Synergistic Actions of Nisin, Sublethal Ultrahigh Pressure, and Reduced Temperature on Bacteria and Yeast

PIETER F. TER STEEG,* JOHAN C. HELLEMONS, AND ANJA E. KOK

Microbiology & Preservation, Unilever Research Vlaardingen, Vlaardingen, The Netherlands

Received 25 March 1999/Accepted 30 June 1999

Nisin in combination with ultrahigh-pressure treatment (UHP) showed strong synergistic effects against *Lactobacillus plantarum* and *Escherichia coli* at reduced temperatures (<15°C). The strongest inactivation effects were observed when nisin was present during pressure treatment and in the recovery medium. Elimination (>6-log reductions) of *L. plantarum* was achieved at 10°C with synergistic combinations of 0.5 µg of nisin per ml at 150 MPa and 0.1 µg of nisin per ml at 200 MPa for 10 min. Additive effects of nisin and UHP accounted for only 1.2- and 3.7-log reductions, respectively. Elimination was also achieved for *E. coli* at 10°C with nisin present at 2 µg/ml, and 10 min of pressure at 200 MPa, whereas the additive effect accounted for only 2.6-log reductions. Slight effects were observed even against the yeast *Saccharomyces cerevisiae* with nisin present at 5 µg/ml and with 200 MPa of pressure. Combining nisin, UHP, and lowered temperature may allow considerable reduction in time and/or pressure of UHP treatments. Kill can be complete without the frequently encountered survival tails in UHP processing. The slightly enhanced synergistic kill with UHP at reduced temperatures was also observed for other antimicrobials, the synthetic peptides MB21 and histatin 5. The postulated mode of action was that the reduced temperature and the binding of peptides to the membrane increased the efficacy of UHP treatment. The increases in fatty acid saturation or diphosphatidylglycerol content and the lysylphosphatidyl content of the cytoplasm membrane of *L. plantarum* were correlated with increased susceptibility to UHP and nisin, respectively.

High hydrostatic pressure offers an attractive alternative to heat pasteurization as a means to produce preservative-free, microbiologically safe and stable foods. Yeasts, molds, and vegetative cells of bacteria can be inactivated by pasteurization pressures in the range of 200 to 700 MPa, while the organoleptic quality of fresh products like fruit juices and jams, guacamole, rice cake, and raw squid will be retained (6, 23, 24, 30, 32, 34). Practical exploitation of high hydrostatic pressure pasteurization has been limited because of economic constraints and the occurrence of pronounced survivor tails of vegetative pathogenic bacteria on death rate graphs (34). To achieve elimination of vegetative cells (pasteurization) without affecting the characteristics of a food, hydrostatic pressure pasteurization may best be conducted at a moderate pressure, which alone will not kill the desired level of vegetative cells. However, along with hydrostatic pressure, other preservation parameters (antimicrobials, pH, and temperature) can be used to enhance the bactericidal effects of pressurization. Nisin is an antimicrobial peptide known to inhibit the growth of a number of gram-positive bacteria, including outgrowth of spores of bacilli and clostridia (7, 15–16). Insight into the synergistic action of such combination preservation systems may assist in the development of cost-effective mild preservation.

Moderate ultrahigh-pressure treatment (UHP) combined with nisin has been investigated as a synergistic combination method for mild food preservation (13, 19–21). Kalchayanand et al. (19–21) suggested the following explanation for the observed synergy: UHP can cause sublethal injury of cells and will sensitize cells of gram-positive and -negative microorganisms to the effects of nisin and other selective agents. The increased efficacy of the synergistic combination of UHP and nisin, how-

ever, may also be explained by changes in membrane fluidity. A clear relation exists between resistance to pressure and/or nisin and the phospholipid composition of the membrane of the susceptible gram-positive microorganisms *Lactobacillus plantarum* and *Listeria monocytogenes* (1, 24, 31, 33). A stiffer membrane of *L. plantarum*, either from an increase of saturated fatty acids at higher growth temperatures (30 to 40°C) or a decrease in phosphatidylglycerol and a corresponding increase in diphosphatidylglycerol (DPG), is known to sensitize the microorganism to UHP (31, 33). On the other hand, a stiffer membrane, however, was claimed to make cells of gram-positive microorganisms less susceptible to pore formation by nisin (1, 9, 17, 18, 31). The first step of the barrel stave mechanism of nisin is a parallel orientation of the molecule and subsequent binding to the membrane surface (4, 9). Our main hypothesis was that this binding of nisin would directly increase the susceptibility of microorganisms during UHP treatment due to an assumed local immobilization of phospholipids. In addition, the UHP treatment may still cause indirect (sublethal) injury by facilitating the access of nisin to the cytoplasm membrane as a result of cell wall (and/or outer membrane for gram-negative microorganisms) permeabilization (13, 30a). The aim of the present study was to obtain evidence of the synergistic action of nisin during and after UHP against *L. plantarum* and to test whether the synergistic effect can be enhanced during a reduced-temperature pressure treatment. Previously published findings (31, 33) that had established the role of growth history and membrane fluidity in UHP susceptibility of *L. plantarum* were complemented with data on nisin susceptibility and culture history. We chose *L. plantarum* as the model organism since it plays a role in food fermentation but is also a well-recognized spoilage microorganism of mildly preserved acidic products, such as processed tomatoes and dressings for salads. Whether any effect of nisin during and/or after UHP was also applicable to other groups of vegetative microorganisms was also tested. The outer membrane of gram-neg-

* Corresponding author. Mailing address: Microbiology & Preservation, Unilever Research Vlaardingen, P.O. Box 114, 3130 AC Vlaardingen, The Netherlands. Phone: 31-10-4605832. Fax: 31-10-4605188. E-mail: Pieter-ter.Steeg@Unilever.com.

TABLE 1. Effect of temperature on log reduction of *L. plantarum*, *E. coli*, and *S. cerevisiae* colonies at pH 4.5 and 7 by 10-min pressure treatments

Microorganism	T (°C)	Pressure (MPa)	-Log (N _t /N ₀) _{UHP}	
			pH 4.5	pH 7.0
<i>L. plantarum</i>	8	150		1.1
	8	200		2.9
	11	150	2.0	0.8
	11	200	4.6	3.3
	21	100	-0.1	-0.1
	25	100	-0.1	-0.1
	25	150	-0.0	-0.1
	25	200	0.5	0.0
	25	250	3.9	0.1
	25	300	5.0	0.1
	35	100	-0.1	0.0
	35	150	0.3	0.0
	35	200		0.0
<i>E. coli</i>	8	150		0.3
	8	200		0.2
	10	150	1.7	1.8
	10	200	>6.2	2.3
	25	150		0.0
	25	200	1.9	0.0
	40	150	2.9	2.4
	40	200	>5.6	3.2
<i>S. cerevisiae</i>	10	150	1.7	1.6
	10	200	>4.6	2.1
	35	150	1.6	0
	40	150	2.9	2.4
	40	200	>4.0	3.4

active bacteria (with *Escherichia coli* as a model) or the thick cell wall of fungi (with *Saccharomyces cerevisiae* as a model) may present extra barriers. These barriers may prevent direct access and binding of nisin to the cytoplasmic membrane (8, 13). A limited study to determine whether synergistic effects could also be observed for synthetic antimicrobial peptides, MB21 and a truncated histatin derivative, with a broad antimicrobial spectrum was carried out. MB21 is a computer-modeled cationic peptide of 15 amino acids which is presumed to form an amphiphilic α -helix upon interaction with the membrane (2). Histatins are salivary histidine-rich cationic peptides, ranging from 7 to 38 amino acid residues in length, that are effective against *Candida albicans*. Histatin 5 (residues 11 to 24; called dh-5) consists of 14 amino acids and has been claimed to have a broad antimicrobial activity (14, 35).

MATERIALS AND METHODS

Microorganisms. *L. plantarum* La10-11, identified by the American Type Culture Collection but with no ATCC number and isolated from onion ketchup, was the gram-positive spoilage model microorganism used. *E. coli* NCTC 9001 and *S. cerevisiae* SU51 were selected as additional model gram-negative and fungal microorganisms.

Antimicrobials. Pure nisin A was kindly provided by Aplin & Barrett (Dorset, United Kingdom). Fifty milligrams of nisin was dissolved in 100 ml of sterile 0.01 M HCl to obtain a concentration of 500 μ g/ml. MB21, a synthetically designed antimicrobial peptide, was synthesized by M. Bhakoo (Unilever Research, Port Sunlight, Bebington, United Kingdom) (2). Its amino acid sequence is FASLL-GKALKALAKQ. A truncated histatin 5 (residues 11 to 24; called dh-5) was synthesized at Commonwealth Biotechnologies, Inc. (Richmond, Va.) and kindly provided by M. Chickendas (5, 14). Stock solutions of MB21 at 10 mg/ml and histatin 5 at 20 mg/ml were made in deionized water. All stock solutions were filter sterilized with a 0.22- μ m-pore-size Millipore filter. Antimicrobials were aseptically added to ready-for-use treatment and recovery media.

Culture conditions. *L. plantarum* was grown on modified de Man-Rogosa-Sharpe medium (mMRS). The mMRS contained (per liter) 10.0 g of proteose

peptone, 10.0 g of beef extract, 5.0 g of yeast extract, 20.0 g of glucose monohydrate, 1.0 g of Tween 80, 0.1 g of magnesium sulfate, 0.05 g of manganese sulfate, and 2.0 g of dipotassium sulfate. To allow experiments to be performed at a reduced pH, acid-precipitable protein was routinely removed. Proteinaceous precipitate may otherwise interfere in turbidimetric growth monitoring. The pH of the pre-medium (MRS) was decreased to pH 3.8 with HCl. The pre-medium was subsequently incubated for 1 h at 100°C. The medium was filtered over a 0.45- μ m-pore-diameter filter (no. 12123; Gelman Science Inc.) to remove any proteinaceous precipitate. The pH was adjusted to 7.0 and 4.5 with 4 N KOH and 4 N HCl, respectively. The addition of 2% agar made the mMRS agar. The mMRS broth and respective agar were subsequently autoclaved for 15 min at 121°C. *E. coli* was grown in brain heart infusion broth or on 2% brain heart infusion agar. The pH had been adjusted to pH 4.5 or 7.0 prior to sterilization for 15 min at 121°C. *S. cerevisiae* was grown on malt extract broth or 2% agar (Oxoid, Basingstoke, United Kingdom). The pH had been adjusted to 4.5 or 7.0 prior to sterilization for 20 min at 115°C. Precultures of all strains were serially diluted in growth medium and incubated overnight at 30°C. Slightly turbid tubes with approximately 1×10^6 to 5×10^7 exponentially growing cells per ml were diluted 10-fold in medium (with or without nisin). The cell suspensions were put in sample bags and stored for up to 30 min on ice until the start of the inactivation experiments.

UHP and nisin treatments. The following treatments were tested to establish whether synergy between nisin and UHP occurred during and/or after the UHP: (i) nisin at atmospheric pressure, i.e., 0.1 MPa (nisin control); (ii) UHP without nisin (UHP or control); (iii) nisin during UHP treatment; (iv) nisin after UHP treatment in recovery agar; (v) nisin during and after UHP. Cells were treated in an isostatic high-pressure 2.2-liter vessel (National Forge, St. Niklaas, Belgium) at 10 to 40°C at pH 4.5 respectively 7.0. Compression was set at 1 to 3 min allowing minimal adiabatic heating. Ramp rates varied from 0.6 to 2.0 MPa $\cdot$ s⁻¹. In a later stage, a Foodlab 900 multivessel (Stansted Fluid Inc., Stansted, United Kingdom) was obtained. The vessel volume was 30 ml, and it allowed better temperature and rapid (de)compression control. Ramp rates were set at 2 MPa $\cdot$ s⁻¹ for compression and 30 MPa $\cdot$ s⁻¹ for decompression. Test pressures were 100 to 300 MPa for *L. plantarum* and 150 to 200 MPa for *E. coli* and *S. cerevisiae*. After UHP or mock treatment, the samples were immediately put on ice and stored for up to 2 h before serial dilution and enumeration on recovery agar for each treatment corresponding to pH 4.5 or 7.0. Samples were plated on two recovery agars containing either no nisin or a corresponding concentration of Nisaplin (0.1, 0.5, 1, 2, or 5 μ g of nisin per ml). Colonies were counted after 5 days of incubation at 30°C. Carryover from nisin treatment did not interfere with correct enumeration. The dilution of treatment samples in ice-cold phosphate-buffered saline gave a 10-fold reduction of nisin carryover. The volume of the agar addition during poor plating led to an additional dilution ($\pm 15\times$) of nisin carryover in the recovery medium. The detection limit was 10 CFU/ml. The effects of UHP combined with nisin at a given temperature were considered synergistic if the net log reduction [$-\log(N_t/N_0)_{\text{UHP} \times \text{nisin}(T)}$] was >0, additive if ± 0 , and antagonistic if <0:

$$-\log(N_t/N_0)_{\text{total}} = -\log(N_t/N_0)_{\text{UHP}(T)} - \log(N_t/N_0)_{\text{nisin}(T)} - \log(N_t/N_0)_{\text{UHP} \times \text{nisin}(T)}$$

$$-\log(N_t/N_0)_{\text{UHP} \times \text{nisin}(T)} = -\log(N_t/N_0)_{\text{total}} + \log(N_t/N_0)_{\text{UHP}(T)} + \log(N_t/N_0)_{\text{nisin}(T)}$$

where N_t is the number of survivors after treatment, N_0 is the number of cells before treatment, and T is temperature.

Phospholipid analyses. *L. plantarum* was precultured in chemically defined medium as described previously (27). Cells had been precultured in a T-pH-NaCl concentration matrix of conditions (T 's of 10, 30, and 40°C; pHs of 4.0, 5.0, 6.0, and 8.0; NaCl concentrations of 1, 3, and 5%). Cells were harvested in exponential phase prior to any significant drop in pH in the culture (33) or under pHstat conditions as described previously (37). Cells harvested from the pHstat conditions were directly frozen in liquid nitrogen and stored at -80°C as described previously (37) until phospholipid analyses or nisin or UHP susceptibility testing.

TABLE 2. Effect of nisin and temperature on log reduction of *L. plantarum* colonies during 10-min control or mock treatment and/or recovery

Nisin concn (μ g/ml)	T (°C)	-Log (N _t /N ₀) _{nisin} at 0.1 MPa		
		10 min	Recovery	Continuous
0.1	11	0.4	1.4	0.4
	25	0.3	0.3	0.6
	35	0.3	0.4	0.5
0.5	8	-0.1	1.1	3.2
	10	0.4	3.5	3.6
	25	0.4	2.4	2.4
	35	0.8	0.6	0.9

TABLE 3. Effects of 10 min of pressurization, the presence of nisin during recovery, temperature, and pH on log reduction of *L. plantarum* and *E. coli* colonies

Microorganism (nisin concn)	Pressure (MPa)	-Log (N_t/N_0)							
		pH 4.5				pH 7.0			
		10°C	25°C	35°C	40°C	10°C	25°C	35°C	40°C
<i>L. plantarum</i> (0.5 µg/ml)	0.1	1.6	1.2	0.6		3.5	2.3	0.6	
	100		0.8	0.4		1.5	1.0		
	150	3.4	1.3	0.6		3.4	1.1	1.2	
	200	4.9	3.6			>6.7	1.4		
	250		4.6					4.9	
	300		5.3				>6.3		
<i>E. coli</i> (5 µg/ml)	0.1	0.3			0.1	0.1			0.1
	150	2.2			0.2	4.1			0.4
	200	>6.2			>6.2	4.9			0.5

Lipid fatty acids were extracted and analyzed by using a modification of the MIDI microbial identification system described previously (28). Phospholipids were extracted by the Bligh-Dyer method modified by Kates (3, 22), followed by thin-layer chromatography and phospholipid staining (10).

RESULTS

The effects of the parameters of pressure (0.1 to 300 MPa), temperature (5 to 40°C), pH (4.5 and 7.0), and nisin level (0 to 5 µg/ml) on three representatives of classes of microorganisms were assessed. The main focus was on the model gram-positive spoilage bacterium *L. plantarum*. Matrices of experiments were executed to investigate specific combined effects. The tables and figures below present selections of the results to demonstrate the trends. The treatment effects were calculated from the enumeration results as a log reduction factor ($\log N_t/N_0$). The effect of UHP-nisin at a given temperature was considered synergistic if the net log reduction [$-\log (N_t/N_0)_{\text{UHP} \times \text{nisin}}(T)$] was >0, additive if this reduction was ± 0 , and antagonistic if this reduction was <0;

UHP, temperature, and pH. Table 1 summarizes the effects of pressure treatment, temperature, and pH without nisin on *L. plantarum*, *E. coli*, and *S. cerevisiae*. *S. cerevisiae* was the most sensitive of the three to pressure. *E. coli* was more sensitive than *L. plantarum* to pressure at pH 4.5. Pressure treatment at reduced temperature or pH 4.5 was more effective than pressure treatment at ambient temperature or pH 7.0.

Effect of nisin without pressure. Nisin (1 to 5 µg/ml) in the absence of pressure was not effective against *S. cerevisiae* or *E.*

coli. The log reduction [$-\log (N_t/N_0)_{\text{nisin}}$] was 0 to 0.4. However, nisin without pressure was effective against *L. plantarum*. The effects of nisin and temperature during the control or mock pressure treatment and/or under recovery conditions against *L. plantarum* are summarized in Table 2.

Effect of nisin and pressure. The presence of nisin during recovery enhanced the efficacy of a UHP treatment against *L. plantarum* and *E. coli*. Pressurization at reduced temperatures at pH 4.5 or 7.0 increased the efficacy of nisin during recovery (Table 3). Nisin was most effective during pressure treatment at pH 7.0. The efficacy increased when nisin was also present in the recovery medium. The net synergistic effects of UHP and nisin at different temperatures against *L. plantarum* and *E. coli* are summarized in Tables 4 and 5, respectively. The inhibitory effect of nisin and UHP was much lower when the experiments were carried out at pH 4.5 (results not shown). The reason for this can most likely be found in the fact that the inoculum was grown at pH 7.0 and treated at pH 4.5. The acid down-shock presumably reduced the transmembrane potential, reducing the efficacy of nisin. This phenomenon may also explain the fact that, generally, stationary-phase cells of *L. plantarum* were more resistant to nisin as well as to pressure than cells in other phases of growth (results not shown) (36). Synergistic effects were clearly demonstrated only at higher pressures (300 MPa) at ambient temperatures. At pH 7, the presence of nisin at a level of 2 µg/ml only when pressure was present was insufficient to exert a synergistic effect on stationary cells.

Surprisingly, low levels of nisin (5 µg/ml) and pressure

TABLE 4. Net effect of nisin during and/or after pressurization for 10 min and of temperature on log reduction of *L. plantarum* at pH 7.0

Nisin concn (µg/ml)	T (°C)	-Log (N_t/N_0) _{UHP × nisin}					
		150 MPa ^a			200 MPa ^b		
		UHP ^c	Recovery ^c	Continuous	UHP ^c	Recovery	Continuous
0.1	11	0.8	-0.4	-0.2	2.5	0.1	>3.0
	25	-0.2	-0.2	-0.5	0.1	0	0.9
	35	0.4	0.0	0.3			
0.5	8	3.7	1.4	1.2	2.5	-1.4	-0.8
	10	4.8	-0.9	2.3	>3.3	-1.3	-0.2
	25	0.4	-1.3	0.0	2.6	-0.9	2.5
	35	0.8	0.6	1.7			

^a -Log (N_t/N_0)_{150 MPa} = 1.1 (8°C), 0.8 (11°C), 0 (25°C), and 0 (35°C).

^b -Log (N_t/N_0)_{200 MPa} = 2.9 (8°C), 3.3 (11°C), 0.1 (25°C), and 0 (35°C).

^c UHP presence of nisin only during UHP treatment; recovery, presence of nisin only after UHP treatment in the recovery agar; continuous, presence of nisin during UHP treatment and in the recovery medium.

TABLE 5. Net effect of 10 min of pressurization, nisin during UHP and/or after recovery, and temperature on log reduction of *E. coli* at pH 7.0

Nisin concn ($\mu\text{g/ml}$)	<i>T</i> ($^{\circ}\text{C}$)	$-\log N_t/N_0)_{\text{UHP} \times \text{nisin}}$					
		150 MPa ^a			200 MPa ^b		
		UHP ^c	Recovery ^c	Continuous ^c	UHP	Recovery	Continuous
1	10	2.9	1.6	2.9	2.0	0.4	3.1
1	40	0.2	0	0.1	-0.1	0	-0.1
2	8	1.7	0.4	1.8	5.1	-0.3	4.8
2	10	3.0	2.1	>3.8	>3.0	0.4	>2.7
2	25	0.9	0.1	0.6	1.5	0.3	1.6
2	40	0.6	0	0.8	0.3	0.2	0.5

^a $-\log N_t/N_0)_{150 \text{ MPa}} = 0.3$ (8°C), 2.0 (10°C), and 0.0 (25°C).

^b $-\log N_t/N_0)_{200 \text{ MPa}} = 0.2$ (8°C), 2.3 (10°C), and 0.0 (25°C).

^c UHP, presence of nisin only during UHP treatment; recovery, presence of nisin only after UHP treatment in the recovery agar; continuous presence of nisin during UHP treatment and in the recovery of medium.

showed some efficacy against *S. cerevisiae*. The presence of nisin during recovery after UHP at 150 MPa had no significant effect at 10 and 40°C . At 200 MPa and 40°C , the log reduction increased 1.6. The presence of nisin during UHP was slightly effective at both temperatures. The log reduction increased 1.5 at 10°C and 1.0 at 40°C . The presence of nisin during and after UHP provided an extra log reduction of 2.0 at both temperatures. One should bear in mind that a pressure treatment of 200 MPa without nisin is sufficient to metabolically inactivate *S. cerevisiae*.

MB21 and histatin 5. MB21, the synthetic design antimicrobial peptide, and the truncated histatin 5 derivative were available only in very limited quantities. The effects of these peptides after pressure treatment to assess any sublethal injury could not be addressed. MB21 (50 $\mu\text{g/ml}$) gave more kill (extra reduction of 2.1) of *L. plantarum* and *E. coli* at reduced temperatures (5 and 10°C) than at 25°C during a 10-min pressure treatment of 200 MPa in the new Stansted equipment. No effects of MB21 (1 to 10 $\mu\text{g/ml}$) against *S. cerevisiae* were observed. Histatin 5 (test level, 250 $\mu\text{g/ml}$) gave more kill (extra log reduction of 1.6) of *L. plantarum* at 5°C than at 25°C during a 200-MPa pressure treatment. No effects at this test level were observed against *E. coli* or *S. cerevisiae*.

Influence of preculture conditions and membrane composition. Preculture temperature had a profound effect on membrane fluidity of *L. plantarum*. The unsaturated/saturated fatty acid ratio changed from 2.08 to 2.2 at 10°C to 0.77 to 1.2 at 30°C and 0.43 to 0.60 at 40°C . The influences of culture temperature on fatty acid composition of the membrane and pressure susceptibility of *L. plantarum* are shown in Tables 6 and 7. An increased membrane fatty acid unsaturation protected against pressure inactivation.

Growth at different pH values hardly affected membrane fluidity. The ratio of unsaturated/saturated fatty acids at 30°C fluctuated between 0.9 and 1.6 at pH values between 4 and 8. At the two extreme pH values, 4 and 8, the amount of lactobacillic acid (9,10-*cis*-methylene octadecanoic acid [$\text{C}_{19:0}$ cyclo]) went up at the expense of octadecenoic acid ($\text{C}_{18:1}$). Membrane fluidity did not provide an explanation for the increased susceptibility of cells grown at pH 4.0 to inactivation by nisin. Exposure to 5 μg of nisin per ml for 5 min at pH 7.0 gave 5-log reductions at 30°C . Exponential cells grown at a higher pH, 5.0 or 6.0, were more nisin resistant (2.5-log reduction). Especially, cells grown at pH 8.0 were the most resistant (1.0-log reduction). Membrane fluidity seemed to play a role in the temperature-dependent effect of nisin, confirming the findings of Abee et al. (1). At 10°C , no inactivation by nisin at pH 7.0

was observed for cells grown at 10, 30, or 40°C . Cells grown at 10°C and treated at 40°C were the most susceptible. Nisin at 5 $\mu\text{g/ml}$ gave complete inactivation at 10°C , whereas a concentration of 20 $\mu\text{g/ml}$ was required to eliminate cells grown at 30 and 40°C .

Phospholipid head group analyses could be performed only for *L. plantarum* cells grown at sufficiently high densities in 8-liter pH-controlled batch fermentors (37). The correlation ($r = 0.65$) between DPG content and susceptibility to UHP is shown in Fig. 1. The correlation can be improved if one compensates for the systematic difference (± 2 -log reductions) between experiments on different days. The pressure treatments were carried out in the less-controlled National Forge equipment. Differences in ambient temperature and/or actual pressure profile may contribute to the systematic deviation. No correlation was observed between susceptibility to nisin and DPG content. Some indication of a correlation between specific phospholipid head groups and susceptibility to nisin was obtained for lysylphosphatidylglycerol. A higher lysylphosphatidylglycerol content in the cytoplasm membrane seemed to increase the susceptibility to nisin (Fig. 2).

TABLE 6. Effect of temperature on membrane fatty acid composition of *L. plantarum*

Fatty acid	% Fatty acid at:		
	10°C	30°C	40°C
16:1	11	18	2
16:0	15	30	56
17:0 cyclopropane	<1	Trace	Trace
18:1	56	34	11
18:0	10	4	13
19:0 cyclopropane	6	8	16

TABLE 7. Susceptibility to pressure of *L. plantarum* precultured at 10, 30, and 40°C , at pH 6, in McFeeters' medium

Pressure (MPa)	$-\log N_t/N_0)_{\text{UHP}}$		
	10°C	30°C	40°C
300	0.4	3.4	3.9
350	2.3	4.4	5.3
400	3.1	>6.1	>6.2

FIG. 1. Observed correlation between DPG content of the cytoplasm membrane of *L. plantarum* and susceptibility to pressure (7 min at 350 MPa) at ambient temperature. Different symbols represent different pressure runs on separate days.

DISCUSSION

This study tested the hypothesis that an assumed reduction in membrane fluidity of *L. plantarum* from lowering the temperature and/or from nisin addition will increase the efficacy of a UHP treatment. The second aim was to test whether such synergistic effects could also be extended to the gram-negative model organism *E. coli* or to the eukaryotic model organism *S. cerevisiae*. Our results clearly provide additional evidence that membrane fluidity may explain the synergistic effects of nisin, UHP, and temperature against microorganisms. Other factors like reduced pH and growth phase of the microbial target will also influence the efficacy of UHP. Most of the observed trends

FIG. 2. Observed correlation between lysylphosphatidylglycerol content of the cytoplasm membrane of *L. plantarum* and the efficacy of a 5-min nisin treatment at 30°C, pH 7.0, *, 0.1 μ g of nisin per ml; —, 0.5 μ g of nisin per ml; ▲, 2 μ g of nisin per ml; ■, 5 μ g of nisin per ml.

have been demonstrated before for other microorganisms. Similar findings have been reported for UHP and reduced pH by Pandya et al. (29), for UHP and reduced temperature (12), for UHP or nisin against stationary-phase cells (24, 26, 33, 36), and for UHP and nisin or pediocin against gram-negative and gram-positive microorganisms (13, 18–20) at ambient or higher temperatures. One can argue that only indirect evidence indicating that relative membrane fluidity plays a key role in the mode of action was obtained. Previous studies, however, which have already provided ample evidence for the importance of membrane fluidity (1, 25, 31, 33), have been complemented by unpublished findings about the effects of preculture conditions on membrane composition and process susceptibility. We did not address the effects on membrane-bound proteins like the F_0F_1 -ATPase since inactivation of the F_0F_1 -ATPase is not the direct cause of cell death (37). We have provided some additional evidence that membrane phospholipid head group composition plays a role in increased nisin (for lysylphosphatidylglycerol) and/or UHP (for DPG) susceptibility.

The most novel observation was that the efficacy of nisin and UHP was enhanced at lower temperatures. On the one hand, the enhanced effect of UHP is not surprising. At or near the growth temperature of microorganisms, the cytoplasm membrane is mostly in the liquid-crystalline state. The membranes of cells far below their growth temperature are in a semicrystalline gel state and are more rigid and UHP sensitive than those of cells closer to their growth temperature (28). UHP treatment was indeed more effective against *L. plantarum* at 5 to 10°C than at ambient or higher temperatures. For *E. coli*, the inactivation rate was at its lowest at 25°C. At reduced (5 to 10°C) and higher (40°C) temperatures, the inactivation rate increased. These results are in agreement with others (24, 25). On the other hand, nisin is known to be far less effective against rigid membranes due to the reduced temperature (1). The pore formation by nisin should be hindered by the increased rigidity due to UHP and/or reduced temperature. We postulated in the introduction that the bound nisin would already increase the susceptibility during UHP inactivation by binding to phospholipid head groups and local immobilization of the membrane. Final proof will be obtained with modified nisin molecules. Site-directed mutagenesis has led to nisin molecules that still have affinity for the phospholipid head groups but lack the ability to form pores (4).

It is also known that UHP and nisin act synergistically during recovery. Kalchayanand et al. claim that UHP causes sublethal injury (19–21). It is also suggested that UHP will facilitate the access of nisin to the cytoplasm membrane. UHP may structurally damage cell wall proteins of microorganisms in general and, more specifically, the outer membranes of gram-negative microorganisms (13, 30a). Reduction of temperature below 15°C reduces the pressure at which synergy with nisin can be observed. Hauben et al. and Kalchayanand et al. observed synergy only at ambient or higher temperatures at pressures above 180 to 210 MPa (13, 19–21). We observed synergy at pressures as low as 100 MPa when nisin was present during and after pressure treatment of *L. plantarum*. Synergy was observed at 150 MPa when nisin was present only during pressure treatment. At 200 MPa, synergy was observed only when nisin was present during recovery only. Elimination (defined here as >6 log reduction) was obtained for *L. plantarum* at 10°C with 0.5 μ g of nisin per ml at 150 MPa or 0.1 μ g of nisin per ml at 200 MPa. For *E. coli*, elimination was achieved at 10°C with 2 μ g of nisin per ml at 150 MPa and 1 μ g of nisin per ml at 200 MPa. The required levels of nisin that synergistically inactivate *E. coli* during an ambient pressure treatment are in the same range as those reported by others. For elimination of *S. cer-*

evisiae and possibly other vegetative spoilage fungi, nisin is not required since moderate pressures of 200 MPa give sufficient inactivation. Most of our results have been obtained with exponentially growing cells. Results with stationary-phase cells of *L. plantarum* do indicate that slightly higher levels of nisin and/or pressure will be required to achieve pasteurization.

Nisin has hardly any antimicrobial effect on yeast or filamentous fungi. Recent studies claim that nisin has antifungal properties if the cell wall is (partially) degraded or lacks protective proteins like the major yeast cell wall protein CWP2 (5, 8). The levels of nisin required to exert such an antifungal effect are, however, high ($>50 \mu\text{g/ml}$). Below these levels, sublethal membrane perturbation of cells with a weakened cell wall can be observed (8). In our study, lower levels of nisin did give some synergistic inactivation of *S. cerevisiae* with pressure. It is, however, unlikely that the inactivation by nisin is due to pore formation caused by a negative transmembrane potential. The phospholipid head group composition of yeast and mechanistic studies do not favor that mode of action (7, 11). We consider it more likely that bound nisin can already increase the susceptibility during UHP inactivation. Nisin may bind to phospholipid head groups and locally immobilize the cytoplasmic membrane once the cell wall, or outer membrane in the case of gram-negative microorganisms, has been permeabilized. The results with synthetic antimicrobial peptides were disappointing. The lantibiotic nisin was much more effective than the synthetically designed or truncated peptides. Only for *L. plantarum* was a synergistic enhancement observed for both histatin and MB21 with pressure treatment at reduced temperatures. The test levels of MB21 against yeast as suggested by other studies (8) were too low. MB21 or nisin has been reported to cause membrane perturbation of *S. cerevisiae* (8). The slightly increased propidium iodide uptake caused by MB21 and nisin was, however, no sign of lethal membrane perturbation, as subsequent cell sorting in a flow cytometer in unpublished follow-up work revealed (36a).

The design of effective combination preservation systems clearly depends on insight into the history of the microbial target, the mode of action of the process, and the likelihood of recovery. Understanding the role of the membrane and its proteins or its protective barrier, the cell wall, in the flexible defense of vegetative microorganisms will assist in the perfection of combination preservation.

ACKNOWLEDGMENTS

We gratefully acknowledge Mohan Bhakoo and Michael Chickendas for providing the synthetic peptides. We thank Nick Russell for training Johan Hellemons in phospholipid analysis. We also thank Alison Hayhurst for her technical contribution in studying the effect of pre-culture conditions of *L. plantarum* and its susceptibility to the effects of pressure and nisin. We thank Stanley Brul, Jan Smelt, and Leon Gorris for stimulating discussions and critically reading the manuscript. Jan Groeneweg is thanked for his technical assistance with the high-pressure equipment.

The AAIR Concerted Action PL920630 provided financial support.

REFERENCES

1. Abee, T., F. H. Rombouts, J. Hugenholtz, G. Guihard, and L. Letellier. 1994. Mode of action of nisin Z against *Listeria monocytogenes* Scott A grown at high and low temperatures. *Appl. Environ. Microbiol.* **60**:1962–1968.
2. Bhakoo, M. September 1996. International patent WO 96/28648.
3. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**:911–917.
4. Breukink, E., C. van Kraaij, A. van Dalen, R. A. Demel, R. J. Siezen, B. de Kruijff, and O. P. Kuipers. 1998. The orientation of nisin in membranes. *Biochemistry* **37**:8153–8162.
5. Brul, S., P. Coote, S. K. Dielbandhoosing, S. C. J. M. Oomes, W. M. Stam, G. Naaktgeboren, and M. Stratford. 1997. Natural composition for combating fungi. International patent WO 1997/16973.
6. Cheftel, J.-C. 1992. Effects of hydrostatic pressure on food constituents: an overview, p. 195–209. In C. Balny, R. Hayashi, K. Heremans, and P. Masson (ed.), *High pressure and biotechnology*, vol. 224. Colloques INSERM/John Libbey Eurotext Ltd., Montrouge, France.
7. Delves-Broughton, J. 1990. Nisin and its application as a food preservative. *J. Soc. Dairy Technol.* **43**:73–76.
8. Dielbandhoosing, S. K., H. Zhang, L. H. P. Caro, J. M. van der Vaart, F. M. Klis, C. T. Verrips, and S. Brul. 1998. Specific yeast cell wall proteins confer upon yeast cells resistance to nisin. *Appl. Environ. Microbiol.* **64**:4047–4052.
9. Driessen, A. J. M., H. W. van den Hooven, W. Kuiper, M. van de Kamp, H.-G. Sahl, R. N. H. Konings, and W. N. Konings. 1994. Mechanistic studies of lantibiotic-induced permeabilization of phospholipid vesicles. *Biochemistry* **34**:1606–1614.
10. Frischer, W., R. A. Laine, and M. Nakano. 1978. On the relationship between glycerophosphoglycerolipids and lipoteichoic acids in Gram-positive bacteria. II Structures of glycerophosphoglycerolipids. *Biochim. Biophys. Acta* **528**:298–308.
11. Greenberg, M. L., and J. M. Lopes. 1996. Genetic regulation of phospholipid biosynthesis in *Saccharomyces cerevisiae*. *Microbiol. Rev.* **60**:1–20.
12. Hashizume, C., K. Kimura, and R. Hayashi. 1995. Kinetic analysis of yeast inactivation by high pressure treatment at low temperatures. *Biosci. Biotechnol. Biochem.* **59**:1455–1458.
13. Hauben, K. J. A., E. Y. Wuytack, C. C. F. Soontjens, and C. W. Michiels. 1996. High-pressure transient sensitization of *Escherichia coli* to lysozyme and nisin by disruption of outer-membrane permeability. *J. Food Prot.* **59**:350–355.
14. Helmerhorst, E. J., W. Van't Hof, E. C. I. Veerman, I. Simoons-Smit, and A. V. N. Amerongen. 1997. Synthetic histatin analogues with broad-spectrum antimicrobial activity. *Biochem. J.* **326**:39–45.
15. Hurst, A. 1981. Nisin. *Adv. Appl. Microbiol.* **27**:85–123.
16. Hurst, A., and D. G. Hoover. 1993. Antimicrobials in food, p. 369–394. Marcel Dekker, Inc., New York, N.Y.
17. Juneja, V. K., and P. M. Davidson. 1992. Influence of altered fatty acid composition on the resistance of *Listeria monocytogenes* to antimicrobials. *J. Food Prot.* **55**:302–305.
18. Juneja, V. K., T. A. Foglia, and B. S. Marmer. 1998. Heat resistance and fatty acid composition of *L. monocytogenes*: effect of pH, acidulant, and growth temperature. *J. Food Prot.* **61**:683–687.
19. Kalchayanand, N., A. Sikes, C. P. Dunne, and B. Ray. 1994. Hydrostatic pressure and electroporation have increased bactericidal efficiency in combination with bacteriocins. *Appl. Environ. Microbiol.* **60**:4174–4177.
20. Kalchayanand, N., A. Sikes, C. P. Dunne, and B. Ray. 1998. Factors influencing death and injury of foodborne pathogens by hydrostatic pressure-pasteurization. *Food Microbiol.* **15**:207–214.
21. Kalchayanand, N., A. Sikes, C. P. Dunne, and B. Ray. 1998. Interaction of hydrostatic pressure, time and temperature of pressurization and pediocin AcH on inactivation of foodborne bacteria. *J. Food Prot.* **61**:425–431.
22. Kates, M. 1972. Lipid extraction, p. 351. In T. S. Work and E. Work (ed.), *Laboratory techniques in biochemistry and molecular biology*. Elsevier, Amsterdam, The Netherlands.
23. Knorr, D. 1995. Hydrostatic pressure treatment of food microbiology, p. 159–175. In G. W. Gould (ed.), *New methods of food preservation*. Blackie, Glasgow, United Kingdom.
24. Ludwig, H., C. Bieler, K. Hallbauer, and W. Scigalla. 1992. Inactivation of microorganisms by hydrostatic pressure, p. 25–32. In C. Balny, R. Hayashi, K. Heremans, and P. Masson (ed.), *High pressure and biotechnology*, vol. 224. Colloques SERM/John Libbey Eurotext Ltd., Montrouge, France.
25. Macdonald, A. G. 1992. Effects of high hydrostatic pressure on natural and artificial membranes, p. 67–75. In C. Balny, R. Hayashi, K. Heremans, and P. Masson (ed.), *High Pressure and Biotechnology Vol. 224. Colloque SERM/John Libbey Eurotext Ltd.*
26. Mackey, B. M., K. Forestiere, and N. Isaacs. 1995. Factors affecting the resistance of *Listeria monocytogenes* to high hydrostatic pressure. *Food Biotechnol.* **9**:1–11.
27. McFeeters, R. F., and K.-H. Chen. 1986. Utilization of electron acceptors for anaerobic mannitol metabolism by *Lactobacillus plantarum*. Compounds which serve as electron acceptors. *Food Microbiol.* **3**:73–81.
28. Olson, W. P., M. J. Groves, and M. E. Klegerman. 1990. Identifying bacterial contaminants in a pharmaceutical manufacturing facility by gas chromatographic fatty acid analysis. *Pharmaceut. Technol.* **1990**:32.
29. Pandya, Y., F. F. Jewett, and D. G. Hoover. 1995. Concurrent effects of high hydrostatic pressure, acidity and heat on the destruction and injury of yeasts. *J. Food Prot.* **58**:301–304.
30. Patterson, M. F., M. Quinn, R. Simpson, and A. Gilmour. 1995. Sensitivity of vegetative pathogens to high hydrostatic pressure treatment in phosphate-buffered saline and foods. *J. Food Prot.* **58**:524–529.
- 30a. Rommen, A., S. Brul, and C. T. Verrips. 1999. Submitted for publication.
31. Russell, N. J., R. I. Evans, P. F. ter Steeg, J. C. Hellemons, A. Verheul, and T. Abee. 1995. Membranes as target for stress adaptation. *Int. J. Food. Microbiol.* **28**:255–261.
32. Sale, J. H., G. W. Gould, and W. A. Hamilton. 1970. Inactivation of spores by hydrostatic pressure. *J. Gen. Microbiol.* **69**:323–334.

33. **Smelt, J. P. P. M., A. G. F. Rijke, and A. Hayhurst.** 1994. Possible mechanism of high pressure inactivation of microorganisms. *High Pressure Res.* **12**:199–203.
34. **Smelt, J. P. P. M.** 1998. Recent advances in the microbiology of high pressure processing. *Trends Food Sci. Technol.* **9**:152–158.
35. **Tsai, H., and L. A. Bobek.** 1998. Human salivary histatins: promising antifungal therapeutic agents. *Crit. Rev. Oral Biol. Med.* **9**:480–497.
36. **Ueckert, J., P. J. Coote, and P. F. ter Steeg.** 1998. Synergistic antibacterial action of nisin and magainin II amide combined with heat. *J. Appl. Microbiol.* **85**:487–494.
- 36a. **Ueckert, J.** Unpublished communication.
37. **Wouters, P. C., E. Glaasker, and J. P. P. M. Smelt.** 1998. Effects of high pressure on inactivation kinetics and events related to proton efflux in *Lactobacillus plantarum*. *Appl. Environ. Microbiol.* **64**:509–514.