COMBINATION WITH PLANT EXTRACTS IMPROVES THE INHIBITORY ACTION OF DIVERGICIN M35 AGAINST *LISTERIA MONOCYTOGENES*

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ABSTRACT

The susceptibility of 11 strains of Listeria monocytogenes *to divergicin M35, a bacteriocin produced by* Carnobacterium divergens *strain M35, and to aqueous extracts of garlic, onion, oregano, red chili and black pepper at 30 and 10C, was evaluated using a microdilution assay. The susceptibility of divergicin-resistant strains to combinations of these agents was also evaluated. Three strains were resistant to divergicin M35 (>500 μg/mL) at 30C but were more susceptible at 10C. Garlic gave the most inhibitory plant extract, followed by onion, while oregano, red chili and black pepper extracts were less active at both temperatures. Garlic extract and divergicin M35 combined or with other extracts increased inhibitory activity against the divergicin-resistant strains. The garlic/divergicin combination was the most effective at inhibiting these strains and was bactericidal at both temperatures. Log-phase cells were the most susceptible to the garlic/divergicin combination. Stationary-phase cells were much more resistant at both incubation temperatures. Furthermore, the effect of the garlic/divergicin combination at inhibit-*

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ing divergicin-resistant L. monocytogenes *in a food system was also studied using cold-smoked salmon as a food model. Results indicated that this combination could efficiently reduce the viability of* L. monocytogenes *in smoked salmon stored at 10C.*

PRACTICAL APPLICATIONS

There is increasing popularity worldwide for chemical preservative-free, ready-to-eat and minimally processed seafood with low salt, fat and sugar content. Bacteriocins produced from lactic acid bacteria can have a potential application to prolong the shelf life of cold-smoked salmon. Also, plant and spice extracts have been shown to contain antibacterial substances with potential for application in foods. Thus, this research explores the combination of divergicin M35, a bacteriocin produced by *Carnobacterium divergens* strain M35, and aqueous extracts of garlic, onion, oregano, red chili and black pepper to inhibit *Listeria monocytogenes* and to prolong the shelf life of cold-smoked salmon.

INTRODUCTION

Lactic acid bacteria (LAB) have been shown to inhibit various food spoilage and pathogenic organisms, including both gram-positive and gram-negative bacteria, yeasts and fungi. The inhibitory effect of LAB has been attributed to their ability to produce various inhibitory substances, including hydrogen peroxide, organic acids, diacetyl and low-molecular-weight proteinaceous molecules known as bacteriocins (Ray and Daeschel 1992; Larsen and Norrung 1993; O'Sullivan *et al.* 2002). These substances, especially bacteriocins, are believed to have potential applications as food preservatives.

Among LAB bacteriocin producers, carnobacteria have been shown to produce several bacteriocins, including carnobacteriocins BM1 and B2 (Quadri *et al.* 1994), divergicin A (Worobo *et al.* 1995), divercin V41 (Métivier *et al.* 1998), divergicin 750 (Holck *et al.* 1996), piscicocin V1a (Bhugaloo-Vial *et al.* 1996) and carnocin CP5 (Herbin *et al.* 1997). Tahiri *et al.* (2004) have characterized a new class IIa bacteriocin called divergicin M35, produced by *Carnobacterium divergens* strain M35, isolated from commercial frozen mussels. Divergicin M35, a peptide of 43 amino acids, has a molecular mass of 4,518.75 Da, a pI value of 8.3, positive net charge (+3) and shares 80.5% homology with divercin V41. This bacteriocin has been shown to have strong inhibitory effect against *Listeria monocytogenes*.

Although effective, the use of bacteriocins for food preservation remains limited by several factors, especially the nature of the food matrix, sensitivity of the peptides to food compounds and development of resistant variants. These limitations have led researchers to seek new bacteriocins that can be used in combination or rotation with existing bacteriocins in order to maximize the inhibitory effect, overcome the appearance of resistant variants and consequently prolong the shelf life of foods. In the case of seafoods, several bacteriocin-producing species have been tested for their ability to suppress pathogens and spoilage microorganisms and to improve overall microbiological quality. However, the use of bacteriocin-producing organisms in ready-to-eat seafoods usually diminishes product organoleptic and sensorial characteristics. To overcome this drawback, purified bacteriocins mixed with inhibitory substances produced from traditional spices naturally present in plant tissues have been proposed. For example, aqueous and oil extracts of traditional ingredients or spices such as garlic and oregano have been shown to contain antibacterial substances with potential for application in foods such as meat and fish as active protection against *L. monocytogenes* (Kumar and Berwal 1998; Seaberg *et al.* 2003; Lin *et al.* 2004). Garlic has been shown to have a synergistic effect with nisin and sakacin K in inhibiting *L. monocytogenes* (Singh *et al.* 2001; Hugas *et al.* 2002).

The aim of the present study was (1) to evaluate the sensitivity of 11 *L. monocytogenes* strains of food origin to divergicin M35 and to aqueous extracts prepared from garlic, onion, oregano and black and red peppers at 30 or 10C; (2) to determine whether combinations of divergicin M35 with any of these extracts act synergistically against divergicin M35-resistant strains of *L. monocytogenes* and (3) to determine the effectiveness of divergicin M35/garlic extract at inhibiting divergicin M35-resistant *L. monocytogenes* in cold-smoked salmon stored at 10C for 21 days.

MATERIALS AND METHODS

Bacterial Strains and Growth Media

All strains were reactivated from frozen stock in 20% glycerol at −80C. Divergicin M35-producing *C. divergens* strain M35 was grown in De Man, Rogosa and Sharpe broth (De Man *et al.* 1960) obtained from Difco Laboratories (Sparks, MD) containing 0.1% (v/v) Tween 80. Food origin *Listeria monocytogenes* strains LSD338, LSD339, LSD340, LSD523, LSD524, LSD525, LSD530, LSD531, LSD532, LSD535 and LSD538, isolated from cheese, egg, milk, ice cream and frozen whole egg, were obtained from the Laboratory Services Division, Canadian Food Inspection Agency (Ottawa,

Ontario, Canada). They were grown in tryptic soy broth (Difco Laboratories) supplemented with 0.6% (w/v) yeast extract (TSBYE). Each bacterial strain was subcultured at least three times (1% transfer, v/v) at 24-h intervals before use.

Purification of Divergicin M35

Divergicin M35 was purified from the culture supernatant of *C. divergens* M35, using the method recently described by Tahiri *et al.* (2004). The pure bacteriocin was freeze-dried and kept at −80C.

Preparation of Aqueous Extracts of Plant Materials

Commercial samples of dry spices, including ground red pepper (*Capsicum annum* L.), black pepper (*Piper nigrum* L.) and oregano (*Origanum vulgare*) were obtained from Encore Gourmet Food Corp. (Montreal, Quebec, Canada). Fresh garlic (*Allium sativum*) and onion (*Allium cepa*) were purchased from a local market in Quebec City (Quebec, Canada), rinsed with sterile distilled water, cut into small pieces, freeze-dried and ground to powder.

Dry material was suspended in distilled water at a final concentration of 10% (w/v) and was stirred for 3 h at 4C. The suspensions were then centrifuged at 7,000 × g for 15 min to produce clear supernatants, which were freeze-dried and stored at −20C.

Sensitivity of *L. monocytogenes* to Plant Extracts and Divergicin M35

The sensitivity of *L. monocytogenes* to divergicin M35 and aqueous extracts of plant matter was determined in terms of minimum inhibitory concentration (*MIC*) and minimum bactericidal concentration (*MBC*) using the microdilution assay described by Mota-Meira *et al.* (2000). Strains were grown in TSBYE for 6 h (mid log phase). The optical density (OD_{650}) of each culture, measured using a Spectronic 20 spectrophotometer (Bausch & Lomb Inc., Rochester, NY), was adjusted to 0.1 with fresh TSBYE, followed by 10-fold dilution in fresh TSBYE. Viable cells in the diluted culture were counted after plating on TSBYE agar (1.4% w/v) and incubating aerobically at 37C for 24 h.

Solutions of freeze-dried divergicin M35 (1 mg/mL) and aqueous extract (10 mg/mL) were prepared in distilled water on the day of testing and were filter sterilized through 0.45-µm pore size membrane (Cameo 25 N, MSI, Westboro, MA).

Serial twofold dilutions of inhibitor were prepared in 96-well polystyrene microplates (Becton Dickinson Labware, Lincoln Park, NJ) containing 150 µL/well of TSBYE. Bacterial suspension (30 µL) standardized to approxi-

mately $2.5–5.0 \times 10^4$ cfu/well was then added to each well. The microplates were incubated at either 30 or 10C for 24 h or 14 days, respectively, and the OD_{650} was read with a Thermomax microplate reader (Molecular Devices, Menlo Park, CA). Controls (wells inoculated with the tested culture without added inhibitor) and blanks (wells containing noninoculated broth medium with added inhibitor) were run on each microplate. The *MIC* corresponds to the lowest concentration of tested inhibitor giving complete inhibition of growth, which is indicated by an optical density similar to that of noninoculated broth (Karakoc and Gerceker 2001). The *MBC* corresponds to the concentration that killed 99.9% of the initial inoculum, based on the National Committee for Clinical Laboratory Standards (1991) method. For the determination of *MBC*, 10 µL was withdrawn from wells showing complete inhibition of tested strains (Kheadr *et al.* 2004), and was plated on TSBYE agar and incubated aerobically at 30C for 24 h. The microdilution assay was repeated four times. The median value of these repetitions provided the *MIC* or *MBC*.

Checkerboard Assay for Sensitivity of *L. monocytogenes* to Inhibitor Combinations

Three *L. monocytogenes* strains (LSD338, LSD525 and LSD535), selected because of their higher resistance to divergicin M35, were tested for sensitivity to combinations of inhibitor. Checkerboard microassays were conducted for garlic extract plus other plant extract and divergicin M35 plus plant extract. Extracts of onion, black pepper, red pepper and oregano were reconstituted in distilled water at initial concentrations of 10.0, 5.0 and 2.5 mg/mL, while garlic extract was reconstituted at concentrations of 2.5, 1.5 and 0.6 mg/mL. Divergicin M35 was reconstituted at concentrations of 0.125, 0.25, 0.50 and 1.0 mg/mL. The combinations were obtained by mixing equal volumes of these concentrations.

Antimicrobial combination (75 µL) was added to each well in a 96-well polystyrene microplate, followed by standardized bacterial suspension. The microplates were then incubated at 30 or 10C for 24 h or 14 days, respectively. The OD_{650} was read with the microplate reader, and *MIC* and *MBC* values were determined as described earlier. The assay was repeated four times. The median value of these repetitions provided the *MIC* and *MBC*.

The fractional inhibitory concentration (*FIC*) index for each inhibitor in each antimicrobial combination was calculated as follows:

$$FIC \text{ index of agent A } (FIC_A) = MIC \text{ of agent A in combination} / MIC \text{ of agent A alone}$$

The *FIC* index of agents A and B in combination is the sum of their respective *FIC* indexes: $FIC_{A+B} = FIC_A + FIC_B$. The interaction between two agents was considered synergistic if FIC_{A+B} was ≤ 0.5, additive if it was from 0.5 to 1.0, indifferent if it was between 1.0 and 4.0, and antagonistic if it was >4.0 (Barchiesi *et al.* 2001).

Death Time Study

The effects of divergicin M35/garlic extract combinations on the viability of *L. monocytogenes* LSD338, LSD525 and LSD535 were determined at two stages of growth. Cells grown for 6 and 18 h (stationary phase) in TSBYE broth (25 mL, inoculated at 1% v/v) were harvested by centrifugation at $7,000 \times g$ for 15 min, washed twice with 0.01 M phosphate-buffered saline (PBS) at pH 6.5 and resuspended to a final concentration of approximately 10^7 cfu/mL. The divergicin/garlic combination in PBS was then added to obtain concentrations twice the *MBC* of each inhibitor, as determined by microdilution assay. Tubes containing 10 mL of bacterial suspension were incubated aerobically at 30C for 3 h or at 10C for 24 h. Samples (100 µL) were withdrawn in duplicate at 0, 1, 2 and 3 h for incubation at 30C, and at 0, 3, 6, 9 and 24 h for incubation at 10C, and were serially diluted 10-fold in peptone water (0.1% w/v). Appropriate dilutions were plated in duplicate on TSBYE agar and were incubated aerobically at 30C for 48 h. Each experiment was repeated three times.

Validation of Inhibition of *L. monocytogenes* LSD535 in Cold-smoked Salmon

L. monocytogenes-free cold-smoked pacific salmon (Sockeye salmon) fillets (25.0 ± 1.0 g each) were obtained from Grizzly Smoke House Company (St. Augustin, Province of Quebec, Canada). Sixty-three fillets were spiked with fresh culture of *L. monocytogenes* LSD535 at a final concentration of 5×10^4 cfu/g and were subjected to the following treatments:

(A) Twenty-one fillets: considered as control.
(B) Twenty-one fillets: freeze-dried divergicin M35 was reconstituted in distilled water, filter sterilized through 0.45-µm pore size membrane and spread on each fillet to obtain a final concentration of 0.125 mg/g.
(C) Twenty-one fillets: freeze-dried divergicin M35 and garlic aqueous extract were reconstituted in distilled water, filter sterilized through 0.45-µm pore size membrane and then spread on each fillet to final concentrations of 0.125 and 1.25 mg/g, respectively.

All fillets were kept in a laminar-flow biological safety cabinet for approximately 10 min in order to dry off excessive liquid. Fillets were vacuum

packed in plastic film (1.3- to 2.2-mil thickness, Cryovac, Lachine, Province of Quebec, Canada) and were kept at 10C for 21 days. Samples were taken in triplicate at 0 (just after applying the treatment), 1, 3, 7, 14 and 21 days for the determination of *L. monocytogenes* viable counts using *Listeria* selective agar medium (Oxoid Ltd., Basingstoke, Hampshire, England) at 37C for 48 h.

Statistical Analysis

Statistical analyses were performed with Statgraphics Plus 4.1 (Manugistics Inc., Rockville, MD). Significant differences among mean values of viable listerial counts determined during death time study or during storage of *L. monocytogenes*-contaminated smoked salmon were tested by analysis of variance. Treatment comparisons were performed using Fisher's least significant difference test, with a P value of ≤ 0.05 considered significant.

RESULTS

Sensitivity of *L. monocytogenes* to Divergicin M35 and Plant Extracts

The *MIC* and *MBC* values for divergicin and plant extracts determined at 30 and 10C against *L. monocytogenes* are given in Table 1. At 30C, *L. monocytogenes* strains showed variable sensitivity to divergicin M35, with *MIC* values ranging from 1 up to >500 μg/mL. Divergicin M35 did not appear to have a bactericidal effect against any strain, even at a concentration of 500 μg/mL.

Among the plant extracts, garlic appeared to be the best inhibitor of *L. monocytogenes*, being effective at 0.6–1.2 mg/mL, while onion extract was inhibitory at 2.5–5.0 mg/mL. Garlic did not show any bactericidal effect against any strain even at a concentration of 5.0 mg/mL (the highest concentration tested). In contrast, onion appeared to be bactericidal against all strains at this concentration, while oregano and black and red pepper did not inhibit any strain.

At 10C, there were remarkable reductions in the *MIC* values for both divergicin M35 and garlic, compared to 30C. All strains were inhibited by divergicin M35 at a concentration of 1 μg/mL, except for LSD525, which resisted concentrations up to 0.5 mg/mL. As was the case at 30C, divergicin M35 did not show any bactericidal effect at 10C even at a concentration of 0.5 mg/mL. Garlic was inhibitory at concentrations ranging from 0.3 to 1.2 mg/mL and became bactericidal at 0.6–1.2 mg/mL. There was a slight difference in *MIC* and *MBC* values for onion at the two temperatures, while oregano and black and red pepper extracts again had no inhibitory effect against any strain, even at 5 mg/mL.

TABLE 1.
MINIMUM INHIBITORY (TOP) AND BACTERICIDAL (BOTTOM) CONCENTRATIONS OF GARLIC, ONION, OREGANO, BLACK PEPPER AND RED PEPPER WATER-SOLUBLE EXTRACTS AND DIVERGICIN M35 FOR *LISTERIA MONOCYTOGENES* AT 30 AND 10C*

Listerial strain	30C						10C					
	Garlic	Onion	Oregano	Black pepper	Red pepper	Divergicin M35	Garlic	Onion	Oregano	Black pepper	Red pepper	Divergicin M35
LSD338	1.2	2.5–5.0	>5.0	>5.0	>5.0	>0.5	0.6	2.5–5.0	>5.0	>5.0	>5.0	0.006
	>5.0	5.0	>5.0	>5.0	>5.0	>0.5	0.6	5.0	>5.0	>5.0	>5.0	>0.5
LSD339	1.2	2.5–5.0	>5.0	>5.0	>5.0	0.031	0.6	2.5–5.0	>5.0	>5.0	>5.0	0.001
	>5.0	5.0	>5.0	>5.0	>5.0	>0.5	1.2	5.0	>5.0	>5.0	>5.0	0.001
LSD340	1.2	2.5–5.0	>5.0	>5.0	>5.0	0.004	0.6	2.5–5.0	>5.0	>5.0	>5.0	0.001
	>5.0	5.0	>5.0	>5.0	>5.0	>0.5	1.2	5.0	>5.0	>5.0	>5.0	>0.5
LSD523	1.2	2.5–5.0	>5.0	>5.0	>5.0	0.015	0.6	2.5–5.0	>5.0	>5.0	>5.0	0.001
	>5.0	5.0	>5.0	>5.0	>5.0	>0.5	1.2	5.0	>5.0	>5.0	>5.0	>0.5
LSD524	1.2	2.5–5.0	>5.0	>5.0	>5.0	0.004	0.6–1.2	2.5–5.0	>5.0	>5.0	>5.0	0.041
	>5.0	5.0	>5.0	>5.0	>5.0	>0.5	1.2	5.0	>5.0	>5.0	>5.0	>0.5
LSD525	1.2	2.5–5.0	>5.0	>5.0	>5.0	>0.5	0.6–1.2	2.5–5.0	>5.0	>5.0	>5.0	>0.5
	>5.0	5.0	>5.0	>5.0	>5.0	>0.5	1.2	5.0	>5.0	>5.0	>5.0	>0.5
LSD530	1.2	2.5–5.0	>5.0	>5.0	>5.0	0.002	0.6–1.2	2.5–5.0	>5.0	>5.0	>5.0	0.001
	>5.0	5.0	>5.0	>5.0	>5.0	>0.5	1.2	5.0	>5.0	>5.0	>5.0	0.5
LSD531	0.6	2.5–5.0	>5.0	>5.0	>5.0	0.031	0.3	2.5–5.0	>5.0	>5.0	>5.0	0.031
	>5.0	5.0	>5.0	>5.0	>5.0	>0.5	1.2	5.0	>5.0	>5.0	>5.0	>0.5
LSD532	0.6	2.5	>5.0	>5.0	>5.0	0.062	0.6	2.5	>5.0	>5.0	>5.0	0.006
	>5.0	5.0	>5.0	>5.0	>5.0	>0.5	1.2	5.0	>5.0	>5.0	>5.0	>0.5
LSD535	1.2	2.5–5.0	>5.0	>5.0	>5.0	>0.5	0.6	2.5–5.0	>5.0	>5.0	>5.0	0.006
	>5.0	5.0	>5.0	>5.0	>5.0	>0.5	1.2	5.0	>5.0	>5.0	>5.0	>0.5

* In milligram per milliter, determined by the microdilution method; median of four repetitions.

Checkerboard Studies of Inhibitor Combinations

Because divergicin M35 and extract of garlic showed the highest antilisterial activity, the checkerboard experiment was carried out to evaluate the effectiveness of combinations of these two inhibitors with the other extracts at inhibiting the divergicin M35-resistant *L. monocytogenes* strains LSD338, LSD525 and LSD535.

The combination garlic extract (1.2 mg/mL)/divergicin M35 (0.06 mg/mL) showed bactericidal activity against the three *L. monocytogenes* divergicin-resistant strains at 30C (Table 2). The garlic/onion combination at 0.3 and 5.0 mg/mL, respectively, showed a bactericidal effect against these strains. Combined with oregano, black and red pepper extracts, garlic extract suppressed the growth of at least one of these strains at a lower concentration than determined for garlic alone. However, none of these latter combinations produced a bactericidal effect. In contrast, at 10C, all combinations of garlic with other inhibitors produced bactericidal effects against the divergicin-resistant strains, and the combination garlic/onion was inhibitory at a lower concentration.

The combination of divergicin M35 (0.06 mg/mL) with aqueous onion extract (5.0 mg/mL) at 30C produced a bacteriostatic inhibitory effect against strains LSD338 and LSD525, and a bactericidal effect against strain LSD535 (Table 3). The same concentrations for the divergicin M35 and oregano combination were inhibitory to LSD338 and LSD525 but not to LSD535. The divergicin/oregano combination did not have a bactericidal effect against either LSD338 or LSD525. Divergicin M35 combined with either aqueous black or red pepper extracts did not have even an inhibitory effect against any of the divergicin-resistant strains.

At 10C, divergicin M35/plant extract combinations were more effective at inhibiting divergicin M35-resistant strains than at 30C. The divergicin/oregano combination at 0.06/1.25 mg/mL inhibited both LSD338 and LSD535. Moreover, increasing the oregano extract concentration to 2.5 mg/mL made this combination bactericidal to both of these strains and inhibitory to strain LSD525. The divergicin/oregano tandem was bactericidal for this latter strain only when the divergicin concentration was also increased to 0.25 mg/mL. Similarly to the results obtained at 30C, the divergicin M35/onion extract combination had mostly a bacteriostatic rather than a bactericidal effect on the divergicin M35-resistant strains, although the effective concentrations of onion extract were lower than those determined at 30C. Two of the three strains were inhibited at divergicin M35/onion extract concentrations of 0.06/1.25 mg/mL. Divergicin M35 in combination with either black or red pepper had bacteriostatic and bactericidal activity against the divergicin-resistant strains. A bactericidal effect was obtained against strain LSD338 at

TABLE 2.
MINIMUM INHIBITORY (TOP) AND BACTERICIDAL (BOTTOM) CONCENTRATIONS OF WATER-SOLUBLE EXTRACT OF GARLIC COMBINED WITH THAT OF ONION, OREGANO, BLACK PEPPER, RED PEPPER OR WITH DIVERGICIN M35-RESISTANT *LISTERIA MONOCYTOGENES* AT 30 AND 10C*

Listerial strain	30C					10C				
	+Onion	+Oregano	+Black pepper	+Red pepper	+Divergicin M35	+Onion	+Oregano	+Black pepper	+Red pepper	+Divergicin M35
LSD338	0.3 + 2.5	0.6 + 1.2	0.6 + 1.2	0.3 + 1.2	0.3 + 0.06	0.3 + 1.2	0.6 + 1.2	0.6 + 1.2	0.3 + 1.2	0.3 + 0.01
	0.3 + 5.0	None	None	None	1.2 + 0.06	0.3 + 5.0	1.2 + 1.2	1.2 + 1.2	0.3 + 1.2	0.3 + 0.06
LSD525	0.3 + 2.5	0.6 + 1.2	0.6 + 1.2	1.2 + 1.2	0.3 + 0.06	0.3 + 1.2	0.3 + 1.2	0.6 + 1.2	0.6 + 1.2	0.3 + 0.06
	0.3 + 5.0	None	None	None	1.2 + 0.06	0.3 + 5.0	1.2 + 1.2	1.2 + 1.2	1.2 + 1.2	0.6 + 0.06
LSD535	0.3 + 2.5	0.6 + 1.2	1.2 + 1.2	1.2 + 1.2	0.3 + 0.06	0.3 + 1.2	0.3 + 2.5	0.6 + 1.2	0.6 + 1.2	0.3 + 0.01
	0.3 + 5.0	None	None	None	1.2 + 0.06	0.3 + 5.0	1.2 + 1.2	1.2 + 1.2	1.2 + 1.2	0.6 + 0.06

* In milligram per milliliter, determined by the microdilution method; median of four repetitions.
None = not bactericidal at any of the tested concentrations.

TABLE 3.
MINIMUM INHIBITORY (TOP) AND BACTERICIDAL (BOTTOM) CONCENTRATIONS OF DIVERGICIN M35 COMBINED WITH WATER-SOLUBLE EXTRACT OF ONION, OREGANO, BLACK PEPPER OR RED PEPPER FOR DIVERGICIN M35-RESISTANT *LISTERIA MONOCYTOGENES* AT 30 AND 10C*

Listerial strain	30C				10C			
	+Onion	+Oregano	+Black pepper	+Red pepper	+Onion	+Oregano	+Black pepper	+Red pepper
LSD338	0.06 + 5.0	0.06 + 5.0	None	None	0.06 + 1.25	0.06 + 1.25	0.06 + 1.25	0.06 + 1.25
	None	None	None	None	None	0.06 + 2.5	0.06 + 1.25	0.06 + 2.5
LSD525	0.06 + 5.0	0.06 + 5.0	None	None	0.06 + 2.5	0.06 + 2.5	0.5 + 5.0	0.5 + 1.25
	None	None	None	None	None	0.25 + 2.5	0.5 + 5.0	None
LSD535	0.06 + 5.0	None	None	None	0.06 + 1.25	0.06 + 1.25	0.06 + 1.25	0.06 + 1.25
	0.06 + 5.0	None	None	None	None	0.06 + 2.5	None	None

* In milligram per milliter, determined by the microdilution method; median of four repetitions.
None = the sought effect did not occur at any of the tested concentrations.

TABLE 4.
EFFECT OF PLANT EXTRACT OR DIVERGICIN M35/PLANT EXTRACT COMBINATION ON DIVERGICIN M35-RESISTANT *LISTERIA MONOCYTOGENES* AT 30 AND 10C, BASED ON FRACTIONAL INHIBITORY CONCENTRATION INDEX

Combination	Temperature (C)	Listerial strain		
		LSD338	LSD525	LSD535
Garlic/onion	30	Additive	Additive	Additive
Garlic/oregano		Additive	Additive	Additive
Garlic/black pepper		Additive	Additive	Indifferent
Garlic/red pepper		Synergistic	Indifferent	Indifferent
Garlic/divergicin M35		Synergistic	Synergistic	Synergistic
Garlic/onion	10	Additive	Synergistic	Additive
Garlic/oregano		Additive	Synergistic	Additive
Garlic/black pepper		Indifferent	Additive	Indifferent
Garlic/red pepper		Additive	Additive	Indifferent
Garlic/divergicin M35		NA	Synergistic	NA
Divergicin M35/onion	30	Indifferent	Indifferent	Indifferent
Divergicin M35/oregano		Indifferent	Indifferent	ND
Divergicin M35/black pepper		ND	ND	ND
Divergicin M35/red pepper		ND	ND	ND
Divergicin M35/onion	10	NA	Synergistic	NA
Divergicin M35/oregano		NA	Synergistic	NA
Divergicin M35/black pepper		NA	Indifferent	NA
Divergicin M35/red pepper		NA	Indifferent	NA

ND, not determined, because no tested combination was inhibitory; NA, not applicable, because the lowest concentration of divergicin M35 tested in combination with plant extract was 10-fold higher than the minimum inhibitory concentration determined for the bacteriocin alone.

0.06/1.25 mg/mL of divergicin M35/black pepper extract and at 0.06/2.5 mg/mL of divergicin M35/red pepper extract. The divergicin M35/black pepper combination was bactericidal to strain LSD525 at the maximal concentration of 0.5/5.0 mg/mL.

In order to characterize the inhibitory action of the various combinations of agents as synergistic, additive, indifferent or antagonistic (Table 4), the *FIC* of each antimicrobial combination was determined. A synergistic interaction was found between garlic extract and divergicin M35 for all three divergicin-resistant *L. monocytogenes* strains at 30C as well as for strain LSD525 at 10C. It is apparent in Table 4 that the type of interaction between the two inhibitors varies widely depending on the target strain and incubation temperature. For example, the effect of garlic/onion and garlic/oregano combinations at 10C was additive for strains LSD338 and LSD535, but was synergistic for strain LSD525. The interaction between divergicin and onion or oregano extracts was indifferent for strain LSD525 at 30C, but was synergistic at 10C.

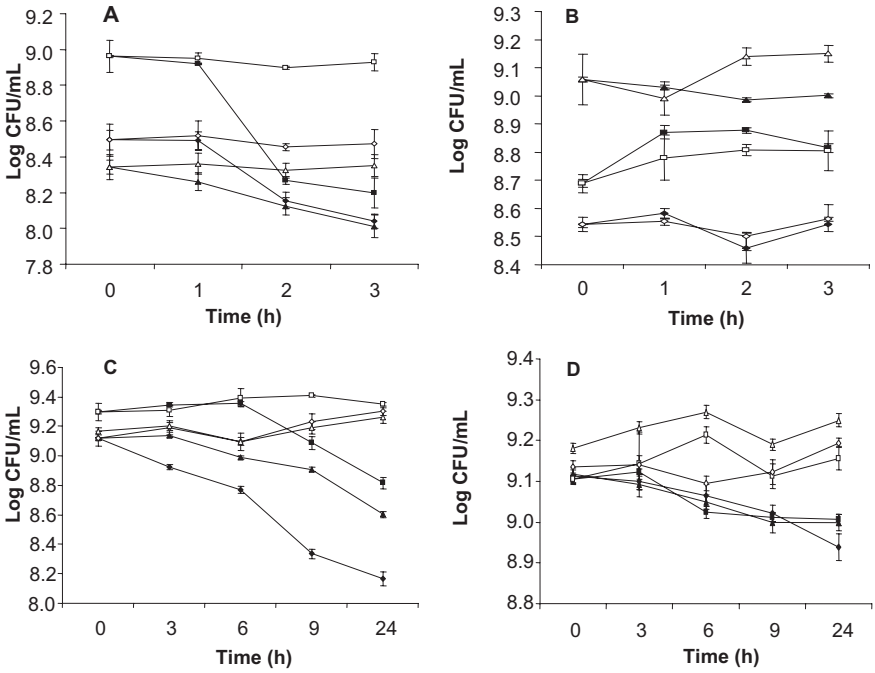

FIG. 1. EFFECT OF DIVERGICIN M35 COMBINED WITH AQUEOUS GARLIC EXTRACT ON THE VIABILITY OF DIVERGICIN-RESISTANT *LISTERIA MONOCYTOGENES* STRAINS LSD338 (■), LSD525 (◆) AND LSD535 (▲) SUSPENDED IN PHOSPHATE-BUFFERED SALINE (PBS) AT TWICE THE PRESPECTIVE MINIMUM INHIBITORY CONCENTRATION VALUE (TABLE 2) AND INCUBATED AEROBICALLY AT 30C FOR 3 H (A,B) OR AT 10C FOR 24 H (C,D)

(A) and (C) represent log-phase cells, while (B) and (D) represent stationary-phase cells. The symbols □, ◇ and △ represent strains LSD338, LSD525 and LSD535, respectively, in PBS without inhibitor.

Death Time Study

Death curves of log and stationary-phase cells of divergicin-resistant *L. monocytogenes* strains LSD338, LSD525 and LSD535 at 30C and 10C in the presence of divergicin M35/aqueous garlic extract at twice the corresponding *MIC* values are shown in Fig. 1. Generally, log-phase cells of the three strains were more sensitive than stationary-phase cells at both temperatures. Log-phase cells of strain LSD338 at 30C decreased by approximately 0.8 log cycle after 3 h compared to 0.5 and 0.35 log reductions for LSD525 and LSD535, respectively (Fig. 1A). No reduction in viability was seen for the corresponding stationary-phase cells (Fig. 1B). At 10C, the viability of log-phase

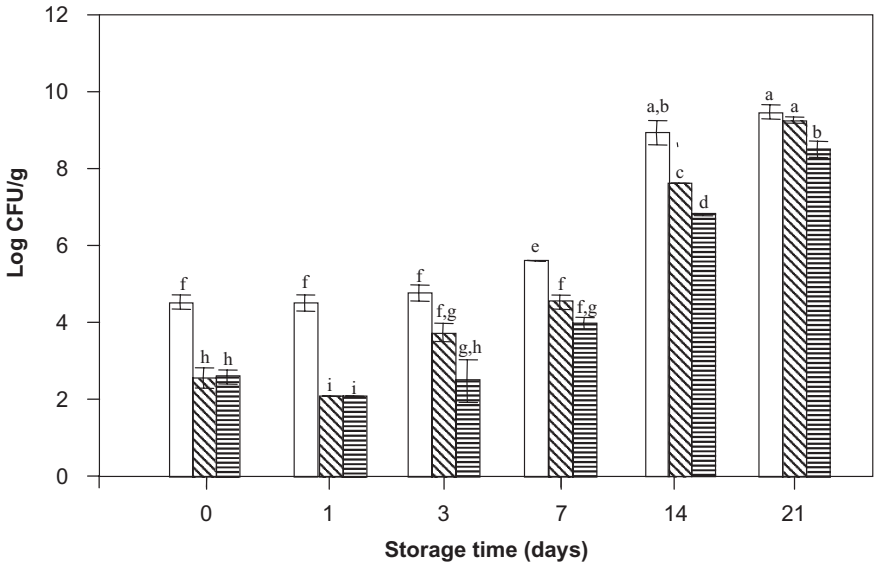

FIG. 2. EVOLUTION OF *LISTERIA MONOCYTOGENES* LSD535 COUNTS IN COLD-SMOKED SALMON DURING STORAGE AT 10C FOR 21 DAYS
All fillets were spiked with *Listeria monocytogenes* LSD535 to a final concentration of 4.5×104 cfu/g. □, untreated fillets; ▨, fillets treated with divergicin M35 (0.125 mg/g) and (▤) fillets treated with a concentration of aqueous garlic extract (1.25 mg/g) and divergicin M35 (0.125 mg/g). Means without common letters are significantly different ($P < 0.05$).

LSD338, LSD525 and LSD535 after 24 h was reduced by 1.4, 1.0 and 0.5 log cfu/mL, respectively (Fig. 1C), while very small decreases in the viability of stationary-phase cells (approximately 0.1–0.2 log cfu/mL) were observed (Fig. 1D).

Inactivation of *L. monocytogenes* in Smoked Salmon

Figure 2 shows the changes in viable counts of *L. monocytogenes* LSD535 in cold-smoked salmon fillets stored at 10C for 21 days. Starting with initial counts of 4.5×10^4 cfu/g, approximately 2 log reductions in counts of *L. monocytogenes* were detected immediately after applying either divergicin M35 alone or in combination with garlic extract. Between days 3 and 21, more reductions in viable counts of LSD535 were observed in fillets treated with divergicin M35/garlic combination compared with those treated with divergicin M35 alone. At day 21, the viable counts of LSD535 strain were approximately 0.2 and 1.0 log cfu/g lower in fillet treated with either divergicin alone or combined with garlic extract, respectively, compared with counts determined in untreated fillets.

DISCUSSION

Of the 11 strains of *L. monocytogenes* tested, three (LSD338, LSD525 and LSD535) were not even inhibited by the highest concentration of divergicin M35 tested. Resistance among the sensitive strains was more apparent at 30C. *L. monocytogenes* is known to be generally susceptible to class IIa bacteriocins (Ennahar *et al.* 2000). Resistance has been attributed to modifications in the structures and composition of the cell envelope (Ming and Daeschel 1995; Davies *et al.* 1996; Mazzotta and Montville 1997). Changes in membrane phospholipid contents and cell surface hydrophobicity in *L. monocytogenes* exposed to bacteriocins have also been reported.

In the present study, aqueous extracts of red chili, black pepper and oregano were not active against *L. monocytogenes* at either incubation temperature, even at the highest concentration tested (5 mg/mL). This may be due to low concentration of the inhibitory active compounds compared to other constituents in the crude aqueous extracts (Cichewicz and Thorpe 1996), to low solubility of such compounds in the aqueous phase and/or the absence of catalysts required for the manifestation of the inhibitory effects of these spices. It is known that the chemical composition of spices extracted from a particular plant species can vary with geographic origin and harvesting period, which may be sufficient to cause variability in the degree of susceptibility of target bacteria (Burt 2004). The inconsistency of commercial samples of spice and the variability of a given herb may be due to protocol design, dosage type, preparation and amounts of critical compounds (e.g., phenolic compounds) during extraction, which could also account for differences in antimicrobial potency (Pandit and Shelef 1994; Hao *et al.* 1998; Ward *et al.* 1998; Ali *et al.* 2000).

In the present study, garlic and onion extracts had a bactericidal effect against *L. monocytogenes*, which was more evident at 10C than at 30C. Similar findings have been reported by Singh *et al.* (2001) and Ali *et al.* (2000). The combination of garlic extract and nisin has previously been shown to act synergistically in inhibiting *L. monocytogenes* (Singh *et al.* 2001). In comparison, the divergicin M35/onion extract combination did not show any bactericidal effect at either incubation temperature, except for strain LSD535 at 30C. This was unexpected, because onion extract either alone or combined with garlic extract did produce a bactericidal effect against all tested *L. monocytogenes* strains at both incubation temperatures. Onion extract thus appears to lose its bactericidal effect when combined with divergicin M35, which raises the possibility of antagonistic effects between divergicin and inhibitory compounds present in onion extract.

The spices used in this study are known to produce a wide variety of antimicrobial inhibitory substances with potential antilisterial activity. Two

antibacterial components in oregano extract, thymol and carvacrol, have been found to be effective at inhibiting the growth of *L. monocytogenes* (Seaberg *et al.* 2003). These inhibitory compounds are believed to act as a transmembrane carrier of monovalent cations by exchanging their hydroxyl proton for ions such as potassium (Ultee *et al.* 2002). Among the components found in garlic aqueous extract, allicin has been identified as a potential antimicrobial compound (Ankri and Mirelman 1999). The antimicrobial activity of allicin has not been fully characterized but has been attributed to its ability to inhibit RNA synthesis (Feldberg *et al.* 1988), in addition to perturbing cell membranes (Miron *et al.* 2000). On the other hand, the antimicrobial activity of red chili and black pepper is attributed mainly to compounds identified as capsaicin and cinnamic acid (Cichewicz and Thorpe 1996; Kouassi and Shelef 1998), which are believed to inhibit enzymes involved in glucose uptake and adenosine triphosphate (ATP) production. The increased antilisterial activity of garlic extract or divergicin M35 when combined with each other or with extracts of oregano, red chili or black pepper suggests that the effects of the substances involved are at least additive. For example, membrane-perturbing effects of divergicin M35 on bacteria may facilitate the penetration of allicin, capsaicin or cinnamic acid into the intracellular medium to inhibit RNA synthesis and glucose uptake enzymes. Bactericidal effects were achieved at lower concentrations of each extract in the combination compared with each extract alone, and at least one extract that was not bactericidal at any tested concentration increased the effectiveness of divergicin, e.g., oregano. Previous studies have shown that the inhibitory effect of sakacin K (pediocin-like bacteriocin) against *L. monocytogenes* is increased by combining it with black pepper extract (Hugas *et al.* 1995; Aymerich *et al.* 2000). This enhancement of sakacin K activity is attributed to manganese ions presented in black pepper extract, which may act as a catalyzing factor for bacteriocin activity (Hugas *et al.* 2002).

Incubation temperature appeared to play an important role in determining the susceptibility of *L. monocytogenes* to the inhibitors and the interactions in the combinations. Almost all of the inhibitors and their combinations were more effective at 10C, and the inhibitory effect was stable for 14 days. They were more bactericidal than bacteriostatic at 10C and were more bacteriostatic at 30C. This may be explained by differences in the fluidity of the cell membrane, which is the primary target for bacteriocins and other inhibitors. At lower temperatures, the proportion of unsaturated fatty acyl chains of the lipids increases, resulting in higher membrane fluidity (Stanley 1991; Abee *et al.* 1994). Such increases in membrane fluidity could facilitate the penetration of lipophilic compounds as divergicin M35 and other inhibitory compounds into the cell membrane.

One of the factors influencing the susceptibility of *Listeria* toward certain biopreservatives, including bacteriocins, is the cell growth phase at the moment of treatment with the antimicrobial substances (Jydegaard *et al.* 2000). In the present study, *L. monocytogenes* cells in the exponential growth phase were more susceptible to divergicin M35/garlic extract than were cells in the stationary growth phase. This is in agreement with previous studies (O'Drissol and Gahan 1996; Luppens *et al.* 2001; Schobitz *et al.* 2003) and may be attributed to differences in cell membrane structure associated with rapid cell division (Madigan *et al.* 2000). Furthermore, cells in the stationary growth phase are adapting to survive adverse conditions, including starvation and exposure to toxic substances (e.g., acid and other metabolites), which may make them less susceptible to inhibitors (Madigan *et al.* 2000).

In the last set of experiments of this study, the efficiency of divergicin M35 either alone or in combination with garlic aqueous extract was studied using cold-smoked salmon as a food model. The lower viable counts of *L. monocytogenes* in fillets treated with divergicin M35/garlic combination, compared with those treated with divergicin alone, may indicate the synergistic relationship between both inhibitors to suppress this pathogen in smoked salmon and confirm the results obtained by checkerboard and death time studies. Similarly, Singh *et al.* (2001) reported that the combination of garlic aqueous extract and nisin was effective at preventing *L. monocytogenes* growth in hummus bi tahini (chickpea-based salad) and could help overcome problems of nisin-resistant strains. The authors attributed this finding to the synergistic action of nisin and allicin, the major antibacterial compound of garlic aqueous extract, on cytoplasmic cell membrane.

The present study has shown that it is possible to improve the antilisterial activity of divergicin and plant extracts by using them in combination, even though these extracts may not produce any antilisterial activity when used alone. The combination of garlic extract/divergicin M35 showed a synergistic effect with a remarkable inhibitory effect against divergicin-resistant *L. monocytogenes* strains. This combination proved also to be efficient to suppress *L. monocytogenes* in cold-smoked salmon and may thus be recommended for applications in those foods that do not receive adequate thermal treatment or do not undergo any thermal treatment. However, more research is needed to determine whether the use of natural antibacterial compounds from plants or spices in purified or partially purified forms can improve the antibacterial effects of bacteriocins and increase their stability and efficacy in food matrixes. More efficient methods for the extraction of naturally occurring antimicrobial compounds in plants and spices are needed in order to recover all potentially useful agents, to characterize these and to maximize their inhibitory effects.

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