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Action of lysozyme and nisin mixtures against lactic acid bacteria

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Abstract

Lysozyme was formulated together with nisin for usage against food spoilage lactobacilli. The mixtures demonstrated improved minimal inhibitory concentrations (MIC), compared to the parent compounds, for many of the bacteria and media tested, including high salt media in which lysozyme lost virtually all of its activity. Synergy was also observed through measurement of the kinetics of bacterial killing of *L. curvatus* 845, in which strain synergy had been observed in MIC assays. The combination of lysozyme and nisin caused more severe cell damage as viewed by scanning electron microscopy, and a consequent change in optical density at 600 nm, compared to the parent compounds, effects that were presumed to reflect the action of lysozyme. In addition, the combination caused more rapid permeabilization (depolarization) of the cytoplasmic membranes of *Staphylococcus aureus*, an effect that reflected the mechanism of action of nisin. Thus, nisin and lysozyme appear to demonstrate synergy against gram-positive bacteria because they reinforce each others mechanisms of bacterial killing.

Keywords: Lysozyme; Nisin; Lactic acid bacteria; Food spoilage

1. Introduction

The antibacterial spectra of lysozyme and nisin have been well documented in the literature with some overlapping applications in the food industry. Lysozyme is a 129-amino acid protein with hydrolytic activity against β (1–4) glycosidic linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine in bacterial peptidoglycan. In addition, it has been reported to have an antibacterial activity that is independent of its enzymatic activity. Generally speaking, the activity spectrum of lysozyme is limited to specific gram-positive bacteria (McKenzie and White, 1991). Lysozyme has proven to be quite ineffective against gram-negative bacteria due to the outer membrane barrier that surrounds and protects the peptidoglycan layer. The majority of commercially prepared lysozyme is purified from hen egg white. Lysozyme presently has a small number of applications in the food industry with the major usage involving the prevention of *Clostridium tyrobutyricum* spore outgrowth in hard cheeses

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(Wasserfall and Teuber, 1979). Lysozyme has been demonstrated to be active throughout a wide pH range of 4–10 (Davies et al., 1969). However, high ionic strength (>0.2 M salt) was shown to have an inhibitory effect on lysozyme activity (Davies et al., 1969; Chang and Carr, 1971).

The lantibiotic nisin is a commercially available 35-amino acid cationic peptide antimicrobial produced by specific lactic acid bacteria (Delves-Broughton et al., 1996). Nisin has been reported to primarily act upon the cytoplasmic membrane of gram-positive bacteria rendering the cell permeable to small ionic components (Bruno et al., 1992). In some bacteria, interaction with the cytoplasmic membrane is potentiated by a primary interaction with lipid II (C55 bactoprenol pyrophosphate – a carrier involved in cell wall biosynthesis) (Brotz et al., 1998). Cell death has been proposed to be due to the loss of cell integrity upon exposure to the lantibiotic. This naturally occurring peptide has shown antibacterial effectiveness against gram-positive bacteria, with reduced or no effectiveness against gram-negative bacteria (Delves-Broughton et al., 1996). Nisin is presently used in the cheese industry to prevent the outgrowth of *Clostridia* spores and subsequent product spoilage (Delves-Broughton et al., 1996). Previous work by Cutter and Siragusa (1996) has demonstrated the ability of nisin and refrigeration to inhibit *Brochothrix thermosphacta* and *Listeria innocua* in vacuum packaged red meat.

Gram-positive bacteria represent a well-defined spoilage problem in the food industry and were thus chosen to demonstrate the synergistic properties of lysozyme and nisin. The major spoilage agents associated with vacuum packaged raw beef have been determined to be *B. thermosphacta, Lactobacillus curvatus* and *L. sake* (Yang and Ray, 1994). Since lysozyme and nisin are reported to have quite different modes of action, we studied here synergy between these two agents, and the possible mechanisms underlying synergy.

2. Materials and methods

2.1. Bacteria

The main bacteria utilized in this study are listed in Table 1. They were obtained from Dr. Rick Holley, University of Manitoba, except for *Brochothrix thermosphacta, Lactobacillus sake* strain 1218 and *Lactobacillus curvatus* strain 845 supplied by Dr. Francis Nattress (Lacombe Research Centre, Agriculture and Agri-food Canada, Alberta). For most experiments, bacteria were grown on modified Difco lactobacillus (MRS) media or the same medium diluted 8-fold (0.125 MRS). In some experiments, sterile filtered pork juice was utilized, as obtained from Dr. Frances Nattress. It was prepared by adding an equal weight of water to ground up pork meat. The mixture was heated for 3 min at 80°C, cooled quickly, and passed through a series of filters until it could pass through a 0.2 μm filter.

2.2. Peptides and proteins

Lysozyme–HCl (24 000 units per mg) was provided by Canadian Inovatech Labs Inc. (Abbotsford, B.C., Canada). Nisin (trade name Chrisin) was obtained from Christen Hansen A/S (Copenhagen, Denmark; 1 000 000 international units per gram). For mixtures, lysozyme and nisin solutions were mixed and co-dried in a pilot scale NIRO spray drier at Inovatech.

2.3. Minimal inhibitory concentration (MIC) determinations

MICs were tested by the broth microdilution procedure (Amsterdam, 1996). Because of the slow growth of lactobacilli at the temperature used, plates were left for 7 days (ambient laboratory growth conditions, 18–19°C) prior to data recording. The MIC was the lowest compound concentration showing inhibition of bacterial growth.

2.4. Scanning electron microscopy

Bacteria were incubated for 22 h at 18°C with 500 μg/ml lysozyme, nisin, or combinations and then fixed, washed and dried. Briefly Nucleopore Track-Etch Membranes (13 mm, 0.4 μm) were installed into Millipore Twinex filters. Bacterial cells were then treated as described and filtered through the membranes using a 5 ml syringe. The bacteria on the filter were then fixed for 30 min in 2.5% glutaraldehyde at 4°C, placed in a Costar 24-well plate, and washed three times for 5 min in 0.1 M sodium phosphate buffer, pH 7.2. Cells were then fixed with

Table 1
Effect of media composition on MICs for nisin, lysozyme and mixtures of the two[a]

Media composition	Ratio nisin: lysozyme	MIC (μg/ml)[b]							
		Brochothrix thermosphacta	*Lactobacillus sake* 22	*L. sake* 6	*L. sake* 1218	*L. curvatus* 5	*L. curvatus* 845	*Pediococcus acidilactici*	*Leuconostoc mesenteroides*
0.125 MRS	1: 0	< 0.5	2	3.9	< 0.5	< 0.5	15.6	< 0.5	< 0.5
	0: 1	< 0.5	1	31.1	< 0.5	< 0.5	62.5	1.0	< 0.5
	1: 2	< 0.5	2	**2**	< 0.5	< 0.5	**3.9**	< 0.5	< 0.5
	1: 3	< 0.5	1	**< 0.5**	< 0.5	< 0.5	**7.8**	< 0.5	< 0.5
MRS	1: 0	< 0.5	15.6	15.6	< 0.5	< 0.5	500	2	< 0.5
	0: 1	3.9	> 500	> 500	500	> 500	500	> 500	500
	1: 2	< 0.5	15.6	62.5	1	< 0.5	500	**< 0.5**	< 0.5
MRS + 2% salt	1: 0	1	62.5	31.3	125	2	125	15.6	3.9
	0: 1	500	> 500	> 500	> 500	> 500	500	> 500	> 500
	1: 2	**< 0.5**	62.5	125	**31.3**	**< 0.5**	500	15.6	**1**
MRS + 4% salt	1: 0	1	15.6	125	31.3	15.6	125	125	15.6
	0: 1	500	> 500	> 500	> 500	> 500	> 500	> 500	> 500
	1: 2	**< 0.5**	31.3	125	31.3	**7.8**	500	**7.8**	**7.8**
Pork juice	1: 0	< 0.5	< 0.5	ND	ND	1	ND	< 0.5	< 0.5
	0: 1	< 0.5	15.6	ND	ND	500	ND	31.3	125
	1: 2	< 0.5	< 0.5	ND	ND	**< 0.5**	ND	< 0.5	< 0.5

[a] Strains utilized and their sources included: *Brochothrix thermoshpacta,* Fresh beef, LaCombe Research Center; *Lactobacillus sake* 1218, University of Alberta, LaCombe Research Center; *Lactobacillus curvatus* 845, CO_2 stored beef, LaCombe Research Center; *Lactobacillus sake* 6, Spoiled cured meat, University of Manitoba; *Lactobacillus sake* 22, DSM 20010, University of Manitoba; *Lactobacillus curvatus* 5, spoiled cured meat, University of Manitoba; *Pediococcus acidilactici*, Lactacel 115 starter culture, University of Manitoba; *Leuconostoc mesenteriodes*, UBC isolate, UBC. Significantly lower MICs observed for the combinations are bolded.

[b] MICs for the mixtures of lysozyme are expressed as the MIC of the mixture. The individual MICs in a, e.g. 1:3 mixture would be one quarter and three quarters, respectively, of the value given.

1% osmium tetroxide for 30 min, rinsed in distilled water for 5 min, fixed with 1% tannic acid for 20 min, rinsed again in distilled water for 5 min, fixed once again with the 1% osmium tetroxide, and finally rinsed one last time with distilled water for 5 min. For dehydration, rinse water was emptied and the sample treated progressively with 50, 70, 80, 95, 95, 100, 100, and 100% alcohol each for 5 min. Samples were then dried in a Ladd Research Industries Inc. (Williston, VT) critical point dryer. They were then sputtered with gold and viewed under a Cambridge Stereo Scan 260 Scanning Electron Microscope (Leica Inc., Deerfield, IL).

2.5. Lysozyme lysis assay

The ability of lysozyme to lyse bacteria was examined by re-suspending washed bacteria in 5 mM sodium Hepes buffer, pH 7.4, containing 5 mM sodium azide and adding lysozyme to 500 μg/ml. The change in optical density at 600 nm was followed continuously using a Perkin Elmer Lambda 3 Spectrophotometer attached to a Perkin Elmer model 561 chart recorder.

2.6. Membrane depolarization assay

After growing the bacteria to an optical density at 600 nm (OD_{600}) of 0.5, the cells were washed with 5 mM Hepes buffer, pH 7.4, and resuspend to an OD_{600} of 0.09. 3,5-Dipropylthiacarbocyanine ($DiSC_35$; Molecular Probes, Eugene, Oregon) (to 0.4 μM) and 20 mM KCl were then added to the resuspended cells and the fluorescence was allowed to quench for 20 min (Wu and Hancock, 1999). Lysozyme, nisin, or combinations were then added, and fluorescence assessed using a Perkin Elmer LS50B Luminescence spectrometer.

2.7. Killing assays

Overnight cultures were diluted to 10^8 cfu/ml in MRS or pork juice and cells diluted and plated on MRS agar to measure the initial viable plate count. The desired concentrations of lysozyme, nisin or

combinations were added to the culture, incubated at 22°C and a viable count taken every hour, by plating onto MRS agar plates at 22°C for 21 h.

3. Results

3.1. Lysozyme:nisin synergy

Preliminary checkerboard analyses with 11 gram-positive bacteria indicated that lysozyme and nisin occasionally demonstrated synergy [as indicated by a Fractional Inhibitory Concentration less than 0.5; Amsterdam, 1996]. Therefore, we formulated a series of defined mixtures of lysozyme and nisin and tested these against a panel of eight strains comprising a broad range of food spoilage organisms (e.g. Table 1; a further 18 representatives of these species gave similar results). The antibacterial activities of these mixtures in one eighth strength MRS occasionally surpassed those of the parent molecules (e.g. N1:L3, a 1:3 mixture of nisin to lysozyme was more active than either parent molecule against *Lactobacillus sake* strain 6 and *L. curvatus* 845). However, taking into account that, for example, a 1:2 mixture of nisin and lysozyme would be only one third by weight nisin, the mixtures were always equal to or better than the equivalent amount of nisin, which was by far the more expensive molecule in this combination.

It has been previously observed that the enzymatic activity of lysozyme is strongly decreased by increasing salt concentrations (Chang and Carr, 1971). Similarly, we observed that an increase to even full strength MRS increased the MIC for lysozyme by up to 1000-fold or more (Table 1). Only for *Brochothrix thermosphacta* was the MIC, 3.9 μg/ml, reasonably low in full strength MRS, although the addition of 2% NaCl raised the MIC to 500 μg/ml. To compare this with a model food system, we measured MICs in pork juice. The MICs for lysozyme in pork juice were lower than those in full strength MRS, but higher than those in one eighth strength MRS. Of great interest, however, was the observation that in full strength MRS with 0–4% NaCl, synergy was seen with lysozyme for the nisin:lysozyme 1:2 combination (Table 1). In nine of the 24 assessments, the MIC of this combination was at least 2-fold and up to 16-fold lower than nisin alone. In another nine cases, MICs were equivalent (but actually lower for nisin when the nisin content, in the combination, of one third was taken into account). The remaining six assessments showed a 2–4-fold increase in MIC that was not significantly different with respect to nisin content. This emphasizes the synergistic benefits of the combinations even in the presence of high salt conditions, which inhibited the activity of lysozyme.

Synergy was also observed through measurement of the kinetics of bacterial killing of *L. curvatus* 845, in which strong synergy between lysozyme and nisin had been observed in MIC assays (Table 1). In this strain, lysozyme at 500 μg/ml killed very slowly (1 log decrease in colony forming units in 3 h), whereas nisin at 500 μg/ml was more effective (4 logs decrease in 2 h) (Fig. 1a). However, a 1:3 combination of nisin:lysozyme (i.e. 125 μg/ml nisin plus 375 μg/ml lysozyme) resulted in a nearly identical level of kill to 500 μg/ml nisin after 60 min, but a much stronger kill after 2 h (i.e. 8 logs of killing). The combinations of nisin:lysozyme of 1:1 and 3:1 showed almost superimposable kill curves to the 1:3 combination (data not shown). The majority of kill curves performed with other bacteria, however, showed more subtle effects (e.g. Fig. 1b), with marginal or no advantages observed in killing over 3 h. We did, however, consistently observe that the rate of kill by combinations was higher for the interval of 1–2 h after addition.

3.2. Morphological effects

Classically, lysozyme is proposed to cause cell lysis (McKenzie and White, 1991). We examined the effects of lysozyme, nisin, and combinations on cell integrity, by measuring the change in optical density (OD_{600}) over time after addition of compound. Against *Lactobacillus curvatus* (Fig. 2a) virtually no lysis was observed over 30 min with either nisin, lysozyme or the 1:3 (Fig. 2a), or 1:1 (data not shown) combinations of these. Very slight reductions in OD_{600} were observed with the 3:1 combination of nisin and lysozyme. Against the more susceptible bacterium, *L. sake* 1218, again lysozyme and nisin showed no effect on OD_{600} whereas combinations of 1:3, 3:1 (Fig. 2b) and 1:1 (data not shown), all showed a 35% increase in OD_{600} over the first 5–10 min followed by a gradual decrease. This change in

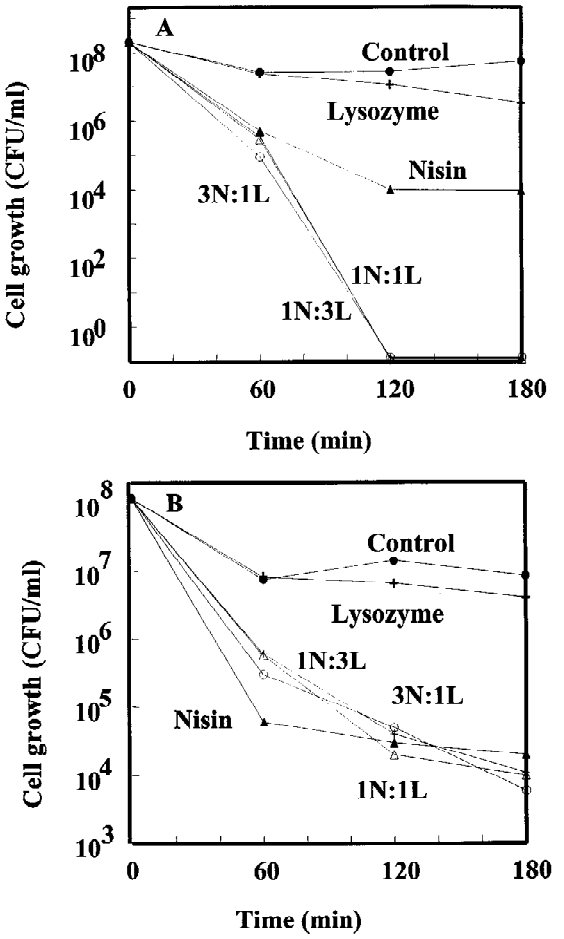

Fig. 1. Killing of lactobacilli by lysozyme (dark plus signs), nisin (filled triangles), combinations of nisin to lysozyme of 1:3 (1N:3L; open triangles), 1:1 (1N:1L; faint plus signs), and 3:1 (3N:1L; open circles) and untreated control (●). (a) Killing of *Lactobacillus curvatus* 845 in MRS medium at 500 μg/ml compound or combination, 22°C. (b) Killing of *Lactobacillus sake* 1218 in pork juice at 50 μg/ml compound or combination, 22°C.

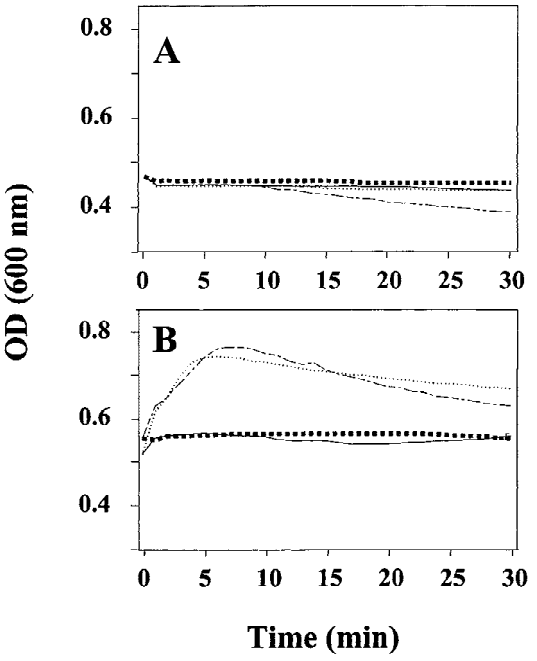

Fig. 2. Optical density changes due to 500 μg/ml lysozyme (plain line), nisin (large squares line), and the combinations of nisin:lysozyme 1:3 (dotted line) and nisin:lysozyme 3:1 (dashed line) in the presence of pork juice. (a) *Lactobacillus curvatus* 845. (b) *Lactobacillus sake* 1218.

OD_{600} could have been caused by swelling of the bacteria, an alteration in refractive index, or the production and release of light scattering material into the supernatant. Nevertheless, these data did seem to indicate that some synergistic action was occurring in the first 5–10 min in *L. sake* 1218.

We probed this further by scanning electron microscopy. Nisin had no apparent effect on cell morphology other than increased surface ruffling (Fig. 3B) and occasional aberrant alterations at the division septum (data not shown). Lysozyme treatment caused the production of small balls of material all over the surface of cells (Fig. 3C). Clearly no lysis was observed in these cells. In contrast, 1:3 and 3:1 combinations of these agents (Fig. 3D–F) caused a major perturbation of cell morphology including apparent holes or craters in the cell surface, drastic abnormalities at the position of septa, and the release of large pieces of material (presumably cell wall). These changes could clearly account for the synergistic effect of these agents on optical density.

3.3. Membrane potential effects

One theory as to how nisin kills bacteria is through the creation of lesions in the cytoplasmic membrane (Bruno et al., 1992). We utilized a fluorescence assay of membrane potential, using the membrane potential sensitive cationic dye $diSC_35$, which has been used to assess the permeabilization of gram-negative bacterial cytoplasmic membranes by cationic antimicrobial peptides (Wu and Hancock, 1999). The dye $diSC_35$ is taken up and concentrates

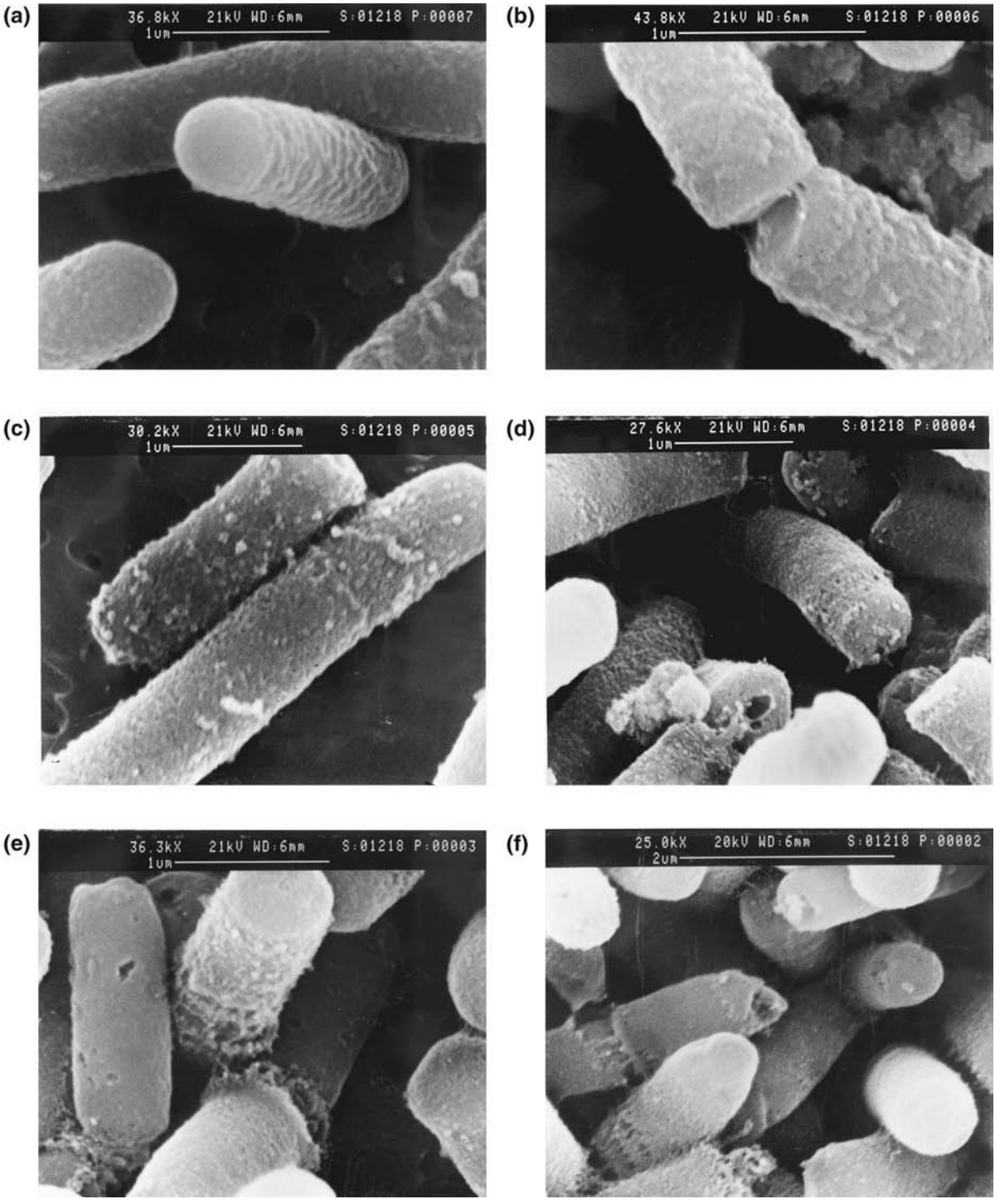

Fig. 3. Influence of lysozyme, nisin and combinations on the morphology of *Lactobacillus sake* 1218, as assessed by scanning electron microscopy. A: untreated control; B: nisin treated; C: lysozyme treated; D: nisin:lysozyme (3:1) combination treated; E and F: nisin:lysozyme (1:3) combination treated.

in the cytoplasmic membrane, according to the membrane potential, and self quenches its own fluorescence. Addition of a substance (e.g. gramicidin S) which causes membrane lesions, leads to the dissipation of the membrane potential and the consequent loss of $diSC_3 5$ from the membrane resulting in a dequenching (i.e. increase) of fluorescence. Attempts to establish this assay in *L. curvatus* 845 and *L. sake* 1218 were unsuccessful. Therefore, we utilized *S. aureus* for these studies. Lysozyme at 150 μg/ml (or 500 μg/ml, not shown) caused no depolarization of the cytoplasmic membrane up to 2 h (Fig. 4). In contrast, nisin at 150 μg/ml cause a rapid increase in fluorescence over the first 4 min to a level of about 55% of maximal dye loss (determined by the addition of valinomycin in the presence of 100 mM K^+). After the first 10–15 min, the fluorescence decreased rapidly. Over the next 15–20 min, the fluorescence declined more slowly and settled to a level of about 30% of maximum. The most likely basis for this initial rapid decline in fluorescence after the primary increase was a partial re-establishment of the membrane potential leading to dye uptake and quenching. The addition of a mixture of 1:3 nisin:lysozyme (i.e. 37.5 μg/ml nisin and 112.5 μg/ml lysozyme) lead to a smaller and slower initial rise in depolarization of the cytoplasmic membrane, but the only decline observed was the slow decline due to the decay of fluorescence in the incident light beam (observed with all fluorescent samples). The mixture of 3:1 nisin:lysozyme (i.e. 12.5 μg/ml nisin and 37.5 μg/ml lysozyme) showed a more dramatic effect compared to nisin alone, with complete depolarization occurring within 5 min (Fig. 4). The 1:1 mixture also showed more rapid depolarization at all concentrations (75, 125 and 500 μg/ml) tested (Fig. 4). The largest effect was observed at 75 μg/ml, at which there was a 2.5-fold greater fluorescence observed (5 vs. 2 units) with the 1:1 mixture than with nisin alone.

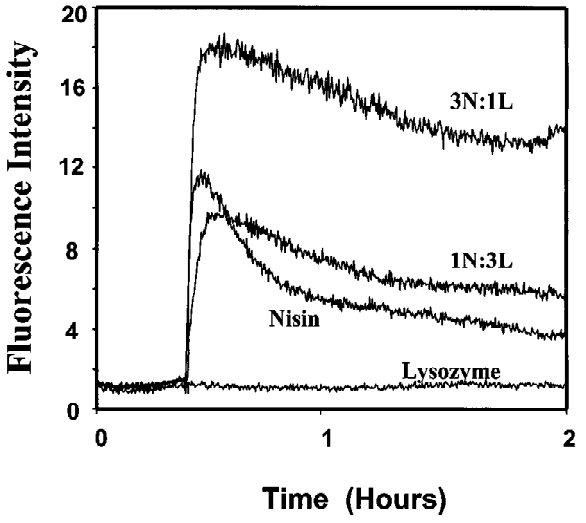

Fig. 4. Permeabilization (depolarization) of the cytoplasmic membrane of *Staphylococcus aureus* ATCC25923 as assessed by the dequenching of $diSC_3 5$ fluorescence by 150 μg/ml lysozyme, nisin, and the combinations of nisin:lysozyme 1:3 and 3:1.

4. Discussion

The results of this paper clearly demonstrate the benefits of using mixtures of nisin and lysozyme against food spoilage bacteria, over the use of the individual agents. This benefit becomes even more obvious when one considers the relative costs of the two agents, with lysozyme being 3-fold cheaper than nisin. Furthermore, and somewhat surprisingly, the benefits of combinations were sustained even at high salt concentration which drastically reduced the activity of lysozyme and had a somewhat lesser, but nevertheless marked effect, on the activity of nisin. Thus, in 50% of the strains described in Table 1, in the presence of 2% or even 4% salt, improved MICs were seen for the 1:2 nisin:lysozyme mixture compared with the most active agent nisin, even though lysozyme was virtually completely inactive at 2% salt (or for seven of the eight strains in MRS medium without salt). While lysozyme retained some activity in a food system, pork juice, the combination was clearly better.

Nisin has been proposed to act on the cytoplasmic membranes of gram-positive bacteria to cause lesions (Montville and Chen, 1998). Conversely, lysozyme has been proposed to act as a muramidase, enzymatically breaking the sugar linkages of peptidoglycan, and causing cells to burst due to the weakened peptidoglycan. Partly consistent with these observations, nisin caused partial depolarization of *S. aureus* cytoplasmic membranes (Fig. 4) while lysozyme caused partial disruption of the smooth surface of *L. sake* 1218. However, these affects seemed incom-

plete, and possibly some other killing mechanism was at least partly operative for each agent. For example, nisin has been shown to trigger autolysis of *Staphylococcus simulans* by activating *N*-acetylmuramoyl-L-alanine amidase (Bierbaum and Sahl, 1987). On the other hand, lysozyme has been demonstrated to be capable of killing cells by a non-enzymatic procedure (During et al., 1999). Nevertheless, it was clear that the combination was more effective at the primary actions of nisin and lysozyme than the individual components. Thus, the combination was superior in causing both surface disruption, as revealed by scanning electron microscopy, and an increase in optical density, as well as cell depolarization.

We do not know the precise mechanism that underlies synergy; however, increased cell lysis is one definite possibility (since cell lysis would destroy the bacterial membrane potential releasing $diSC_35$). Conversely, nisin could be inhibiting energy dependent processes that repair lysozyme damage. However, it does seem that this combination is beneficial against food spoilage organisms and that the combination has potential to prevent spoilage in food systems. We have also observed good activity against the food pathogen *Listeria monocytogenes*, but activity against other food pathogens was much weaker and there was no activity for gram-negative food pathogens and food spoilage organisms.

Acknowledgements

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