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Effect of Vanillin, Ethyl Vanillin, and Vanillic Acid on the Growth and Heat Resistance of *Cronobacter* Species

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ABSTRACT

Preservatives could be part of an effective intervention strategy for the control of *Cronobacter* species in foods, but few compounds with the desired antimicrobial properties have been identified to date. We examined the antibacterial activity of vanillin, ethyl vanillin, and vanillic acid against seven *Cronobacter* spp. in quarter-strength tryptic soy broth with 5 g/liter yeast extract (TSBYE) adjusted to pH 5.0, 6.0, and 7.0 at 10, 21, and 37°C. All compounds exhibited pH- and temperature-dependant bacteriostatic and bactericidal activity. MICs of vanillin and ethyl vanillin consistently increased with decreasing pH and temperature, but vanillic acid had little activity at pH values of 6.0 and 7.0. The MICs for all temperatures, pH values, and bacterial strains tested were 2 mg/ml ethyl vanillin, 3 mg/ml vanillin, and >8 mg/ml vanillic acid. MBCs also were influenced by pH, although significantly higher concentrations were needed to inactivate the bacteria at 21°C than at 10 or 37°C. Survivor curves for *Cronobacter sakazakii* strains at the MBCs of each compound revealed that all treatments resulted in immediate loss of cell viability at 37°C. Measurements of propidium iodide uptake indicated that the cell membranes were damaged by exposure to all three compounds. The thermal resistance of *C. sakazakii* was examined at 58°C in TSBYE supplemented with MBCs of each compound at pH 5.0 and 6.0. *D*-values at pH 5.0 were reduced from 14.56 ± 0.60 min to 0.93 ± 0.01, 0.63 ± 0.01, and 0.98 ± 0.02 min for vanillin, ethyl vanillin, and vanillic acid, respectively. These results suggest that vanillin, ethyl vanillin, and vanillic acid may be useful for the control of *Cronobacter* spp. in food during preparation and storage.

Cronobacter (which includes biogroups formerly known as *Enterobacter sakazakii*) is a genus in the family *Enterobacteriaceae* that includes several emerging opportunistic pathogenic species which can cause severe and often lethal infections in neonates, the elderly, and immunocompromised adults *(17)*. The normal habitat of *Cronobacter* is unknown, but various species have been isolated from the natural environment, food processing or preparation facilities, and households *(5, 18, 22, 45, 47)*. Microbiological examination of foods has revealed occasional contamination of raw milk, cheeses, meats, fresh vegetables, and a range of dried products including milk or whey, herbs, spices, bread, tea, cereals, flours, dehydrated processed foods, and powdered infant formula *(7, 16, 17)*. The health risk associated with powdered infant formula has become widely recognized due to strong epidemiological evidence linking reconstituted infant formula to *Cronobacter* infections in neonates *(17, 37)*. Available data suggest that the pathogen occurs infrequently and at low levels in commercial dried food products. For example, Muytjens et al. *(35)*, using a most-probable-number procedure, recovered *Cronobacter* at 0.36 to 66 CFU/100 g from 20 powdered infant formulas from 13 countries. *Cronobacter* can persist for long periods of time at low water activity (a_w) and survive rehydration with hot water, a common method applied in the preparation of dried foods. Growth has been reported in diverse food matrices at 6 to 47°C *(19, 23, 24)*. Hence, outgrowth during storage subsequent to rehydration is a potential health risk, notably in household refrigerators where temperatures can be as high as 7 to 10°C *(37)*. The persistence of *Cronobacter* species in dried foods has been ascribed to their resistance to a broad range of stresses, including desiccation, heat, cold, acid, and osmotic shock *(6, 7, 13)*. The inherent thermotolerance of *Cronobacter* species, which appears to be highest among the *Enterobacteriaceae,* has been the focus of several investigations *(2, 19, 38, 41)*. Decimal reduction times (*D*-values) for *Cronobacter* species differ depending on strain or prior exposure to stresses such as heat, desiccation. or antimicrobial agents and range from 0.27 to 9.87 min at 58°C *(11, 20, 48)*. Consequently, the control of *Cronobacter* during the manufacture, preparation, and storage of some foods is challenging, notably in dried products that require rehydration before consumption.

Various strategies have been proposed to control the risk associated with *Cronobacter* contamination, including the use of antimicrobial compounds that can reduce thermal

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tolerance during rehydration in hot water and/or inhibit growth in reconstituted dried foods *(1, 3, 20, 25, 26, 28, 36, 43)*. Vanilla extracted from the bean or pod of the vanilla orchid (*Vanilla planifolia* Andrews, syn. *V. fragrans* (Salisb. Ames)) contains a number of molecules, principally the phenolic aldehyde vanillin (4-hydroxy-3-methoxybenzaldehyde) and minor amounts of ethyl vanillin (3-ethoxy-4-hydroxybenzaldehyde) and vanillic acid (4-hydroxy-3-methoxybenzoic acid). These compounds are generally regarded as safe, are widely used as flavoring agents in food products such as ice cream, beverages, pastries, and confectionery, and are known to have antimicrobial activity against a wide range of bacteria *(12, 15, 21, 32–34, 39, 45)*, yeasts, and molds *(8, 14, 29–31)*. To our knowledge, activity against *Cronobacter* species has not been reported. Hence, the objectives of this study were to determine whether vanillin, ethyl vanillin, or vanillic acid exert antimicrobial effects against *Cronobacter* species; to quantify the activity of each compound over a range of pH values and temperatures; to establish whether inhibition is due to bacteriostatic or bactericidal effects; and to determine the influence of each compound on the heat resistance of *Cronobacter* species. This research was carried out to establish the potential value of vanillin, ethyl vanillin, or vanillic acid as preservatives for the formulation of safer dried food products.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Seven *Cronobacter* strains (*C. sakazakii* HPB 2855, *C. sakazakii* HPB 2871, *C. sakazakii* HPB 3290, *C. dublinensis* HPB 3169, *C. malonaticus* HPB 3267, *C. muytjensii* HPB 3270, and *Cronobacter* genomospecies 1 HPB 3287) were obtained from the Bureau of Microbial Hazards culture collection (Health Canada, Ottawa, Ontario, Canada). Stock cultures were maintained at −80°C in tryptic soy broth (TSB; BBL, BD, Sparks, MD) containing 20% glycerol. Working cultures were grown in TSB supplemented with 5 g/liter yeast extract (TSBYE) for 24 h at 37°C.

Chemicals and preparation of stock solutions. Vanillin and vanillic acid were obtained from Sigma-Aldrich (St. Louis, MO), and ethyl vanillin was obtained from SAFC Supply Solutions (St. Louis, MO). Stock solutions of each compound were prepared in quarter-strength TSBYE. The compounds were solubilized by heating to approximately 100°C. When needed, pH was adjusted to 5.0, 6.0, or 7.0 with 5 N HCl or 10 N NaOH, and the solutions were sterilized by passage through 0.45-μm-pore-size membrane filters (Fisher Scientific, Ottawa, Ontario, Canada).

Measurement of MICs and MBCs at different pH values and temperatures. MICs and MBCs were determined with the use-dilution method as described by Delaquis et al. *(12)*. Each *Cronobacter* strain was grown in TSBYE for 24 h at 37°C. Inocula were prepared by dilution of the culture with quarter-strength TSBYE adjusted to the experimental pH with either 5 N HCl or 10 N NaOH and sterilization by membrane filtration. Appropriate amounts of the stock solution and quarter-strength TSBYE were dispensed into the wells of microtiter plates (Cellstar, Greiner Bio-One, Monroe, NC) with a multichannel micropipettor to achieve concentrations of 1 to 8 mg/ml vanillin and vanillic acid and 1 to 4 mg/ml ethyl vanillin. Each well was then inoculated with 100 μl of *Cronobacter* culture (final level of approximately 5 log CFU/ml). The wells were examined for evidence of growth after 48 h at 37°C, 48 h at 21°C, and 8 days at 10°C. The MIC was defined as the lowest concentration that prevented visible growth. A loopful of medium from wells without evidence of growth was transferred to tryptic soy agar supplemented with 5 g/liter yeast extract (TSAYE), which was incubated for 24 h at 37°C to determine the MBCs. Three independent replicates were performed at 37, 21, and 10°C in media adjusted to pH 5.0, 6.0, and 7.0.

Survivor curves for *C. sakazakii* at different pH values and temperatures in media containing vanillin, ethyl vanillin, and vanillic acid. Stock solutions of vanillin, ethyl vanillin, and vanillic acid were prepared in quarter-strength TSBYE adjusted to pH 5.0 and 6.0 as described above. Duplicate Erlenmeyer flasks containing quarter-strength TSBYE adjusted to pH 5.0 were supplemented with the stock solutions to achieve final concentrations (after inoculation) of 3, 2, and 2 mg/ml vanillin, ethyl vanillin, and vanillic acid respectively. A second series of Erlenmeyer flasks with quarter-strength TSBYE at pH 6.0 was supplemented with 4, 3, and 6 mg/ml concentrations of each compound. Three *C. sakazakii* strains (HPB 2855, HPB 2871, and HPB 3290) were grown separately in TSBYE for 24 h at 37°C. Equal volumes of each culture were combined and diluted in quarter-strength TSBYE adjusted to pH 5.0 or 6.0. The mixed culture was added to the test medium to achieve an initial cell density of approximately 5 log CFU/ml. Inoculated samples without any added vanillin, ethyl vanillin, and vanillic acid were used as controls. One series of flasks was incubated at 37°C for 48 h, and the second series was incubated at 10°C for 8 days. Samples were periodically withdrawn from each flask to estimate surviving cell populations. Suitable dilutions were spread onto duplicate TSAYE plates, which were incubated at 37°C for 24 h. One milliliter from each sample was also added to 10 ml of TSBYE to bring the detection limit to 1 CFU/ml. The medium was examined for evidence of turbidity after 24 h of incubation at 37°C.

Effect of vanillin, vanillic acid, and ethyl vanillin on the heat resistance of *C. sakazakii*. A cocktail of the three *C. sakazakii* strains was prepared as described previously. Stock solutions of the test compounds were prepared in quarter-strength TSBYE adjusted to pH 5.0 or 6.0. Test solutions were prepared with vanillin, ethyl vanillin, and vanillic acid at concentrations of 3, 2, and 2 mg/ml, respectively, at pH 5.0 and concentrations of 4, 3, and 6 mg/ml, respectively, at pH 6.0. Thermal inactivation experiments were carried out essentially as described by Osaili et al. *(42)*. Fifty milliliters of each test solution in a sterile 100-ml Pyrex bottle (Duran, Wertheim/Main, Germany) were preheated to 58°C in a temperature-controlled shaking water bath (Precision Scientific Inc., Chicago, IL). Inoculum (500 μl) was added to each test bottle to achieve initial cell densities of approximately 7 log CFU/ml. Samples (3 ml) were withdrawn from each bottle at various time intervals and immediately placed in an ice-water bath. Surviving cell populations in the samples were determined by the spread plate method on TSAYE incubated at 37°C for 24 h. Recovery of cells below the detection limit afforded by direct plating was ensured by enrichment of 1 ml sample in 10 ml of TSBYE at 37°C for 24 h. The *D*-values were determined from the negative reciprocal of the slopes of the regression lines, using the linear portions of the survivor curves.

One bottle containing 50 ml of quarter-strength TSBYE was fitted with a thermocouple (Omega Engineering, Inc., Stamford, CT), and a second was placed in the water bath to monitor temperatures during each experiment. Addition of inoculum and switching off the agitation in the water bath during sampling did

TABLE 1. *MICs and MBCs of vanillin against* Cronobacter *species in quarter-strength TSBYE adjusted to pH 5.0, 6.0, and 7.0 at 10, 21, and 37°C*[a]

Temp (°C)	*Cronobacter* strain	MIC (mg/ml)			MBC (mg/ml)		
		pH 5.0	pH 6.0	pH 7.0	pH 5.0	pH 6.0	pH 7.0
10	*C. sakazakii* HPB 2855	0.50 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	4.00 ± 0.00	4.33 ± 0.47	4.67 ± 0.47
	C. sakazakii HPB 2871	0.50 ± 0.00	1.00 ± 0.00	1.33 ± 0.47	4.00 ± 0.00	5.00 ± 0.00	4.67 ± 0.47
	C. sakazakii HPB 3290	0.50 ± 0.00	1.00 ± 0.00	1.33 ± 0.47	2.67 ± 0.47	4.00 ± 0.00	4.00 ± 0.82
	C. dublinensis HPB 3169	0.50 ± 0.00	1.00 ± 0.00	1.33 ± 0.47	3.33 ± 0.47	3.67 ± 0.47	4.00 ± 0.00
	C. malonaticus HPB 3267	0.50 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	3.67 ± 0.47	4.00 ± 0.00	4.33 ± 0.47
	C. muytjensii HPB 3270	0.50 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	3.00 ± 0.00	3.33 ± 0.47	3.33 ± 0.47
	C. genomospecies 1 HPB 3287	0.50 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	5.00 ± 0.00	5.67 ± 0.47	5.67 ± 1.70
21	*C. sakazakii* HPB 2855	1.33 ± 0.47	2.33 ± 0.47	2.67 ± 0.47	5.33 ± 0.47	5.67 ± 0.47	5.67 ± 0.47
	C. sakazakii HPB 2871	1.00 ± 0.00	2.33 ± 0.47	2.67 ± 0.94	5.33 ± 0.94	5.67 ± 0.47	6.00 ± 0.82
	C. sakazakii HPB 3290	1.67 ± 0.47	2.33 ± 0.47	2.67 ± 0.47	4.33 ± 0.47	5.67 ± 0.47	5.00 ± 0.82
	C. dublinensis HPB 3169	1.00 ± 0.00	2.00 ± 0.00	2.67 ± 0.47	5.00 ± 0.82	5.33 ± 0.47	5.33 ± 0.94
	C. malonaticus HPB 3267	1.33 ± 0.47	2.33 ± 0.47	2.67 ± 0.47	5.00 ± 0.82	5.67 ± 0.47	5.00 ± 1.41
	C. muytjensii HPB 3270	1.33 ± 0.47	2.33 ± 0.47	2.67 ± 0.47	5.00 ± 0.82	5.33 ± 0.47	5.33 ± 0.94
	C. genomospecies 1 HPB 3287	1.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	4.67 ± 1.25	6.33 ± 0.94	8.00 ± 0.00
37	*C. sakazakii* HPB 2855	2.00 ± 0.00	2.33 ± 0.47	3.00 ± 0.00	3.00 ± 0.00	3.33 ± 0.47	4.00 ± 0.82
	C. sakazakii HPB 2871	2.00 ± 0.00	2.33 ± 0.47	3.00 ± 0.00	3.00 ± 0.00	3.67 ± 0.47	3.67 ± 0.47
	C. sakazakii HPB 3290	2.00 ± 0.00	2.33 ± 0.47	3.00 ± 0.00	3.00 ± 0.00	3.33 ± 0.47	3.67 ± 0.47
	C. dublinensis HPB 3169	1.67 ± 0.47	2.33 ± 0.47	3.00 ± 0.00	2.67 ± 0.47	3.33 ± 0.47	3.33 ± 0.47
	C. malonaticus HPB 3267	1.67 ± 0.47	2.00 ± 0.00	3.00 ± 0.00	2.67 ± 0.47	3.00 ± 0.00	3.33 ± 0.47
	C. muytjensii HPB 3270	1.67 ± 0.47	2.00 ± 0.00	3.00 ± 0.00	2.67 ± 0.47	2.67 ± 0.47	3.33 ± 0.47
	C. genomospecies 1 HPB 3287	1.67 ± 0.47	2.00 ± 0.00	3.00 ± 0.00	2.67 ± 0.47	3.33 ± 0.94	3.67 ± 0.47

[a] Each value is the mean ± standard error of three independent replicates.

not cause a measurable decline in the temperatures of the test solution.

Determination of membrane damage by propidium iodide uptake. Propidium iodide uptake by cells exposed to vanillin, vanillic acid, and ethyl vanillin was determined essentially as described by Niven and Mulholland *(40)*. Two strains of *C. sakazakii* (HPB 2855 and HPB 2871) were cultured individually in TSBYE at 37°C for 24 h. The cells were harvested by centrifugation at 6,000 × *g* for 10 min, washed twice with phosphate-buffered saline (PBS; pH 5.0), and resuspended in an equal volume of PBS (pH 5.0). Aliquots (10 ml) of the cell suspensions were added to glass test tubes containing 10 ml of a stock solution prepared with the test compounds. Aliquots (4 ml) were then transferred to test tubes with 4 ml of a propidium iodide solution (100 μM) after 6 and 24 h of incubation at 37°C. The samples were held in the dark for 10 min at room temperature (21 ± 1°C) and then spun at 6,000 × *g* for 10 min. The cells in the pellets were resuspended in fresh PBS (pH 5.0) and spun again at the same speed. Aliquots of the final pellet suspended in PBS (200 μl, pH 5.0) were added to the wells of a 96-well black microtiter plate (Fluotrac 200, Greiner Bio-one, Frickenhausen, Germany). Fluorescence was measured with a spectrofluorometer (Gemini EM, Molecular Device Corp., Sunnyvale, CA) with an excitation wavelength of 490 nm and an emission wavelength of 610 nm. Cell cultures treated with cetyl trimethylammonium bromide (CTAB; 1 mM) or without addition of antimicrobial were subjected to the same procedures for use as positive and negative controls, respectively.

Statistical analyses. Three independent replicates were performed for each experimental condition. A one-way analysis of variance was performed using procedures in the SPSS Statistics Standard software (SPSS, Chicago, IL), and means were compared using Tukey's honest significant differences post-hoc test (IBM Corp, Sommers, NY).

RESULTS AND DISCUSSION

The antibacterial activity of vanillin, ethyl vanillin, and vanillic acid against *Cronobacter* species was examined at pH 5.0, 6.0, and 7.0 and 10, 21, and 37°C. Mean MICs and MBCs (Tables 1 through 3) indicate that the three compounds exerted both bacteriostatic and bactericidal effects against all the test strains. The consistency in measurements achieved within specific pH-temperature combinations suggested that none of the strains were resistant to the compounds. Mean values were therefore calculated from measurements obtained with all strains to shed light on the influence of pH and temperature on antimicrobial activity. Temperature and pH both influenced the nature and strength of the antibacterial effects associated with individual compounds (Table 4). The bacteriostatic activity of vanillin and ethyl vanillin consistently increased with decreasing pH and temperature. Bactericidal activity was likewise influenced by pH, although significantly higher concentrations of both compounds were needed to inactivate the bacteria at 21°C than at 10 or 37°C ($P < 0.05$). Vanillic acid had comparatively weak bacteriostatic and bactericidal effects at pH 6.0 and 7.0, but MICs at pH 5.0 were similar to those of vanillin and ethyl vanillin, and MBCs were the lowest measured during this study.

Enhanced antimicrobial activity at reduced pH has been described for various natural antimicrobial compounds from plant sources, including vanillin *(12, 29, 30, 33, 44)*. Activity is often derived from weak acids or molecules with

TABLE 2. *MICs and MBCs of ethyl vanillin against* Cronobacter *species in quarter-strength TSBYE adjusted to pH 5.0, 6.0, and 7.0 at 10, 21, and 37°C*[a]

Temp (°C)	*Cronobacter* strain	MIC (mg/ml)			MBC (mg/ml)		
		pH 5.0	pH 6.0	pH 7.0	pH 5.0	pH 6.0	pH 7.0
10	*C. sakazakii* HPB 2855	0.50 ± 0.00	0.50 ± 0.00	0.50 ± 0.00	3.00 ± 0.00	3.00 ± 0.00	3.33 ± 0.47
	C. sakazakii HPB 2871	0.50 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	3.00 ± 0.00	3.33 ± 0.47	3.33 ± 0.47
	C. sakazakii HPB 3290	0.50 ± 0.00	0.67 ± 0.24	1.00 ± 0.00	2.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00
	C. dublinensis HPB 3169	0.50 ± 0.00	0.83 ± 0.24	1.00 ± 0.00	2.00 ± 0.00	2.67 ± 0.47	2.67 ± 0.47
	C. malonaticus HPB 3267	0.50 ± 0.00	0.50 ± 0.00	0.50 ± 0.00	2.00 ± 0.00	2.67 ± 0.47	3.00 ± 0.00
	C. muytjensii HPB 3270	0.50 ± 0.00	0.50 ± 0.00	0.50 ± 0.00	2.00 ± 0.00	2.33 ± 0.47	2.67 ± 0.47
	C. genomospecies 1 HPB 3287	0.50 ± 0.00	0.83 ± 0.24	0.83 ± 0.24	3.00 ± 0.00	4.00 ± 0.00	4.00 ± 0.00
21	*C. sakazakii* HPB 2855	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	3.33 ± 0.47	4.00 ± 0.00	4.00 ± 0.00
	C. sakazakii HPB 2871	1.00 ± 0.00	1.33 ± 0.47	2.00 ± 0.00	4.00 ± 0.00	4.00 ± 0.00	>4.00
	C. sakazakii HPB 3290	1.00 ± 0.00	1.00 ± 0.00	2.00 ± 0.00	3.00 ± 0.00	4.00 ± 0.00	4.00 ± 0.00
	C. dublinensis HPB 3169	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	3.00 ± 0.00	3.67 ± 0.47	4.00 ± 0.00
	C. malonaticus HPB 3267	1.00 ± 0.00	1.33 ± 0.47	2.00 ± 0.00	3.00 ± 0.00	4.00 ± 0.00	4.00 ± 0.00
	C. muytjensii HPB 3270	0.67 ± 0.24	0.83 ± 0.24	1.00 ± 0.00	3.00 ± 0.00	4.00 ± 0.00	4.00 ± 0.00
	C. genomospecies 1 HPB 3287	0.83 ± 0.24	0.83 ± 0.24	1.00 ± 0.00	3.67 ± 0.47	4.00 ± 0.00	>4.00
37	*C. sakazakii* HPB 2855	1.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00
	C. sakazakii HPB 2871	1.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.67 ± 0.47	3.33 ± 0.47
	C. sakazakii HPB 3290	1.00 ± 0.00	1.67 ± 0.47	2.00 ± 0.00	2.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00
	C. dublinensis HPB 3169	1.00 ± 0.00	1.33 ± 0.47	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	3.00 ± 0.00
	C. malonaticus HPB 3267	1.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.67 ± 0.47
	C. muytjensii HPB 3270	1.00 ± 0.00	1.33 ± 0.47	2.00 ± 0.00	2.00 ± 0.00	2.67 ± 0.47	3.00 ± 0.00
	C. genomospecies 1 HPB 3287	1.00 ± 0.00	1.67 ± 0.47	2.00 ± 0.00	2.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00

[a] Each value is the mean ± standard error of three independent replicates.

side groups that can become dissociated and acquire a charge. The pH of the test medium or food can therefore influence activity because the undissociated form of the molecules can penetrate the negatively charged cell membrane more readily than can the dissociated form *(12, 44)*. Vanillic acid is a classical weak acid with a pKa of 4.51 at 25°C. Increased activity at pH 5.0 can therefore be ascribed to the acid-base behavior of the compound. In

TABLE 3. *MICs and MBCs of vanillic acid against* Cronobacter *species in quarter-strength TSBYE adjusted to pH 5.0, 6.0, and 7.0 at 10, 21, and 37°C*[a]

Temp (°C)	*Cronobacter* strain	MIC (mg/ml)			MBC (mg/ml)		
		pH 5.0	pH 6.0	pH 7.0	pH 5.0	pH 6.0	pH 7.0
10	*C. sakazakii* HPB 2855	0.50 ± 0.00	3.00 ± 0.00	>8.00	1.00 ± 0.00	8.00 ± 0.00	>8.00
	C. sakazakii HPB 2871	0.83 ± 0.24	6.00 ± 0.00	>8.00	1.00 ± 0.00	8.00 ± 0.00	>8.00
	C. sakazakii HPB 3290	0.50 ± 0.00	3.00 ± 0.00	>8.00	1.00 ± 0.00	8.00 ± 0.00	>8.00
	C. dublinensis HPB 3169	1.00 ± 0.00	5.00 ± 0.00	>8.00	1.00 ± 0.00	8.00 ± 0.00	>8.00
	C. malonaticus HPB 3267	0.50 ± 0.00	2.33 ± 0.47	>8.00	1.00 ± 0.00	8.00 ± 0.00	>8.00
	C. muytjensii HPB 3270	0.50 ± 0.00	2.00 ± 0.00	>8.00	0.50 ± 0.00	8.00 ± 0.00	>8.00
	C. genomospecies 1 HPB 3287	0.50 ± 0.00	4.67 ± 0.47	>8.00	2.00 ± 0.00	>8.00	>8.00
21	*C. sakazakii* HPB 2855	1.00 ± 0.00	5.67 ± 0.47	>8.00	3.00 ± 0.00	>8.00	6.67 ± 0.47
	C. sakazakii HPB 2871	2.00 ± 0.00	7.33 ± 0.47	>8.00	3.33 ± 0.47	>8.00	7.00 ± 0.82
	C. sakazakii HPB 3290	1.00 ± 0.00	6.33 ± 0.47	>8.00	4.00 ± 0.00	>8.00	6.00 ± 0.00
	C. dublinensis HPB 3169	1.33 ± 0.47	7.33 ± 0.47	>8.00	2.00 ± 0.00	>8.00	6.67 ± 0.47
	C. malonaticus HPB 3267	1.00 ± 0.00	5.33 ± 0.47	>8.00	3.00 ± 0.00	>8.00	7.50 ± 0.50
	C. muytjensii HPB 3270	1.00 ± 0.00	5.33 ± 0.47	>8.00	2.33 ± 0.47	>8.00	7.00 ± 0.00
	C. genomospecies 1 HPB 3287	1.33 ± 0.47	>8.00	>8.00	5.00 ± 0.00	>8.00	7.00 ± 0.00
37	*C. sakazakii* HPB 2855	2.00 ± 0.00	5.00 ± 0.00	4.00 ± 0.00	2.00 ± 0.00	5.67 ± 0.47	4.00 ± 0.00
	C. sakazakii HPB 2871	2.00 ± 0.00	5.00 ± 0.00	4.00 ± 0.00	2.00 ± 0.00	5.67 ± 0.47	4.00 ± 0.00
	C. sakazakii HPB 3290	2.00 ± 0.00	5.00 ± 0.00	4.00 ± 0.00	2.00 ± 0.00	6.33 ± 0.47	4.00 ± 0.00
	C. dublinensis HPB 3169	1.00 ± 0.00	5.67 ± 0.47	4.00 ± 0.00	1.00 ± 0.00	6.33 ± 0.47	4.00 ± 0.00
	C. malonaticus HPB 3267	1.00 ± 0.00	5.00 ± 0.00	4.00 ± 0.00	1.00 ± 0.00	6.33 ± 0.47	4.33 ± 0.47
	C. muytjensii HPB 3270	1.00 ± 0.00	5.00 ± 0.00	4.00 ± 0.00	1.00 ± 0.00	5.33 ± 0.47	5.00 ± 0.00
	C. genomospecies 1 HPB 3287	2.00 ± 0.00	7.00 ± 0.00	4.00 ± 0.00	2.00 ± 0.00	7.00 ± 0.00	5.33 ± 0.47

[a] Each value is the mean ± standard error of three independent replicates.

TABLE 4. *MICs and MBCs of vanillin, ethyl vanillin, and vanillic acid for all* Cronobacter *strains*[a]

		MIC (mg/ml)			MBC (mg/ml)		
Compound	Temp (°C)	pH 5.0	pH 6.0	pH 7.0	pH 5.0	pH 6.0	pH 7.0
Vanillin	10	C 0.50 ± 0.00 Y	B 1.00 ± 0.00 X	C 1.14 ± 0.20 X	B 3.67 ± 0.13 Y	B 4.29 ± 0.23 X	B 4.38 ± 0.59 X
	21	B 1.24 ± 0.24 Z	A 2.24 ± 0.34 Y	B 2.57 ± 0.42 X	A 4.95 ± 0.71 Y	A 5.67 ± 0.49 X	A 5.76 ± 0.74 X
	37	A 1.81 ± 0.13 Z	A 2.19 ± 0.27 Y	A 3.00 ± 0.00 X	C 2.81 ± 0.27 Y	C 3.24 ± 0.37 XY	C 3.57 ± 0.42 X
Ethyl vanillin	10	C 0.50 ± 0.00 Y	C 0.69 ± 0.09 X	C 0.76 ± 0.03 X	B 2.43 ± 0.00 Y	B 3.00 ± 0.12 X	B 3.14 ± 0.20 X
	21	B 0.93 ± 0.06 Y	B 1.05 ± 0.17 Y	B 1.43 ± 0.00 X	A 3.29 ± 0.12 Y	A 3.95 ± 0.07 X	A 4.00 ± 0.00 X
	37	A 1.00 ± 0.00 Z	A 1.71 ± 0.20 Y	A 2.00 ± 0.00 X	C 2.00 ± 0.00 Z	C 2.62 ± 0.13 Y	B 3.00 ± 0.12 X
Vanillic acid	10	C 0.62 ± 0.03 Z	C 3.71 ± 0.12 Y	A 8.00 ± 0.00 X	C 1.07 ± 0.00 Y	A 8.00 ± 0.00 X	A 8.00 ± 0.00 X
	21	B 1.24 ± 0.07 Z	A 6.48 ± 0.37 Y	A 8.00 ± 0.00 X	A 3.24 ± 0.13 Z	A 8.00 ± 0.00 X	B 7.05 ± 0.45 Y
	37	A 1.57 ± 0.00 Z	B 5.38 ± 0.07 X	B 4.00 ± 0.00 Y	B 1.57 ± 0.00 Z	B 6.14 ± 0.12 X	C 4.38 ± 0.07 Y

[a] MICs and MBCs from Tables 1 through 3 were averaged for comparative purposes. Each value represents the mean ± standard error of three independent replicates for each *Cronobacter* strain. Within a column and for each compound, means preceded by different letters are significantly different ($P < 0.05$). Within a row and for each measure (MIC or MBC), means followed by different letters are significantly different ($P < 0.05$).

contrast, the influence of incubation temperature on the antimicrobial effects is unclear. The results of previous investigations suggested that the antimicrobial activity of vanillin should have increased with decreasing incubation temperature *(14, 29, 46)*. Differences in the fatty acid profile and fluidity of cell membranes at different temperatures affect permeability to various compounds *(3)*, and the solubility of the test compounds in water may be limited, especially at low temperatures *(12)*. Hence, the reduced bactericidal activity observed at 21°C could be the result of physiological factors that affect membrane stability and differences in the solubility of the compounds at various temperatures.

Quarter-strength TSBYE containing vanillin, ethyl vanillin, or vanillic acid at concentrations approaching the MBC for each condition was inoculated with *C. sakazakii* to examine the rate and extent of inhibition at pH 5.0 and 6.0 at 10 and 37°C. Cell viability began to decline immediately upon exposure to all the compounds at 37°C (Fig. 1). There was little difference in the responses observed to vanillin or ethyl vanillin at either pH, but the decline was slightly faster in quarter-strength TSBYE containing vanillic acid at pH 5.0. Figure 2 shows similar immediate reductions in viability at 10°C, although the effect of pH was more pronounced, particularly with vanillin and vanillic acid. *Cronobacter* is a moderately acid-resistant member of the *Enterobacteriaceae*. Some isolates are able to resist exposure to pH 3.5 for >5 h, and growth has been demonstrated in tomato juice (pH 4.4), watermelon juice (pH 5.1), and cantaloupe juice (pH 6.8) stored at 25°C *(12, 23)*. The present findings suggest that all three compounds may be useful for the control of *Cronobacter* species in acidic foods that are normally preserved by refrigeration.

Phenolic compounds such as vanillin, ethyl vanillin, and vanillic acid are known to affect the cell membranes of bacterial cells. Fitzgerald et al. *(15)* found that vanillin caused loss of cytoplasmic membrane integrity, leading to cellular leakage in gram-positive and gram-negative bacteria. Inhibition is believed to result in part from increased membrane permeability, leading to the loss of intracellular

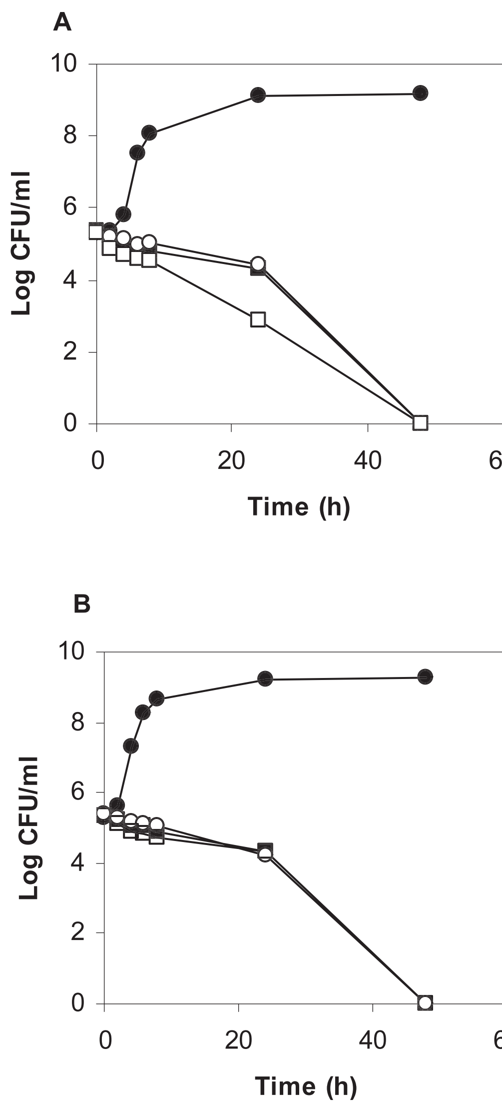

FIGURE 1. *Effects of vanillin, ethyl vanillin, and vanillic acid on the fate of a three-strain* C. sakazakii *cocktail in quarter-strength TSBYE adjusted to pH 5.0 (A) or pH 6.0 (B) at 37°C. Control (untreated) (●); ethyl vanillin 2 mg/ml at pH 5.0, 3 mg/ml at pH 6.0 (○); vanillin 3 mg/ml at pH 5.0, 4 mg/ml at pH 6.0 (■); vanillic acid 2 mg/ml at pH 5.0, 6 mg/ml at pH 6.0 (□).*

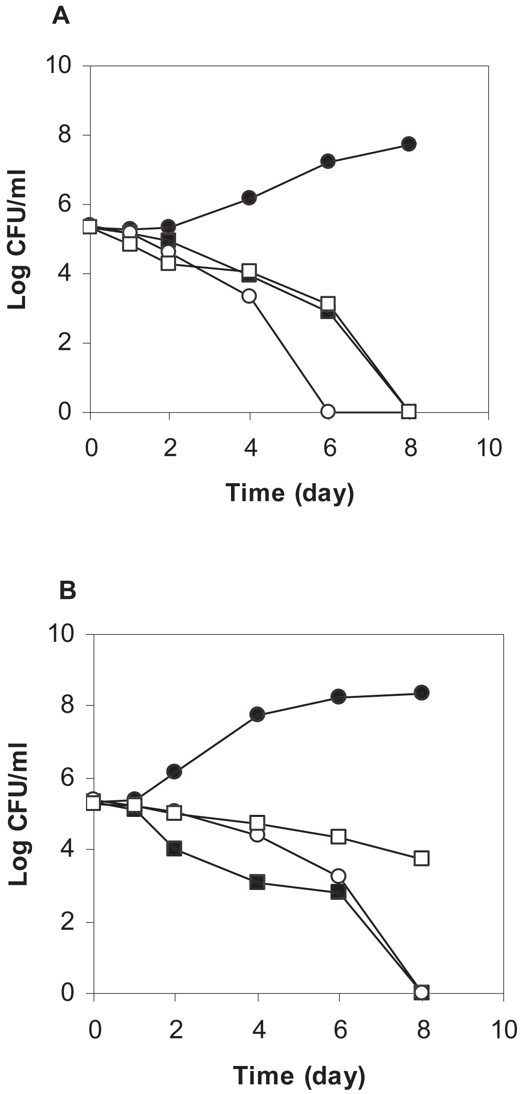

FIGURE 2. *Effects of vanillin, ethyl vanillin, and vanillic acid on the fate of a three-strain* C. sakazakii *cocktail in quarter-strength TSBYE adjusted to pH 5.0 (A) or pH 6.0 (B) at 10°C. Control (untreated) (●); ethyl vanillin 2 mg/ml at pH 5.0, 3 mg/ml at pH 6.0 (○); vanillin 3 mg/ml at pH 5.0, 4 mg/ml at pH 6.0 (■); vanillic acid 2 mg/ml at pH 5.0, 6 mg/ml at pH 6.0 (□).*

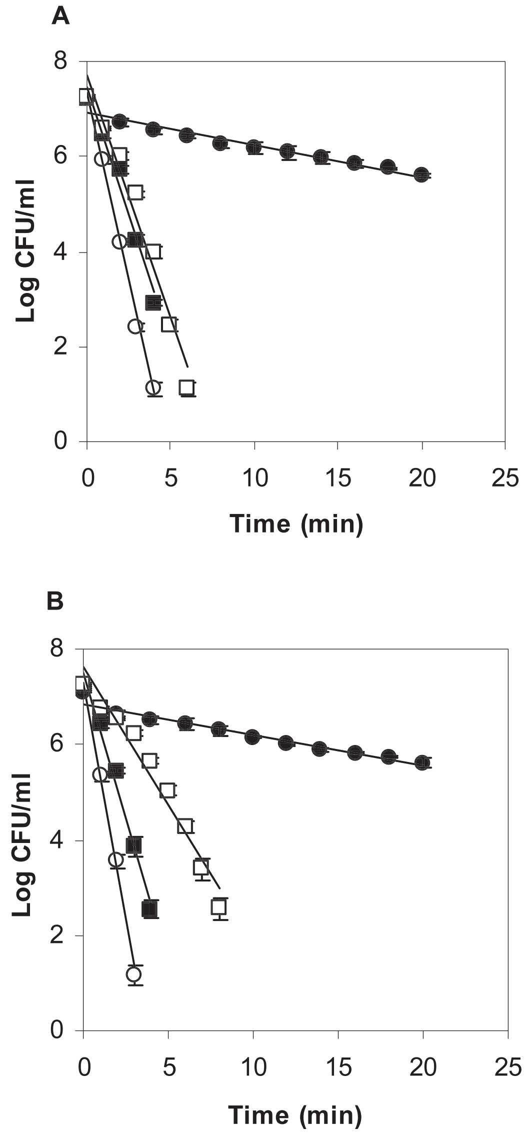

FIGURE 3. *Thermal inactivation curves at 58°C for a three-strain* C. sakazakii *cocktail in quarter-strength TSBYE containing vanillin, ethyl vanillin, and vanillic acid adjusted to pH 5.0 (A) or pH 6.0 (B). Control (untreated) (●); ethyl vanillin 2 mg/ml at pH 5.0, 3 mg/ml at pH 6.0 (○); vanillin 3 mg/ml at pH 5.0, 4 mg/ml at pH 6.0 (■); vanillic acid 2 mg/ml at pH 5.0, 6 mg/ml at pH 6.0 (□). Error bars represent the standard error of the mean.*

molecules such as protein, nucleic acids, inorganic ions, and ATP *(27)*. The effects of the test compounds on the permeability of two *C. sakazakii* strains were verified using a method based on the uptake of propidium iodide. This compound is able to enter a cell with a damaged membrane and bind to nucleic acids, producing red fluorescence *(48)*. CTAB is a strong cationic surfactant widely used to permeabilize cell membranes *(40)*. Treatment with CTAB led to an immediate uptake of propidium iodide (Table 5). The change in fluorescence was more gradual and increased over 24 h in *C. sakazakii* cells exposed to vanillin, ethyl vanillin, or vanillic acid. Nevertheless, this fluorescence

TABLE 5. *Propidium iodide uptake by* C. sakazakii *cell suspensions treated with vanillin, ethyl vanillin, and vanillic acid*

		Propidium iodide uptake (fluorescence units)				
Strain	Time (h)	Negative control	Positive control	Vanillin	Ethyl vanillin	Vanillic acid
C. sakazakii HPB 2855	6	5.62 ± 0.17	75.13 ± 2.44	24.67 ± 0.84	56.97 ± 0.92	5.73 ± 0.10
	24	26.31 ± 0.64	50.06 ± 0.97	129.70 ± 1.38	148.51 ± 1.73	42.14 ± 0.81
C. sakazakii HPB 2871	6	12.33 ± 0.26	60.57 ± 1.32	42.66 ± 0.93	66.73 ± 1.58	12.22 ± 0.21
	24	20.46 ± 0.49	38.38 ± 1.21	79.02 ± 1.29	85.07 ± 1.89	33.84 ± 0.72

TABLE 6. D-*values at 58°C for a three-strain cocktail of* C. sakazakii *in quarter-strength TSBYE adjusted to pH 5.0 or pH 6.0 and containing vanillin, ethyl vanillin, and vanillic acid*

		D-values (min)		
pH	Control	Vanillin	Ethyl vanillin	Vanillic acid
5.0	A 14.56 ± 0.60 X	B 0.93 ± 0.01 X	B 0.63 ± 0.01 X	B 0.98 ± 0.02 Y
6.0	A 15.13 ± 0.54 X	C 0.84 ± 0.04 X	C 0.50 ± 0.02 Y	B 1.74 ± 0.10 X

[a] Each value represents the mean ± standard error of three independent replicates. Within a row, means preceded by different letters are significantly different ($P < 0.05$). Within a column, means followed by different letters are significantly different ($P < 0.05$).

experiment provided clear evidence of changes in cell permeability indicative of membrane damage.

Damage to the cell membrane reduces the heat resistance of vegetative bacteria *(16)*. Exposure to sublethal stresses that could damage the membrane can lessen the thermal resistance of *Cronobacter* species. Shaker et al. *(48)* and Osaili et al. *(42)* reported that desiccation, heat, detergent, and sanitizer stresses significantly reduced the *D*-values of *Cronobacter* strains at 58°C in reconstituted infant formula. Jang and Rhee *(20)* reported that mild heating synergistically enhanced the bactericidal effect of caprylic acid against *Cronobacter* species in a similar medium. Exposure to *trans*-cinnamaldehyde reduced resistance to environmental stresses, including temperature (50, 55, and 60°C), acidic pH (3.3), high osmotic pressure (a_w of 0.81), and desiccation in *C. sakazakii (4)*. Vanillin also reduced the heat resistance of *Listeria innocua* in orange juice *(9, 10)*. Therefore, the finding that vanillin, ethyl vanillin, and vanillic acid damaged the cell membrane hinted that the compounds could enhance the lethal effect of heat against *C. sakazakii*. This hypothesis was tested by heating cell suspensions to 58°C in quarter-strength TSBYE supplemented with MBCs of each compound. Survivor curves are presented in Figure 3, and the associated lethalities were expressed as *D*-values (Table 6). The thermal resistance of *C. sakazakii* was clearly diminished by the presence of vanillin, ethyl vanillin, and vanillic acid in the heating menstruum at both pH 5.0 and pH 6.0. *D*-values obtained at pH 5.0 for vanillin (0.93 ± 0.01 min), ethyl vanillin (0.63 ± 0.01 min) and vanillic acid (0.98 ± 0.02 min) were significantly lower ($P < 0.05$) than that of the control (14.56 ± 0.60 min), but there were no difference in *D*-values between the individual compounds. At pH 6.0, *D*-values obtained with vanillic acid were significantly higher ($P < 0.05$) than those obtained with the other compounds but still much lower than that of the control. Increased ionization of the carboxyl group and the consequent negative charge of the molecule at pH 6.0 likely reduced penetration of vanillic acid into bacterial cells, leading to less lethality than that at pH 5.0.

The present investigations revealed that vanillin, ethyl vanillin, and vanillic acid have direct bactericidal effects, inhibit the growth, and lessen the heat resistance of *Cronobacter* species in a microbiological medium. These compounds could therefore prove useful for the control of the pathogen in dried products, particularly during home preparation and subsequent storage. Further studies are needed with food products to determine whether the compounds exert their effects in complex food matrices.

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