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Research Note

Effect of pH on the Inhibition of *Listeria* spp. by Vanillin and Vanillic Acid

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ABSTRACT

The antimicrobial effects of vanillin and vanillic acid were verified against several species and strains of *Listeria monocytogenes, Listeria innocua, Listeria grayi,* and *Listeria seeligeri* in a laboratory medium adjusted to pH values ranging from 5.0 to 8.0. Medium pH had little influence on the MIC of vanillin as determined by a broth dilution assay, and growth of all test strains was inhibited by concentrations ranging from 23 to 33 mM. In contrast, none of the strains were inhibited by 100 mM vanillic acid at pH $>$ 6.0, but complete inhibition was achieved at pH 5.0 with 10 mM. The effect of pH was further characterized by incubation of *L. monocytogenes, L. innocua,* and *L. grayi* in media containing 30 mM vanillin or 60 mM vanillic acid at pH 5.0, 6.0, and 7.0. Bactericidal effects increased with pH in media supplemented with vanillin. An inverse relationship was found for vanillic acid, and the lethality of the compound increased with declining pH. Mixtures of vanillin and vanillic acid exhibited additive inhibitory effects, particularly at lower pH. These natural antimicrobial compounds could prove useful either alone or in mixtures for the control of *Listeria* spp. in food products.

Natural vanilla is derived from the bean or pod of the *Vanilla* orchid (*Vanilla planifolia* Andrews, syn. *Vanilla fragrans* (Salisb. Ames)), a plant native to Mexico *(25).* Commercial extracts are obtained by alcoholic extraction of the fermented or cured vanilla bean. The main constituent of natural vanilla extract is the phenolic aldehyde vanillin (4-hydroxy-3-methoxybenzaldehyde), combined with minor amounts of vanillic acid (4-hydroxy-3-methoxybenzoic acid) and up to 200 trace components *(4).* Vanilla, vanillin, and to a lesser extent vanillic acid are important flavoring agents in Western food products. Natural vanilla extracts tend to be expensive due to limited worldwide production and labor-intensive manufacturing methods. Market demand for vanilla extracts destined for home use or food manufacturing is therefore partially filled by preparations formulated with chemically synthesized pure vanillin or ethyl vanillin.

Vanillin and related compounds derived from benzaldehyde are known to have antimicrobial properties. Vanillic acid inhibits the yeast *Saccharomyces cerevisiae,* and high background levels in wood hydrolysates may impede fermentation of such substrates for the production of ethanol *(1, 7).* The antifungal and antimycotic activities of vanillin have been widely documented in microbiological media *(5, 13, 14, 18–21)* and in food products including fruit purees, soft drinks, and fruit juices *(6, 12).* Activity at low pH suggests that the compound may be of value for the control of yeast and molds in other high-acid foods, including minimally processed fresh fruit *(5).* Comparatively less is known about the effect of vanillin and vanillic acid on bacteria or their potential usefulness for the control of bacterial contaminants in foods. Vanillic acid inhibits growth and reduces ethanol yield in *Zymomonas mobilis (22)* and an ethanolenic *Escherichia coli* construct designed for the fermentation of hemicelluloses *(27).* Katayama and Nagai *(17)* reported on the antibacterial activity of vanillin against a broad range of gram-positive and -negative bacteria including *Bacillus subtilis, Salmonella* Enteritidis, *Staphylococcus aureus, Pseudomonas aeruginosa,* and *E. coli.* Jay and Rivers *(16)* observed strong inhibition of gram-positive bacteria, but little activity against gram-negative test strains. More recently, Fitzgerald et al. *(13)* reported MICs of 15, 75, and 35 mM for vanillin against *E. coli, Lactobacillus plantarum,* and *Listeria innocua,* respectively. Furthermore, Tipparaju et al. *(24)* provided evidence that natural vanilla flavor enhances the destruction of *Listeria monocytogenes* in yogurt, a high-acid food product. These observations suggest that vanillin and related phenolic compounds may be of value for the control of acid tolerant foodborne pathogens. Such applications will require characterization of antimicrobial activity over a broad range of pH values and an understanding of their mode of action. We report here on the antimicrobial effects of vanillin and vanillic acid against several species of the genus *Listeria* in laboratory media adjusted to pH values between 5.0 and 8.0.

MATERIALS AND METHODS

Microorganisms. The *Listeria* strains used in this work are described in Table 1. Stock cultures were maintained at 4°C on

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TABLE 1. *Experimental* Listeria *strains used in the experiments*

Listeria strain	Source
L. grayi	Beef isolate, Lacombe Research Centre, Alberta, Canada
Listeria sp.	Chinese parsley isolate, HPB 197, Health Canada, Ottawa, Ontario, Canada
L. innocua	Watercress isolate, HPB 198, Health Canada
L. seeligeri	Lettuce isolate, Pacific Agri-Food Research Centre, Summerland, British Columbia, Canada
L. monocytogenes I	Cabbage isolate, LCDC 81-861, Nova Scotia, Canada
L. monocytogenes II	Frozen egg yolk isolate, Agriculture and Agri-Food Canada (AAFC), Guelph, Ontario, Canada
L. monocytogenes III	Chicken wiener isolate, AAFC
L. monocytogenes IV	Institut Pasteur, Paris, France

plates of Trypticase soy broth (BBL, Becton Dickinson, Sparks, Md.) amended with 5 g/liter yeast extract and 15 g/liter agar (TSAYE). Cultures for experiments were prepared by transferring a loop of cells from the stock cultures to 10 ml Trypticase soy broth amended with 5 g/liter yeast extract (TSBYE), followed by incubation for 24 h at 30°C. Inocula were prepared by dilution of the cultures with ¼-strength TSBYE adjusted to experimental pH with either 5 N HCl or 5 N NaOH prior to sterile filtration through

TABLE 2. *MICs of vanillin and vanillic acid against* Listeria *strains in test medium adjusted to pH 5.0, 6.0, 7.0, and 8.0*

	Vanillin, mM			Vanillic acid, mM		
Listeria strain	24 h	48 h	SD[a]	24 h	48 h	SD
pH 8.0						
L. grayi	27	27	6	>100	>100	
Listeria sp.	27	27	6	>100	>100	
L. innocua	37	30	0	>100	>100	
L. seeligeri	23	30	0	>100	>100	
L. monocytogenes I	20	20	0	>100	>100	
L. monocytogenes II	27	27	6	>100	>100	
L. monocytogenes III	27	27	6	>100	>100	
L. monocytogenes IV	23	27	6	>100	>100	
pH 7.0						
L. grayi	33	33	6	>100	>100	
Listeria sp.	30	30	0	>100	>100	
L. innocua	30	30	0	>100	>100	
L. seeligeri	30	30	0	>100	>100	
L. monocytogenes I	20	27	6	>100	>100	
L. monocytogenes II	30	30	0	>100	>100	
L. monocytogenes III	30	30	0	>100	>100	
L. monocytogenes IV	20	20	0	>100	>100	
pH 6.0						
L. grayi	30	30	0	20	27	6
Listeria sp.	30	30	0	20	27	6
L. innocua	30	30	0	23	30	0
L. seeligeri	27	27	6	20	23	6
L. monocytogenes I	20	27	6	17	23	6
L. monocytogenes II	30	30	0	17	27	6
L. monocytogenes III	30	30	0	17	30	0
L. monocytogenes IV	33	23	6	17	23	6
pH 5.0						
L. grayi	23	23	6	<10	<10	0
Listeria sp.	23	23	6	<10	<10	0
L. innocua	23	23	6	<10	<10	0
L. seeligeri	23	23	6	<10	<10	0
L. monocytogenes I	20	20	0	<10	<10	0
L. monocytogenes II	20	23	6	<10	<10	0
L. monocytogenes III	20	23	6	<10	<10	0
L. monocytogenes IV	13	20	10	<10	<10	0

[a] SD, standard deviation for trials read after 48 h.

0.22-μm membranes. Inocula with the desired cell density were obtained by spectrophotometric adjustment of the suspension (620 nm) against standard curves prepared previously (data not shown).

Determination of MICs. MICs were determined by means of a miniaturized broth dilution assay performed in microtiter plates *(9).* Vanillin and vanillic acid (Sigma, St. Louis, Mo.) stock solutions were prepared in ¼-strength TSBYE adjusted to the desired pH as described above. The test compounds were solubilized by heating to 50°C (vanillin) and 80°C (vanillic acid) prior to pH adjustment and sterile filtration. Aliquots (100 μl) were dispensed into rows of wells in microtiter plates (96 by 320 μl wells, Becton Dickinson) with a multichannel micropipettor to achieve concentrations from 0 to 100 mM in 10-mM increments after inoculation. Individual bacterial cultures (100 μl) were added to the wells to yield an inoculum level of 1.0×10^6 CFU/ml. All plates were incubated at 30°C, and the wells were examined for visual evidence of growth after 24 and 48 h. MICs were determined as the lowest antimicrobial concentration that inhibited growth. Three replicates were performed with each strain.

Behavior of *Listeria* species in growth media supplemented with vanillin and vanillic acid. Cell suspensions of *L. monocytogenes* I, *L. innocua,* and *Listeria grayi* were prepared as described above. Screw-capped Erlenmeyer flasks filled with 100 ml ¼-strength TSBYE adjusted to pH 5.0, 6.0, and 7.0 with and without 30 mM vanillin or 60 mM vanillic acid were separately inoculated with each test strain (0.25 ml) to achieve an initial cell density of approximately 1×10^5 CFU/ml. The flasks were incubated at 30°C in an agitating waterbath (150 rpm). Surviving cell populations in samples withdrawn after 0, 1, 4, 8, 24, and 48 h were estimated by spreading suitably diluted samples onto TSAYE, followed by incubation at 30°C for 48 h. Recovery of cells below the detection limit (10 CFU/ml) was ensured by enrichment of a 1.0-ml sample in 100 ml TSBYE at 30°C for up to 7 days. Fluids from positive enrichments were spread onto PALCAM agar (Difco, Becton Dickinson). The plates were examined for typical *Listeria* colonies (i.e., esculin hydrolysis) after 48 h incubation at 30°C.

Antimicrobial effect of vanillin and vanillic acid mixtures. The effect of mixtures of vanillin and vanillic acid on *L. monocytogenes* strain I, *L. innocua,* and *L. grayi* were determined by a modification of the checkerboard assay described by Barry *(3)* and Delaquis et al. *(9).* Stock solutions of each compound were prepared in ¼-strength TSBYE as before. Aliquots (0 to 50 μl) of the first stock solution were dispensed into the wells of microtitre plates along the horizontal axis in 5-μl increments. Aliquots of the second stock solution were delivered along the vertical axis to obtain a series of wells containing 50 μl vanillin and vanillic acid mixtures. Each well was inoculated with 100 μl of the test culture prepared as described earlier, and the contents were mixed with the micropipettor. After 48 h incubation at 30°C the wells were examined for visual evidence of growth and MICs were determined for each mixture. Isobolograms were prepared by plotting measured MICs for vanillin against MICs for vanillic acid as described by Davidson and Parish *(8)* and Delaquis et al. *(9).*

RESULTS AND DISCUSSION

MICs of vanillin and vanillic acid toward several species of *Listeria* were measured in ¼-strength TSBYE adjusted to pH 5.0, 6.0, 7.0 and 8.0. Results provided in Table 2 show that vanillin inhibited growth of the test strains at all pH values. The range in effective MICs was narrow (20 to 33 mM), suggesting that medium pH did not influence

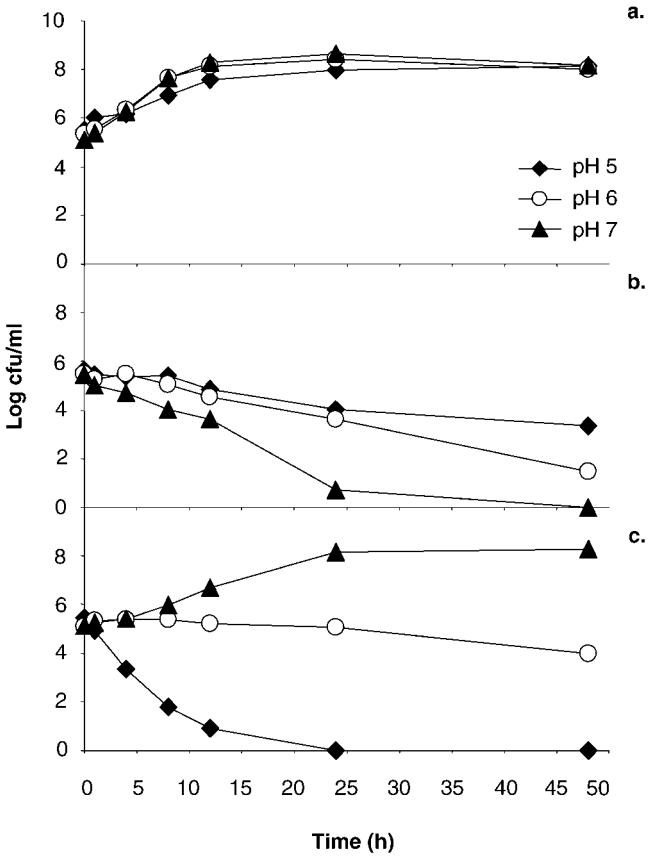

FIGURE 1. *Effect of 30 mM vanillin and 60 mM vanillic acid on the fate of* Listeria monocytogenes *I incubated at 30°C in TSBYE adjusted to pH 5.0, 6.0, and 7.0. (a) Controls. (b) 30 mM vanillin. (c) 60 mM vanillic acid.*

activity and that the *Listeria* species and strains chosen for this work have comparable sensitivity to the compound. In contrast, MICs for vanillic acid were strongly affected by medium pH. While no inhibitory activity was evident at a concentration of 100 mM, MICs were approximately equivalent to those achieved with vanillin at pH 6.0, and <10 mM was sufficient to prevent growth of all the test strains at pH 5.0. The effect of prolonged exposure to vanillin and vanillic acid was verified by inoculation of *L. monocytogenes* I, *L. innocua,* and *L. grayi* in ¼-strength TSBYE medium adjusted to different pH values. Plots of viable cell populations over time showed that the inhibitory effects of both vanillin and vanillic acid were clearly time and pH dependent (Figs. 1 through 3). The consequences of exposure to 30 mM of vanillin ranged from growth stasis to pronounced declines in cell viability. An immediate reduction in the population of *L. monocytogenes* I was evident at all pH values, and this species was slightly more sensitive to the effect of the compound than *L. grayi* or *L. innocua. L. grayi* populations remained unchanged at pH 5.0 and 6.0 but declined gradually at pH 7.0. An intermediate response was observed with *L. innocua,* and a decrease in cell populations occurred only at pH 6.0 and 7.0. None of the test strains were recovered by enrichment of 1-ml samples withdrawn from cultures where direct plating failed to detect viable cells (detection limit = 10 CFU/ml). Hence vanillin exhibited inhibitory effects ranging from bacterio-

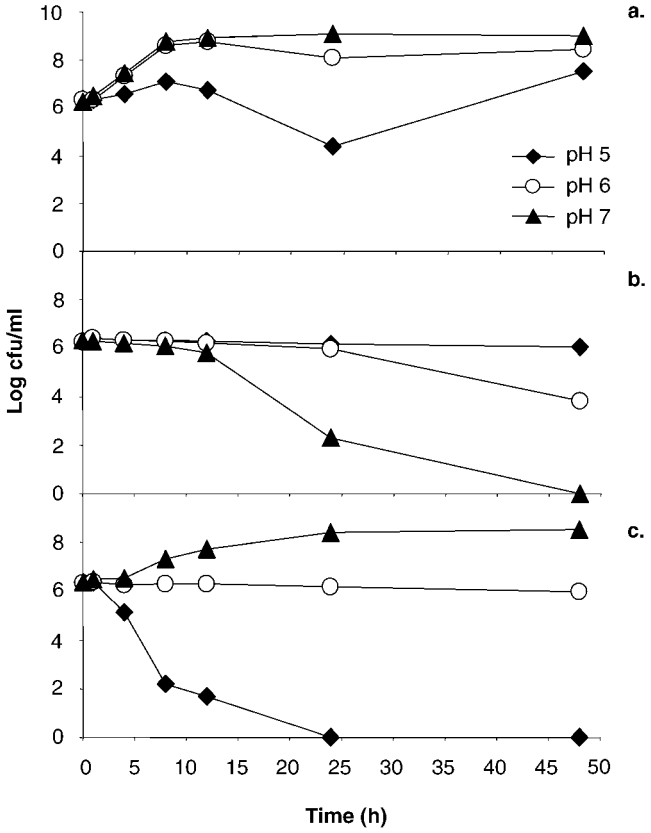

FIGURE 2. *Effect of 30 mM vanillin and 60 mM vanillic acid on* Listeria innocua *incubated at 30°C in TSBYE adjusted to pH 5.0, 6.0, and 7.0. (a) Controls. (b) 30 mM vanillin. (c) 60 mM vanillic acid.*

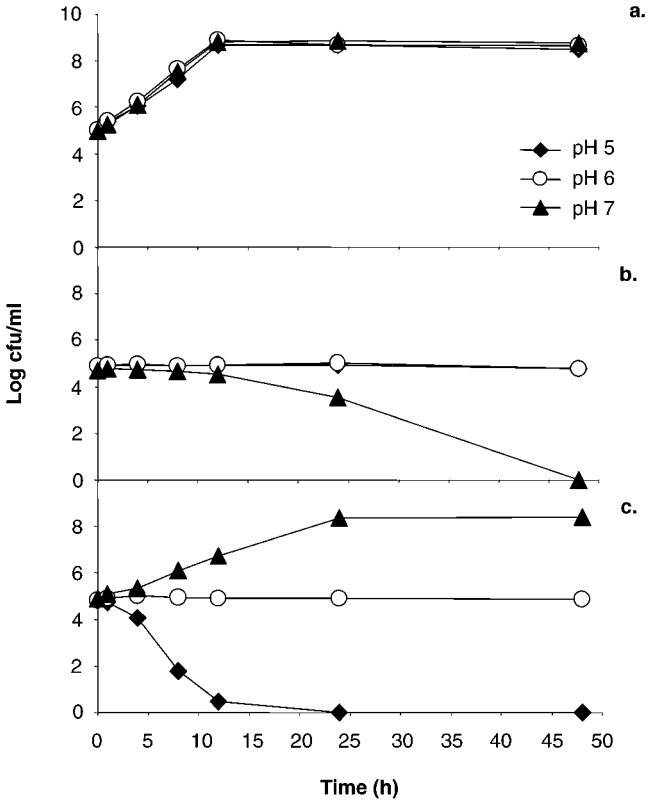

FIGURE 3. *Effect of 30 mM vanillin and 60 mM vanillic acid on* Listeria grayi *incubated at 30°C in TSBYE adjusted to pH 5.0, 6.0, and 7.0. (a) Controls. (b) 30 mM vanillin. (c) 60 mM vanillic acid.*

static to bactericidal activity as medium pH increased from 5.0 to 7.0. Bacteriostatic and bactericidal activity were also evident in media supplemented with vanillic acid, although the relationship between pH and inhibition was reversed and inhibition increased with decreasing pH. There was no evidence of differences in sensitivity to the effects of vanillic acid among the three *Listeria* species tested.

Antimicrobial activity was also examined in mixtures of the two compounds at pH 5.0 and 6.0. Isobolograms plotted using MIC measurements obtained with the mixtures against *L. monocytogenes* I, *L. innocua,* and *L. grayi* are shown in Figure 4. MICs for both vanillin and vanillic acid declined when the compounds were tested in mixtures. The linear responses observed with all test strains were indicative of an additive inhibitory effect *(8).* MICs were lower at pH 5.0 than pH 6.0, particularly for vanillic acid. Concentrations ≤5 mM alone were sufficient to fully inhibit the experimental strains and enhance the activity of vanillin. The results also indicated that *L. monocytogenes* I was more susceptible to the effect of the mixtures than *L. innocua* or *L. grayi.*

There are few reports on the effect of vanillin and vanillic acid on *Listeria* spp. in the scientific literature. Fitzgerald et al. *(13)* presented convincing evidence that the cytoplasmic membrane of *L. innocua* is compromised by exposure to vanillin. The MIC for this species was 35 mM, a value similar to that reported here. However, the observations of Fitzgerald et al. *(13)* differ in several respects from those derived from the present study. The nature of the inhibition was described as bacteriostatic, but the data herein show that vanillin (and vanillic acid) can also be lethal to *Listeria* spp. In addition, inhibition was strongly

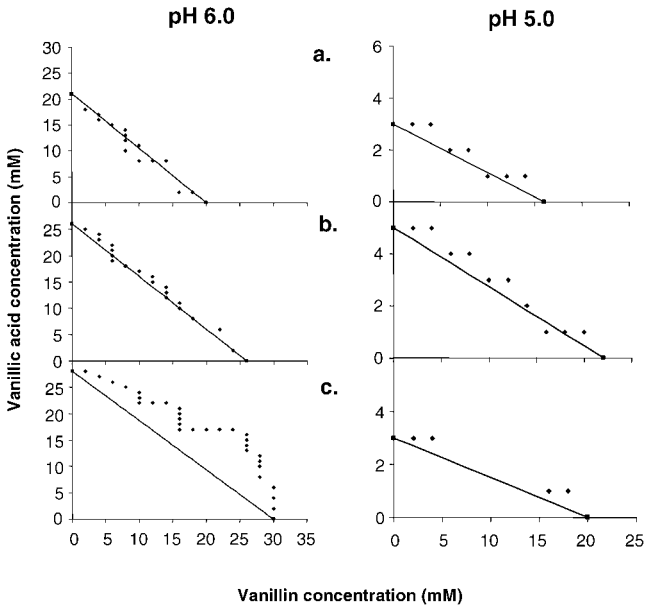

FIGURE 4. *Isobolograms for mixtures of vanillin and vanillic acid in TSBYE adjusted to pH 5.0 and 6.0.* Listeria monocytogenes *I (a),* Listeria innocua *(b), and* Listeria grayi *(c).*

pH dependent, a characteristic of antimicrobial organic acids including other phenolic acids. These tend to exhibit greater activity at pH values near the pK_a due to an increase in the proportion of the undissociated acid, the more active antimicrobial form *(26).* Vanillic acid has two pK_a values at 25°C (pK_{a1} = 4.51, pK_{a2} = 9.39) *(2)*. The enhanced activity of this compound at pH 5.0 may therefore be due to an increase in the proportion of molecules in the undissociated state, since the latter would predominate under these conditions. Vanillin, although an aldehyde, also exhibits acid-base behavior at 25°C (pK_a = 7.4) *(2)*. Enhanced potency of vanillin at pH 7.0 suggests that dissociation of the ionizable hydroxyl radical may be responsible for greater activity. Hence the acid-base behavior of both compounds must be considered in further attempts to elucidate the mechanisms that underlie their mode of action.

From a practical point of view, an understanding of the acid-base behavior and the relative potency of vanillin and vanillic acid at various pH values will be imperative for the appropriate application of these antimicrobials in food products. Our results suggest that vanillic acid may be more suitable for the control of *Listeria* spp. in products with pH approaching 4.5 and vanillin where pH is nearer neutrality. Evidently, specific applications in food products must take into account the organoleptic properties of vanillin, a highly fragrant compound with a low taste threshold (0.5 ppm in water) *(11)*. On the other hand, vanillic acid is nearly odorless and has a taste threshold of 30 ppm *(11)*. Mixtures of the two compounds could therefore prove useful in applications where a strong vanilla flavor is expected to be deleterious to the overall sensory profile of the target product. It should also be noted that both compounds are widely used in food manufacture and are regarded as nutritionally beneficial components of plant foods. Indeed, the vanillins have elicited considerable interest due to their antimutagenic properties and a putative role in cancer prevention *(10, 23).* According to the Joint Food and Agriculture Organization of the World Health Organization Expert Committee on Food Additives, both compounds benefit from the designation ''No safety concern at current levels of intake when used as a flavouring agent'' *(15).* Hence there should be few regulatory obstacles to the application of either compound in food preservation.

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