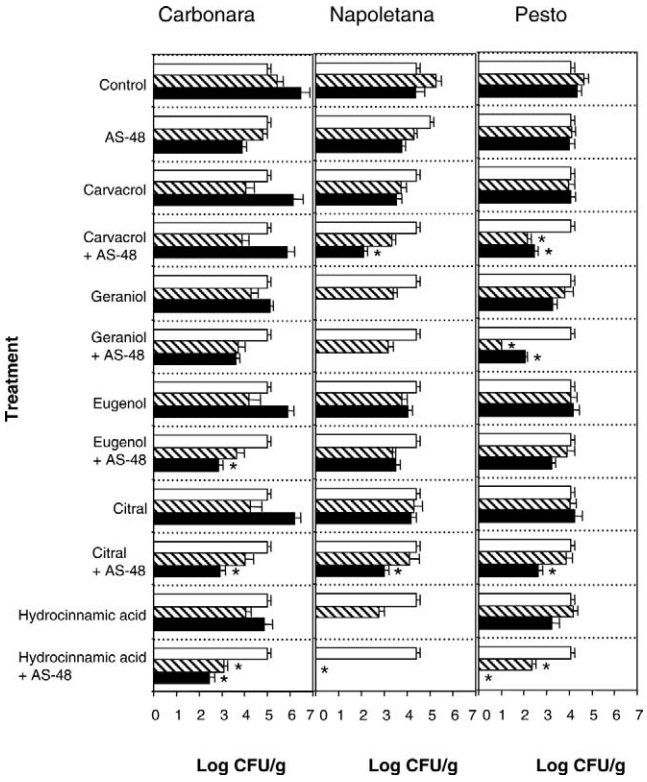

FIGURE 3. *Effect of enterocin AS-48 (15 μg/ml) on* S. aureus *CECT 976 inoculated in carbonara, napoletana, and pesto sauces, in combination with different phenolic compounds. Viable cell counts were determined after 8 h (dashed bars) and 24 h (solid bars) of incubation at 22°C. Controls without added preservative are represented by clear bars. Asterisks denote a statistically significant reduction of viable counts for the combined treatment compared with treatment with the phenolic compound alone. Data represent the average of duplicate assays ± standard deviations (error bars).*

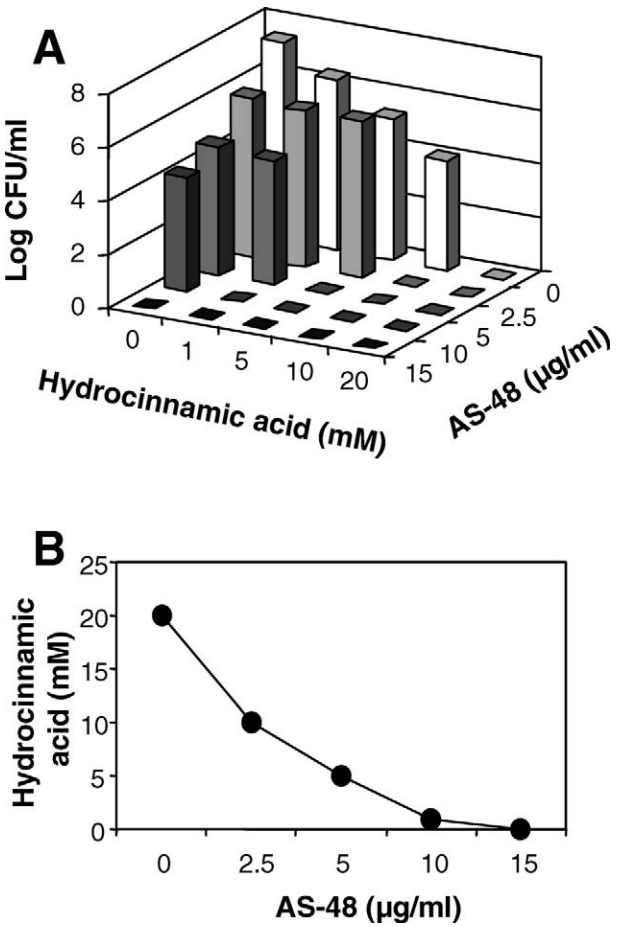

FIGURE 4. *(A) Effect of different concentrations of enterocin AS-48 and hydrocinnamic acid on survival of* S. aureus *CECT 976 in BHI broth. (B) The different combinations of enterocin AS-48 and hydrocinnamic acid that caused complete inactivation of* S. aureus *after 24 h incubation at 22°C are shown. Samples were incubated at 22°C for 24 h.*

citral or hydrocinnamic acid in the three sauces (Fig. 3). The best results were obtained for AS-48 plus hydrocinnamic acid (especially in pesto and napoletana sauces), not only because of the large amount of cells inactivated by this treatment, but also because of the low concentration of the phenolic compound required for inactivation (5 mM). The activity of AS-48 plus eugenol was significantly higher ($P < 0.05$) in carbonara sauce but not in napoletana or in pesto sauces, whereas the combination of AS-48 and geraniol showed a significantly higher activity only in pesto sauce. Addition of geraniol alone inactivated all detectable staphylococci in napoletana sauce after 24 h of incubation, but it had a much more limited effect in carbonara sauce.

The concentration dependency of antimicrobial activity of hydrocinnamic acid in combination with AS-48 was tested on *S. aureus* inoculated onto BHI broth. Several combinations of enterocin AS-48 and hydrocinnamic acid were able to reduce viable counts of staphylococci below detection limits at 24 h incubation (Fig. 4A). The graphic representation of the minimal concentrations of each compound that caused complete inactivation of staphylococci indicated a direct relationship between the concentrations of each compound in the combination (Fig. 4B). As the concentration of hydrocinnamic acid increased, a lower bacteriocin concentration was required to completely inactivate *S. aureus* and vice versa.

In carbonara sauce—where the bacteriocin showed a lower efficacy—the combined effects of AS-48 plus hydrocinnamic acid or carvacrol were tested for a 30-day storage period at 22°C (Fig. 5). Hydrocinnamic acid alone did not eliminate staphylococci from carbonara sauce, even at the highest concentration of 20 mM tested (Fig. 5A). However, combined treatments of enterocin AS-48 and hydrocinnamic acid showed higher efficacy as the concentration of each inhibitor increased. A concentration of 20 mM hydrocinnamic acid reduced viable counts of staphylococci in carbonara sauce below detection limits for up to 5, 7, or 30 days of storage when used in combination with bacteriocin concentrations of 20, 50, or 80 μg/ml, respectively (Fig. 5B through 5D). At 10 mM hydrocinnamic acid and 80 μg/ml AS-48, counts of staphylococci were also reduced below detection limits for up to 5 days of storage (Fig. 5D).

Carvacrol alone only caused a slight reduction of viable counts (Fig. 5E), and this was achieved at a much higher concentration of 126 mM, as compared with hydrocinnamic acid. For the combination of 126 mM carvacrol and AS-48, counts of staphylococci were also reduced below

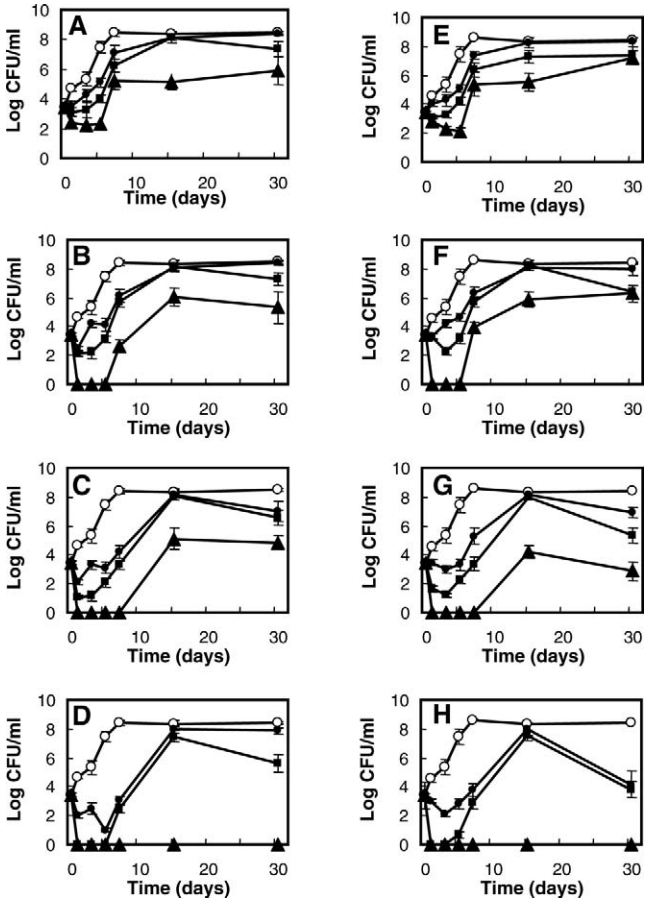

FIGURE 5. *Combined effect of enterocin AS-48 and hydrocinnamic acid (A through D) or carvacrol (E through H) on survival of* S. aureus *CECT 976 in carbonara sauce. Sauce samples were supplemented with hydrocinnamic acid at final concentrations of 5 (●), 10 (■) and 20 mM (▲), either alone (A) or in combination with enterocin AS-48 at final concentrations of 20 (B), 50 (C), or 80 μg/ml (D). Sauce samples were supplemented with carvacrol at final concentrations of 31.5 (●), 63 (■), and 126 mM (▲), either alone (E) or in combination with enterocin AS-48 at final concentrations of 20 (F), 50 (G), or 80 μg/ml (H). After inoculation with* S. aureus *CECT 976, samples were stored at 22°C for various periods of time. Untreated controls (○). Data represent the average of duplicate assays ± standard deviations (error bars).*

detection limits for 5, 7, and 30 day for bacteriocin concentrations of 20, 50, or 80 μg/ml respectively (Fig. 5F through 5H). When carvacrol was tested at a lower concentration (63 mM), it only reduced viable counts below detection limits for 3 days (Fig. 5G).

DISCUSSION

Little is known about the incidence of *S. aureus* in vegetable foods. In the present study, the enterotoxin A–producer strain *S. aureus* CECT 976 was able to proliferate in 6 of the 11 different vegetable sauces tested, both at 22°C and at moderate refrigeration. Because food handlers are the most common source of *S. aureus* contamination in outbreaks of staphylococcal food poisoning *(20),* vegetable sauces that become contaminated during handling may be considered as a potential risk and also as vehicles for transmission of staphylococci to other foods. Therefore, the use of additional hurdles such as bacteriocins to lower the risks for contamination should be recommended. The results obtained in the present study indicate that the efficacy of AS-48 to control *S. aureus* greatly depends on the type of sauce. Whereas a bacteriocin concentration of 25 μg/ml completely eliminated staphylococci in napoletana and pesto sauces stored at 22°C, the same effect was achieved in green sauce for fish at much higher bacteriocin concentration (80 μg/ml), and even this high concentration only produced some growth inhibition in carbonara sauce. It has been reported recently that the AS-48 concentration required to inhibit *S. aureus* CECT 976 in a meat sausage model system (40 μg/g) is also much higher as compared with the minimal bactericidal concentration of 15 μg/ml established in BHI broth *(6, 7).* These results are in agreement with previous observations indicating that the chemical composition and the physical conditions of food may have a great influence on the activity of bacteriocins *(12).* Results from the present study also indicate that storage temperature can have an influence on the efficacy of enterocin AS-48 against *S. aureus.* This was more clearly in napoletana and pesto sauces stored at 10°C, where a bacteriocin concentration of 25 μg/ml was not sufficient to inhibit staphylococci, compared with results obtained at 22°C. On the contrary, a very high bacteriocin concentration seemed to be slightly more active on staphylococci in carbonara sauce at 10°C as compared with 22°C, presumably because the slower growth of staphylococci compensated for the slow diffusion of bacteriocin molecules from the food matrix.

The activity of bacteriocins can be potentiated when they are applied in combination with other antimicrobial substances or treatments, an interesting approach in the hurdle technology concept. One group of candidate antimicrobial substances for food preservation is the phenolic compounds, which are naturally found in many different essential oils *(11).* Several works have described the antimicrobial activity of phenolic compounds such as α-terpineol, citral, carvacrol, thymol, eugenol, and others against *S. aureus (14, 23–25, 32).* Therefore, different combinations of enterocin AS-48 and phenolic compounds were tested in the present work in order to increase the antimicrobial activity against *S. aureus.* In most cases, the combinations of AS-48 and the phenolic compound had much higher antimicrobial activity than did each of the components separately, indicating additive or synergistic effects. Interestingly, some of the combinations of enterocin AS-48 and phenolic compounds served to completely inactivate *S. aureus* in sauces, although this effect depended largely on the type of food, which in turn had a great influence on the activity of AS-48 as well as the phenolic compounds tested individually. It has been reported that the antibacterial activity of phenolic compounds is influenced by food factors, especially the fat and protein content *(11).* In addition, the activity of phenolic compounds also increases as the pH decreases *(11).* At low pH the hydrophobicity of phenolic compounds increases, enabling them to more easily dissolve in the lipids of the cell membrane of target bacteria

(22). This could explain the higher activity of some phenolic compounds (such as hydrocinnamic acid) and their combinations in napoletana and pesto sauces, with lower pH values as compared with carbonara and green sauce for fish. In other cases (such as eugenol) a high activity was also detected at higher pH (like carbonara sauce). Therefore, the food composition seems to play a key role on the final effect of phenolic compounds and AS-48 against *S. aureus.*

We should also remark on the low concentration of hydrocinnamic acid required to potentiate the activity of AS-48 (5 mM), compared with the other phenolic compounds (at least 20 mM or above), especially in napoletana and pesto sauces. In carbonara sauce, where both AS-48 and the phenolic compounds had a much more limited activity, the reduction of staphylococcal counts below detection levels during prolonged storage at 22°C for some combinations of hydrocinnamic acid and AS-48 is of technological interest in order to develop naturally preserved low-acid sauces, and also to provide an additional hurdle against *S. aureus* proliferation during accidental interruption of the refrigeration chain.

Results from the present work clearly indicate that both the concentration of enterocin AS-48 and the phenolic compounds required to inhibit *S. aureus* in food could be lowered by combined addition of the two types of inhibitors. There are few reports on the combined action of bacteriocins and phenolic compounds. The simultaneous application of nisin (0.15 μg/ml) and carvacrol or thymol (0.3 mM or 45 μg/ml) caused a larger decline in viable counts for strains of *Bacillus cereus* as compared with application of the same antimicrobials individually *(33).* Although the mechanism of synergy is not known, it has been suggested that it may lie in the enhanced dissipation of the membrane potential and a reduction in the pH gradient and intracellular ATP *(34).* Because enterocin AS-48 also dissipates the bacterial membrane potential *(16),* the potentiating effect of enterocin AS-48 and phenolic compounds may rely on a similar mechanism of action. However, the precise mechanism of potentiation needs to be investigated further.

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