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Interactive inhibition of meat spoilage and pathogenic bacteria by lysozyme, nisin and EDTA in the presence of nitrite and sodium chloride at 24 °C

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Abstract

To develop a nisin- and lysozyme-based antimicrobial treatment for use with processed ham and bologna, *in vitro* experiments were conducted to determine whether inhibition enhancing interactions occur between the antimicrobials lysozyme, chrisin (a commercial nisin preparation), EDTA, NaCl and $NaNO_2$. Inhibitory interactions were observed between a number of agents when used against specific pathogenic and spoilage bacteria. The observed interactions included lysozyme with EDTA (*Enterococcus faecalis* and *Weissella viridescens*), chrisin with EDTA (all Gram-positive organisms), EDTA with NaCl (*Escherichia coli*, *Salmonella enterica* serovar Typhimurium, *Serratia grimesii*), EDTA with nitrite (*E. coli*, *Lactobacillus curvatus*, *Leuconostoc mesenteroides*, *Listeria monocytogenes*, *S.* Typhimurium), chrisin with nitrite (*Lc. mesenteroides*, *L. monocytogenes*), and NaCl with nitrite (*S.* Typhimurium, *Shewanella putrefaciens*). Previous reports have described interactions between nisin with EDTA that resulted in enhanced antimicrobial effect against Gram-negative bacteria, or lysozyme with nisin against Gram-positive bacteria. These interactions were not observed in these experiments. We observed that unlike previous studies, these experiments were conducted on growing cells in nutrient broth, rather than under conditions of nutrient limitation. We propose that screening of antimicrobials for use in food systems in nutrient-deficient systems is inappropriate and that new protocols should be developed.

Keywords: Lysozyme; Nisin; EDTA; Nitrite; Microbiology

1. Introduction

In developing novel antimicrobial treatments for use in the food industry there are two persistent problems, the limited range of bacteria which are sensitive to particular agents and the high concentrations of agents that are required to inhibit growth. Two proteins that are of interest for use in such applications are lysozyme and nisin. It has been claimed that the antimicrobial spectrum and potency of lysozyme and nisin can be increased when used in combination with one another (Chung and Hancock, 2000) or other antimicrobials (Shively and Hartsell, 1964a,b; Hughey and Johnson, 1987; Ellison and Giehl, 1991; Stevens et al., 1991, 1992; Payne et al., 1994; Cutter and Siragusa, 1995a; Park, 1997; Carneiro De

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Melo et al., 1998). The aim of the present experiments was to evaluate in vitro a number of antimicrobial agents and to determine whether interactions, which enhance antimicrobial activity, occur between these agents. The results of these experiments were used in developing lysozyme and nisin-based antimicrobial treatments for processed ham and bologna which have been described in Gill and Holley (2000a,b).

The rationale for the increased effectiveness of combinations of antimicrobials is that a simultaneous attack on different targets in the bacterial cell is more difficult for the bacteria to overcome. The use of antimicrobials with different mechanisms can also be expected to expand the range of organisms that may be inhibited.

Lysozyme has enzymatic activity against the (1–4) glycosidic linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine of cell wall peptidoglycan (Proctor and Cunningham, 1988). The high resistance of Gram-negative organisms whose cell wall is protected from lysozyme by the outer membrane currently limits the application of lysozyme in foods.

The bacteriocin, nisin, has been shown to alter the cell membrane of sensitive organisms resulting in leakage of low molecular weight cytoplasmic components and the destruction of the proton motive force (PMF) (Bruno et al., 1992). Nisin is most effective as an antimicrobial against lactic acid bacteria (LAB) and other Gram-positive bacteria, notably *Clostridium* spp. (Delves-Broughton et al., 1996).

Diacetyl has been demonstrated to be an effective antimicrobial against a wide range of Gram-negative and Gram-positive bacteria, although LAB are generally resistant (Jay, 1982a,b; Jay et al., 1983; Jay and Rivers, 1984). However, the mechanism by which diacetyl inhibits growth is unknown, although Jay and Rivers (1984) suggested that it may result from interaction of diacetyl with arginine residues at the active site of enzymes.

It has been recognized since the 1960s that the susceptibility of Gram-negative organisms to lysis by lysozyme can be increased by the use of membrane disrupting agents, such as detergents and chelators (Shively and Hartsell, 1964a,b). Chelators, such as sodium phosphate tripoly and EDTA, can destabilize the cell membranes of bacteria by complexing the divalent cations which act as salt bridges between membrane macromolecules such as lipopolysaccharides (for reviews see, Vaara, 1992; Shelef and Seiter, 1993).

Preliminary experiments were conducted to determine the minimum inhibitory concentration in broth media of the antimicrobials lysozyme, EDTA, diacetyl, sodium phosphate tripoly and chrisin (a commercial food grade nisin preparation), against a wide range of spoilage or pathogenic bacteria found in cured meats. The antimicrobials lysozyme, chrisin, and EDTA were selected for further study to determine whether any interactions could be observed when used in combination in broth. Also included in the interaction studies were sodium nitrite (180 mg/l) and NaCl (2.7% w/v), as these chemicals are ingredients of cured meat products that are ascribed growth inhibitory properties. Additionally, it has been reported that high ionic concentrations have a negative effect on the activity of lysozyme (Davies et al., 1969) and the presence of numerous freely available ions in complex media can also be expected to reduce the membrane disruption effect of chelators. The maximum permitted content of nitrite in cured meats in Canada is 200 ppm, except for side bacon where 120 ppm is permitted at formulation (Health Canada, 2001). These levels would be reduced after thermal processing; nonetheless, tests reported here were done near the theoretical maximum.

All experiments were conducted at 24 °C, as this is a non-restrictive temperature for all of the bacteria studied, and is in the range of temperatures experienced by food products during temperature abuse. In screening for interactions, each agent was tested individually and with 1–4 other agents against the 14 test bacteria. Culture media absorbance data were collected and used to develop a two-level, five-factorial model to screen for interactions.

2. Materials and methods

2.1. Materials

Lysozyme HCl and chrisin (2.5 g pure nisin/kg w/w) were provided by Canadian Inovatech, Abbotsford, BC. Morpholinoethane sulfonic acid, MES (2-4-morpholino-ethane sulfonic acid), EDTA (disodium ethylenediamine tetraacetate) and tripolyphosphate (sodium phosphate tripoly) were obtained from Fisher Scien-

tific, Toronto, ON. Diacetyl (2,3-butadione) was from Aldrich, Oakville, ON. The all purpose Tween broth (APT broth) was from BBL, Becton-Dickinson, Franklin Lakes, NJ. The brain heart infusion broth (BHI broth) and granulated agar, were from Difco, Detroit, MI. The M17 broth was from Oxoid, Nepean, ON. GasPak Jars (BBL, Becton-Dickinson) were made anaerobic using the GasPak Plus Anaerobic System with palladium catalyst. An anaerobic incubator (National Appliance, Portland, OR) flushed three times with CO_2 was also used for anaerobic incubations. Growth was monitored in 96-well polypropylene micro-titre plates (Corning-Costar #3790, Corning, Acton, MA), using a Titretek Multiskan MCC/340 Mk II type 347 spectrophotometer (Flow Laboratories International in Switzerland), at a wavelength of 450 nm.

2.2. Cultures used, source and incubation conditions

The tests were conducted in BHI or APT broth, as appropriate, buffered to pH 6.0 with morpholinoethane sulphonic acid (MES) (Buncic et al., 1995) as this pH is with in the range of the target products, processed ham and bologna (Gill and Holley, 2000a,b) and is non-inhibitory to the bacteria studied. The specific media and incubation conditions used for each organism are described in Table 1.

Brochothrix thermosphacta B2 was from Dr G. Greer Agriculture and Agri-Food Canada (AAFC) Research Station, Lacombe, AB. *Escherichia coli* O157:H7 strain E318 was a human clinical isolate provided by Dr. R. Johnson, Health Canada, Guelph, ON. *Lactobacillus sakei* #7, *Lactobacillus curvatus* #15 and *Leuconostoc mesenteroides* #11, were isolated from spoiled cured meats (Holley et al., 1996). *Listeria monocytogenes* was from the University of Manitoba, Department of Food Science culture collection. *Pediococcus acidilactici* 122P and *Pediococcus pentosaceus* 116P were isolated from commercial meat starter cultures. *Serratia grimesii* S12 was isolated from cooked roast beef, AAFC collection, Summerland, BC. *Shewanella putrefaciens* was ATCC #8071. *Enterococcus faecalis* #28 (ATCC #7080), *Salmonella enterica* serovar Typhimurium #98, *Staphylococcus aureus* #427 and *Weissella viridescens* #13 (ATCC #12706) were provided by the AAFC collection, Guelph, ON.

Cultures were maintained monthly on agar slants at 4 °C and as frozen glycerol stocks. Cultures were streaked onto agar plates once a week and cultures for experiments were inoculated into media from a single colony and incubated overnight under the appropriate atmospheric conditions. All cultures were maintained in either APT or BHI media, except for *L. sakei* #7, which was maintained on M17 agar but grown in APT. Incubations were conducted at 24 °C.

Table 1
Culture media and incubation atmosphere for test bacteria

Organism	Media	Atmosphere
Brochothrix thermosphacta	BHI[a]	Aerobic
Enterococcus faecalis	BHI	CO_2
Escherichia coli	BHI	Aerobic
Lactobacillus curvatus	APT[b]	CO_2
Lactobacillus sakei	APT	CO_2
Leuconostoc mesenteroides	APT	CO_2
Listeria monocytogenes	BHI	Aerobic
Pediococcus acidilactici	APT	CO_2
Pediococcus pentosaceus	APT	CO_2
Salmonella Typhimurium	BHI	CO_2
Serratia grimesii	BHI	CO_2
Shewanella putrefaciens	BHI	Aerobic
Staphylococcus aureus	BHI	Aerobic
Weissella viridescens	APT	CO_2

[a] BHI—brain heart infusion broth.
[b] APT—all purpose Tween broth.

2.3. Determination of MIC

The protocol described here was adapted from that of Maclean et al. (1997a,b).

The media used in these experiments were either APT or BHI broth, and both were prepared with 10 g/l MES buffer, titrated to pH 6.0 with 0.1 mol/l HCl or NaOH.

Each well of a sterile 96 micro-titre plate was filled with 100 μl of APT or BHI broth. All media containing the agents to be tested were prepared freshly each day. Lysozyme, chrisin and diacetyl were prepared as 1000 mg/l solutions, dissolved in previously sterilized media broth. Standard stock solutions of EDTA and tripolyphosphate were prepared in deionized water and filter sterilized when added to previously autoclaved broth media, to achieve the desired final concentration.

The first column of wells in each micro-titre plate received 100 μl of broth media containing the agent to be tested. After mixing, 100 μl was transferred to the

next column of wells in a process of 1:1 serial dilution until column #11 was reached. Column #12 was left without the agent.

Each strain to be tested was inoculated from a single colony into APT or BHI broth in a test tube and incubated overnight. Incubation was at 24 °C under aerobic or anaerobic conditions, as appropriate. An inoculum was prepared by diluting the overnight culture to 10^5 CFU/ml in fresh broth media. The inoculum was enumerated by surface plating agar plates that were incubated for 2 days at 24 °C and then counted. Each well in the micro-titre plates, except four in column #12, were inoculated with 10 μl of the inoculumn to be tested. Four wells (inoculated, no agent), in column #12, served as a positive control for growth and four (uninoculated, no agent) as a negative control for growth.

The inoculated micro-titre plates were then incubated for 60 h at 24 °C, under the appropriate atmospheric conditions. The wells were scored visually as growth or no growth. The concentration in the lowest serial dilution of the agent at which growth did not occur was recorded as the minimal inhibitory concentration (MIC). Experiments were repeated four times.

2.4. Interaction screening

The media used in these experiments were APT or BHI broths and both were prepared with 10 g/l MES buffer, titrated to pH 6.0 with 0.1 mol/l HCl or NaOH.

The experiments were conducted in 96-well polypropylene micro-titre plates, with four plates being used for each organism. In columns 1–10 of each plate, appropriate volumes of the six inhibitor test agent solutions and growth media were added to each well to produce all 32 combinations of the five agents at minimal or maximal concentration, with a final volume of 100 μl. With a 10-μl inoculum, the final concentrations of the five agents were: lysozyme, absent or 450 mg/l; chrisin, absent or 450 mg/l; EDTA, absent or 900 mg/l; $NaNO_2$, absent or 180 mg/l; NaCl, absent or 270 mg/l. Column 11 contained 100 μl media without added test agents and was inoculated as a positive control. Column 12 contained 110 μl of media and was not inoculated so as to serve as a negative control.

The inoculum was prepared as in the MIC experiments. Each well in the micro-titre plates was charged with 10 μl of the inoculum, except for the wells of column 12. The inoculated plates were then incubated for 3 days at 24 °C.

After incubation had been completed, the absorbance of the media in each microtitre well was measured with a Titretek Multiscanner at 450 nm. By the deduction of the average absorbance of the negative control wells on each plate (110 μl of uninoculated media), the net absorbance of the wells for each treatment was determined. These values were used to calculate the average relative absorbance of treatment wells compared to the positive controls (110 μl

Table 2
Minimum inhibitory concentration of single agents tested in mg/l

Organism	Lysozyme	Chrisin	EDTA	Diacetyl	Tripoly-phosphate
Brochothrix thermosphacta	10	5.6	250	>500[a]	>4000
Enterococcus faecalis	>500	500	500	>500	>4000
Escherichia coli	>500	>500	1000	250	>4000
Lactobacillus curvatus	>500	5	1000	>500	>4000
Lactobacillus sakei	>500	500	500	>500	>4000
Leuconostoc mesenteroides	>500	1.3	2000	>500	>4000
Listeria monocytogenes	>500	250	250	>500	>4000
Pediococcus acidilactici	>500	0.63	500	>500	>4000
Pediococcus pentosaceus	>500	5	1000	>500	>4000
Salmonella Typhimurium	>500	>500	>2000	250	>4000
Serratia grimesii	>500	>500	>2000	>500	>4000
Shewanella putrefaciens	>500	>500	>2000	NT[b]	>4000
Staphylococcus aureus	>500	250	250	>500	1000
Weissella viridescens	>500	7.8	1000	>500	>4000

[a] Where ">" is used, the number following represents the highest concentration tested.
[b] NT—not tested.

Table 3
Probability[a] of single agent inhibition or two-agent inhibitory interactions against several Gram-positive bacteria studied in five-factor, two-level tests

Effect	*Brochothrix thermosphacta*	*Weissella viridescens*	*Lactobacillus sakei*	*Lactobacillus curvatus*	*Leuconostoc mesenteroides*
Lysozyme	0.0049[a]	0.0070[a]	0.2122	0.1594	0.7718
Chrisin	0.0003[a]	<0.0001[a]	<0.0001[a]	<0.0001[a]	<0.0001[a]
Lysozyme×chrisin	0.0949	0.0137	0.0923	0.2445	0.9386
EDTA	0.0004[a]	<0.0001[a]	<0.0001[a]	<0.0001[a]	<0.0001[a]
Lysozyme×EDTA	0.0957	0.0128	0.0698	0.2251	0.5303
Chrisin×EDTA	0.0035[a]	<0.0001[a]	<0.0001[a]	<0.0001[a]	<0.0001[a]
NaCl	0.6873	0.7237	0.0833	0.2326	0.5434
Lysozyme×NaCl	0.3060	0.4386	0.1305	0.8052	0.6660
Chrisin×NaCl	0.6435	0.7390	0.6723	0.2684	0.5170
EDTA×NaCl	0.5884	0.7806	0.6588	0.4510	0.9362
Nitrite	0.0109	0.0183	0.0196	0.0027[a]	<0.0001[a]
Lysozyme×nitrite	0.3662	0.6233	0.4331	0.8209	0.5613
Chrisin×nitrite	0.0496	0.0293	0.1327	0.0309	0.0004[a]
EDTA×nitrite	0.0588	0.0273	0.1827	0.0058[a]	0.0035[a]
NaCl×nitrite	0.7533	0.9801	0.3915	0.9259	0.8058

[a] Values tabulated are Prob>*F*. These represent the probability of being wrong if an effect is declared non-null (inhibitory). The effect of an agent or its interaction with a second agent upon absorbance was considered significant using an alpha (α) value of 0.01. In all instances of statistical significance (denoted by superscript "a"), the interaction had a negative effect on bacterial growth.

of inoculated media). The values for relative absorbance were then entered into the statistical model for analysis. Values for relative absorbance were calculated from 10 replicates.

2.5. Statistical analysis

The experiment was designed with 32 treatments (or combinations of the agents), as this was the mini-

Table 4
Probability[a] of single agent inhibition or two-agent inhibitory interactions against several Gram-positive bacteria studied in five-factor, two-level tests

Effect	*Enterococcus faecalis*	*Pediococcus pentosaceus*	*Pediococcus acidilactici*	*Staphylococcus aureus*	*Listeria monocytogenes*
Lysozyme	0.0022[a]	0.0790	0.0038[a]	0.7608	0.0544
Chrisin	<0.0001[a]	<0.0001[a]	<0.0001[a]	0.0004[a]	<0.0001[a]
Lysozyme×chrisin	0.3207	0.0321	0.1231	0.8561	0.1758
EDTA	<0.0001[a]	<0.0001[a]	<0.0001[a]	0.0004[a]	<0.0001[a]
Lysozyme×EDTA	0.0044[a]	0.0479	0.0919	0.6563	0.2220
Chrisin×EDTA	<0.0001[a]	<0.0001[a]	<0.0001[a]	0.0109	0.0006[a]
NaCl	0.2079	0.6658	0.9644	0.1095	0.3041
Lysozyme×NaCl	0.8906	0.1463	0.0835	0.5417	0.7413
Chrisin×NaCl	0.7709	0.6232	0.1231	0.2992	0.5362
EDTA×NaCl	0.3687	0.4476	0.6874	0.3980	0.5913
Nitrite	0.2293	0.5280	0.5266	0.0005[a]	0.0002[a]
Lysozyme×nitrite	0.5111	0.3399	0.1512	0.5340	0.5281
Chrisin×nitrite	0.5906	0.0920	0.8620	0.0109	0.0036[a]
EDTA×nitrite	0.2128	0.1493	0.6983	0.0132	0.0041[a]
NaCl×nitrite	0.4727	0.3341	0.4653	0.2711	0.9169

[a] Values tabulated are Prob>*F*. These represent the probability of being wrong if an effect is declared non-null (inhibitory). The effect of an agent or its interaction with a second agent upon absorbance was considered significant using an alpha (α) value of 0.01. In all instances of statistical significance (denoted by superscript "a"), the interaction had a negative effect on bacterial growth.

mum number that would allow resolution of two-way interactions between agents without confounding effects between agents. The pattern of treatments has been described in the previous section. The results were subjected to ANOVA to screen for paired interactions among the five antimicrobial agents (lysozyme, chrisin, EDTA, NaCl and nitrite).

The values for relative absorbance were determined for each treatment and entered into the experimental design as the response. ANOVA by the JMP program (SAS Institute) was used to evaluate the probability that individual agents significantly affected the response (relative absorbance) by conducting an F test, the results of which are described by the value Prob > F. Prob>F is the "probability of being wrong if you declare an effect to be non null" (Sall and Lehman, 1996). This can be interpreted as the probability that an agent or combination of agents does not affect the relative absorbance of the inoculated media after incubation. It is assumed that the relative absorption of inoculated media is related to the growth of the test organism.

The apparent effect of an agent or its interaction with a second agent on the relative absorbance was determined using an alpha value of 0.01. In other words, it was determined that the agent or interaction had no effect on the relative absorbance when there was a greater than 0.01 probability of being wrong if the null hypothesis (no effect) were rejected. The effect on growth was characterized as negative (decreasing absorption or growth) or positive (increasing absorption or growth) by the direction of the slope of the response predicted by the statistically generated model. A confidence level of P=0.01 was chosen, as the use of a high maximal level (i.e. test concentration) and zero minimal level should result in a highly significant effect upon the response.

3. Results

The MICs determined for inhibition of growth by lysozyme, chrisin, EDTA, tripolyphosphate and diacetyl are reported in Table 2 as mg/l. Where no inhibition of growth was observed, the highest concentration of agent tested was reported. Of the organisms tested, only *B. thermosphacta* was inhibited by concentrations of lysozyme of 500 mg/l or less. Chrisin was effective against all Gram-positive organisms tested, at varied concentrations, but was ineffective against all of the Gram-negative organisms at the concentrations tested. EDTA was inhibitory against all of the organisms tested except *S.* Typhimurium and *Ser. grimesii*. Diacetyl was inhibitory towards *S.* Typhimurium and *E. coli* O157:H7 at 250 mg/l, while tripolyphosphate was ineffective as an antimicrobial agent at the concentrations tested.

Interaction screening experiments were not conducted using tripolyphosphate due to its poor antimicrobial activity. Diacetyl was rejected for further investigation as the concentrations required for inhibition caused sensory changes (buttery odor) that made it unsuitable for application to meat products.

Table 5
Probability[a] of single agent inhibition or two-agent inhibitory interactions against several Gram-negative bacteria studied in five-factor, two-level tests

Effect	*Salmonella* Typhimurium	*Escherichia coli*	*Serratia grimesii*	*Shewanella putrefaciens*
Lysozyme	0.3189	0.0153	0.1556	0.1784
Chrisin	0.5993	0.6437	0.6618	0.2516
Lysozyme× chrisin	0.0748	0.1529	0.3384	0.7111
EDTA	<0.0001[a]	<0.0001[a]	<0.0001[a]	0.0003[a]
Lysozyme× EDTA	0.5999	0.0892	0.3206	0.3501
Chrisin× EDTA	0.8898	0.7299	0.4322	0.4261
NaCl	<0.0001[a]	<0.0001[a]	0.0018[a]	0.0003[a]
Lysozyme× NaCl	0.5224	0.5352	0.9663	0.5006
Chrisin× NaCl	0.8079	0.7937	0.3522	0.4651
EDTA× NaCl	0.0049[a]	<0.0001[a]	0.0029[a]	0.0138
Nitrite	<0.0001[a]	<0.0001[a]	<0.0001[a]	0.0004[a]
Lysozyme× nitrite	0.4991	0.3633	0.5022	0.3490
Chrisin× nitrite	0.5303	0.8590	0.7857	0.5420
EDTA× nitrite	<0.0001[a]	<0.0001[a]	0.1146	0.0114
NaCl× nitrite	<0.0001[a]	0.8488	0.5689	0.0076[a]

[a] Values tabulated are Prob>F. These represent the probability of being wrong if an effect is declared non-null (inhibitory). The effect of an agent or its interaction with a second agent upon absorbance was considered significant using an alpha (α) value of 0.01. In all instances of statistical significance (denoted by superscript "a"), the interaction had a negative effect on bacterial growth.

The results of the two-level, five-factor study are presented in Tables 3–5. Lysozyme alone was observed to be effective against *B. thermosphacta*, *P. acidilactici*, *En. faecalis* and *W. viridescens*. The results also showed growth inhibitory effects by both NaCl and nitrite on *E. coli* O157:H7, *S.* Typhimurium, *Ser. grimesii*, and *Sh. putrefaciens*. The following organisms were sensitive to the presence of nitrite, but not NaCl: *Lb. curvatus*, *Lc. mesenteroides*, *L. monocytogenes*, and *St. aureus*.

Statistical analysis of the results indicated that negative interactions (increased inhibition of growth) could occur with combinations of lysozyme or chrisin with EDTA. For both *En. faecalis* and *W. viridescens*, an interaction was observed between lysozyme and EDTA. An interaction between chrisin and EDTA was observed with all of the Gram-positive organisms tested. Various patterns of interactions were observed among the organisms tested including: EDTA and NaCl (*E. coli*, *S.* Typhimurium, *Ser. grimesii*); EDTA and nitrite (*E. coli*, *Lb. curvatus*, *Lc. mesenteroides*, *L. monocytogenes*, *S.* Typhimurium); chrisin and nitrite (*Lc. mesenteroides*, *L. monocytogenes*); and NaCl and nitrite (*S.* Typhimurium, *Sh. putrefaciens*). No antagonistic interactions were observed between the factors tested.

4. Discussion

In this study, no increase in antimicrobial activity was observed when chrisin was used with EDTA or when lysozyme was used with EDTA against any of the Gram-negative organisms tested. In contrast, a number of authors have reported observation of increased antimicrobial activity by lysozyme (Shively and Hartsell, 1964a,b; Hughey and Johnson, 1987; Ellison and Giehl, 1991; Payne et al., 1994; Park, 1997; Carneiro De Melo et al., 1998) or nisin (Stevens et al., 1991, 1992; Cutter and Siragusa, 1995a; Carneiro De Melo et al., 1998) against Gram-negative and Gram-positive organisms in the presence of membrane disrupting agents or sub-lethal injury (Kalachyanand et al., 1992). The majority of these reports were based upon observation of organisms suspended in a buffer, rather than growing in nutrient media (Shively and Hartsell, 1964a,b; Ellison and Giehl, 1991; Stevens et al., 1991, 1992; Cutter and Siragusa, 1995a; Kalachyanand et al., 1992), although in one study (Carneiro De Melo et al., 1998), the authors used distilled water as a suspension medium. Where studies have been conducted in media capable of supporting growth, no activity against gram-negative organisms has been reported, except where unrealistically high levels of agents for food applications have been used. Park (1997) observed lysozyme activity against *E. coli* in nutrient broth and vegetable juice at levels of 0.1 to 0.4 g/l. Cutter and Siragusa (1995a) reported antimicrobial activity of nisin plus chelators against *E. coli* and *Salmonella* spp. in buffer, but reported no significant effect upon the same organisms when trials were conducted on beef (Cutter and Siragusa, 1995b).

Since food products provide a nutrient rich environment, tests of antimicrobials under conditions of cell starvation would appear to be of limited value in evaluating them for application in food products. For this reason, our experiments were designed to examine the interactions between the antimicrobial agents of interest without nutrient restriction. The failure to observe interaction between lysozyme or chrisin with EDTA in this study may indicate that the observed interaction between these antimicrobials in buffer systems (Stevens et al., 1991, 1992; Cutter and Siragusa, 1995a,b; Kalachyanand et al., 1992; Carneiro De Melo et al., 1998) are a consequence of cell starvation which prevents cell repair. Additionally, the use of cells harvested from log or stationary phase cultures for the assessment of antimicrobials is a poor model for the inoculation of bacteria to a food product. Bacteria will more often be introduced, at a low population level, to a food product in which inhibitory agents have already been formulated. The bacteria will then undergo a lag phase or decline as they attempt to adapt to the new environment. The physiological state of bacteria recovered from log phase or peak population stationary phase should not be expected to be equivalent to cells in lag adaption phase.

In a recently published study, Chung and Hancock (2000) reported apparent interactions between lysozyme and nisin against lactic acid bacteria. No such interactions were observed in our study, which may be a consequence of the different growth media used in these two studies (deMann Rogosa Sharpe, MRS, and APT). The antimicrobial effect of lysozyme, nisin,

and any interactions against lactic acid bacteria seems to be highly dependent on the growth media. Chung and Hancock (2000) reported enhancement of the antimicrobial effect of lysozyme and nisin when MRS was diluted to 1/8 (a nutrient-deficient environment), and an enhanced bactericidal effect of a combination of these two agents in MRS was not observed in a pork juice medium under the same conditions.

The two-level, five-factor experimental design used in this study allowed identification of each factor which may contribute significantly to the response, and whether any two-way interaction between factors occurred. This approach provided certain advantages over the checker-board method (Maclean et al., 1997a,b; Chung and Hancock, 2000) for preliminary evaluation of antimicrobials, prior to studies in food systems. This method was as rapid and inexpensive as the checker-board method, but can provide considerably more information by allowing prediciton of responses in systems containing more than two antimicrobial agents. Additionally, evaluation of results based on a variable response rather than the growth/no-growth scoring of the checker-board method allowed the determination of effects on growth in cases where the growth rate was slowed, as well as where growth was inhibited. This accounts for the observation that lysozyme negatively affected the growth of *B. thermosphacta*, *P. acidilactici*, *En. faecalis* and *W. viridescens* as determined by the absorption analysis (Tables 3 and 4), whereas MIC determinations showed only *B. thermosphacta* to be sensitive (Table 2).

The method presented here possesses certain limitations. Interactions between factors can only be detected where the effect of one factor alone does not exceed the maximum response. Furthermore, if a single agent inhibits growth below the detectable level, then any further inhibition by other factors will not allow detection of the initial inhibitory event and will not contribute to the statistical model. In addition, although the relative absorbance of the media can be related to the bacterial population level due to increasing turbidity, bacterial populations below 10^5 CFU/ml have little or no effect on turbidity.

The results of these experiments indicated a complex pattern of interactions between pairs of agents, which differed between different organisms. However, there were no interactions observed between lysozyme, chrisin and EDTA that enhanced their effectiveness against any of the Gram-negative organisms. In contrast, several interactions were observed between lysozyme or chrisin and EDTA that resulted in enhanced activity against several gram-positive organisms.

There was no evidence that the presence of nitrite and NaCl prevented the antimicrobial activity of lysozyme, chrisin and EDTA against any of the organisms used in this study. In fact, several interactions, which enhanced inhibition, were observed between chrisin or EDTA and nitrite or NaCl. These interactions included enhancement of activity against Gram-negative organisms such as EDTA and NaCl (*E. coli*, *S.* Typhimurium, *Ser. grimesii*); EDTA and nitrite (*E. coli*, *S.* Typhimurium); and NaCl and nitrite (*S.* Typhimurium, *Sh. putrefaciens*).

These results indicate that the activity of lysozyme and chrisin was enhanced against the tested gram-positive organisms in the presence of EDTA under conditions in which growth was not restricted by starvation. There was no protective effect of nitrite or NaCl upon the inhibitory action of lysozyme, nisin and EDTA. In addition, the results indicated that the addition of lysozyme, nisin and EDTA to cured meat products containing NaCl and nitrite should result in a wider spectrum of antimicrobial activity.

The results of these experiments show that methodologies for the assessment of antimicrobials that use a non-growing population of cells will give different results from studies in which a growing population of cells are used. Since bacterial populations on most food products are growing, the use of static cell populations for the assessment of antimicrobials for food applications is of questionable value. We would suggest that for purposes of screening antimicrobials for use in food systems, methodologies that do not use growth-restricting conditions be adopted.

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