

Controlling cabbage fermentations with nisin and nisin-resistant *Leuconostoc mesenteroides**

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A paired starter culture system for sauerkraut fermentation was proposed previously by our laboratory, consisting of a nisin-producing strain of Lactococcus lactis and a nisin-resistant strain of Leuconostoc mesenteroides. The objectives of this study were to determine nisin production and stability by a genetically marked culture of L. lactis NCK400 in brined cabbage fermentations and to study the effect of purified nisin on the natural microflora in brined cabbage. We found that NCK400 produced up to 700 IU ml⁻¹ of nisin in brined cabbage within 24 h, but by 72 h nisin activity was no longer detectable. The nisin produced was shown to have an effect on the microflora of the fermentation, however, resulting in a 40% reduction in the population of homofermentative lactic acid bacteria (LAB) at 33 days. We also found that the addition of purified nisin to brined cabbage (up to 12 000 IU ml⁻¹) resulted in a reduction in the cell counts of LAB, as compared to a non-treated cabbage brine, to the extent that fermentation by Gram-negative bacteria occurred. The addition of a nisin-resistant L. mesenteroides strain to the nisin-treated cabbage resulted in a heterolactic fermentation by the added culture, with no evidence of a homolactic fermentation for at least 20 days. We concluded that nisin produced in situ or added to brined cabbage can direct the progression of the species in the resultant fermentations, by preventing the growth of naturally present LAB.

Introduction

Commercial production and storage of sauerkraut in the United States is commonly carried out in bulk tanks of 45–150 ton (fresh

cabbage) capacity (Fleming et al. 1988). These fermentation tanks are used for the bulk storage of the fermented cabbage, while many European manufacturers package and pasteurize the fermented cabbage after 7–9 days of fermentation (Pederson and Albury 1969, Hammes 1990). Bulk storage of sauerkraut offers economic advantages, but can result in unpredictable concentrations of fermentation end-products, depending upon the time of storage and other factors (Fleming and McFeeters 1985, Fleming 1987). High concentrations of lactic acid (in excess

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of 200 mM) which result from long-term storage can make the final product too acidic for consumption without dilution. Dilution of the brine to reduce acidity results in loss of flavour and nutritional value and creates waste disposal problems. It is, therefore, desirable to maximize hexose utilization by heterofermentative organisms, which produce acetic acid and mannitol, before they are displaced by homolactic fermenters. Our research efforts have focused on the use of nisin to delay, but not eliminate, the homolactic (terminal) phase of the sauerkraut fermentation.

Previous work in our laboratory led to the proposal of a paired starter culture system for use in sauerkraut production (Harris et al. 1992). This system involved use of a nisin-resistant *Leuconostoc mesenteroides* strain and a nisin-producing *Lactococcus lactis* strain. Harris et al. (1992) demonstrated that sufficient nisin could be produced in mixed culture cabbage juice broth (CJB) fermentations to reduce the numbers of *Lactobacillus plantarum* below the level of detection and allow the nisin-resistant *L. mesenteroides* strain to reach a maximum cell density of approximately 10^9 cfu ml⁻¹. Breidt et al. (1993) developed *L. mesenteroides* strains resistant to high levels of nisin (up to 25 000 IU ml⁻¹) for use in nisin-treated cabbage fermentations. In this report, we have investigated the use of nisin in cabbage fermentations, either produced by *L. lactis* or by the addition of purified nisin. Our goals were to determine the survival of *L. lactis* in cabbage fermentations and the ability of this strain to produce nisin in fermentations. We also investigated how nisin affects the indigenous microflora of cabbage fermentations, both with and without a nisin-resistant *L. mesenteroides* strain.

Materials and Methods

Strains

L. lactis NCK400-CE (LA152), NCK403-CE (LA167), and *L. mesenteroides* LA113M3-CE (LA189) all contain pGK12 (Kok et al. 1984), which confers erythromycin and chloramphenicol resistance on the host cells (transformed as described below). NCK400 and NCK403 were previously described

by Harris et al. (1992); these are isogenic strains, with the exception that NCK400 produces nisin and NCK403 lacks that ability. The nisin-resistant *L. mesenteroides* strain LA113M3 was characterized by Breidt et al. (1993). *L. lactis* subsp. *cremoris* LA120 (ATCC 14365) was used as the indicator strain for nisin bioassays.

Media preparation and fermentation procedures

Lactobacilli MRS broth, M17 broth, plate count agar (PCA), and MacConkey agar (all from Difco Laboratories, Detroit, MI, USA) were prepared according to the supplier's instructions. All chemicals, unless otherwise noted, were obtained from Sigma Chemical Company (St Louis, MO, USA). Glucose was added to M17 medium at 1% (GM17). Agar (Difco) was added to MRS and GM17 broth at 1.5% for plating on solid medium. Chloramphenicol was added to MRS or GM17 agar as noted below at 6 µg ml⁻¹. LBS agar (BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) was prepared according to instructions. Cabbage used for all experiments was Cecile cultivar from commercial sources. CJB was prepared by the method of Kyung and Fleming (1994). Cabbage fermentations were prepared by brining shredded cabbage with a 55:45 (cabbage to brine) pack-out ratio in 375 ml glass jars (obtained from a local pickle company). Cabbage was shredded to an average thickness of 3 mm. The jars were capped with metal lids containing a rubber septum (Serum Stopper no. 7123-00, Rusch Inc., Duluth, GA, USA) to allow sampling of the brine over time with a syringe. Approximate equilibration of NaCl between the brine and the cabbage at 2% was determined to have occurred within 1 h of brining (data not shown). Purified nisin was obtained from Applied Microbiology, Inc. (Brooklyn, NY, USA, batch NP28), at a potency of 50×10^6 IU g⁻¹. Nisin stock solutions were prepared using 0.02 N HCl at a concentration of 1 mg ml⁻¹ and stored at -20°C. The initial brine concentration was 4.5 NaCl and the brine contained 0.0037 N HCl. The HCl was added to the brine because nisin was added to some fermentations, and nisin is stored in an HCl solution. This resulted in an initial cabbage brine pH of 5.7, instead of 6.0, which was typically found in brined cabbage prior to fermentation. This change in pH was not found to affect the course of the fermentation (see below). LA113M3-CE was inoculated into cabbage fermentations from an overnight culture that was grown in the presence of 5 µg ml⁻¹ of chloramphenicol and 5000 IU ml⁻¹ of nisin. The overnight culture was harvested by centrifugation and resuspended in an equal volume of saline prior to inoculation of cabbage brines. *L. lactis* strains NCK400-CE and

NCK403-CE were prepared as described for LA113M3-CE, except no nisin was used in the overnight culture. Cells, at 10^6 cfu ml⁻¹ equilibrated concentration, and/or nisin were added to the brine as noted in the text. Samples (2 ml total) for cell counts, nisin bioassay, and HPLC analysis of cabbage fermentations were removed at each time point as indicated below, with initial samples being taken after 1–2 h of brining to allow for equilibration.

Electroporation

NCK400, NCK403 and LA113M3 were transformed with pGK12 by electroporation (Cell-Porator with Voltage Booster, Gibco BRL Life Technologies, Inc., Grand Island, NY, USA) using a modification (Breidt and Fleming 1992) of the method of Luchansky et al. (1988). The resulting strains, NCK400-CE, NCK403-CE and LA113M3-CE were then checked to determine the stability of the plasmids. Plasmid stability was determined by serial passage of the plasmid-carrying strains under non-selective conditions as described (Breidt and Fleming 1992). The pGK12 was stably maintained in all strains for over 50 generations.

Assays

For the nisin bioassays, a modification (Breidt et al. 1993) of the method of Tramer and Fowler (1964) was used. HPLC separation of organic acids and sugars was carried out using an anion exchange column (Phenomenex Resex, 30 cm × 7.8 mm, Phenomenex, Rancho Palos Verdes, CA, USA) with a conductivity detector and a pulsed amperometric detector (Dionex CDM-II and PAD, respectively, Dionex Corporation, Sunnyvale, CA, USA) by the single injection method of McFeeters (1993). Cell counts from cabbage fermentations were determined with a spiral plater (Spiral Systems model D2, Spiral Systems Inc., Cincinnati, OH, USA). The lower limit of detection for enumerating bacterial cell populations was 10^3 cfu ml⁻¹. Total cell counts from cabbage fermentations were determined either on PCA or GM17 agar plates. *Enterobacteriaceae* counts were determined by plating on MacConkey agar. Gas production by heterolactic fermenting organisms was determined by using Durham tubes (tube number S32185, VWR Scientific, West Chester, PA) with MRS broth. Lactic acid bacterial counts were determined on LBS agar. MRS agar containing $6 \mu\text{g ml}^{-1}$ of chloramphenicol was used to enumerate LA113M3-CE in cabbage fermentations. NCK400-CM and NCK403-CM were enumerated on GM17 agar containing $6 \mu\text{g ml}^{-1}$ chloramphenicol. Data from each experiment represents the mean of two

or more identical treatments. The pH data were converted to antilog values prior to averaging. NaCl concentrations were determined by Fajans' method (Brown and Sallee 1963), as described by Etchells et al. (1964). pH values were determined using a Fisher pH meter (model 825MP, Fisher Scientific, Pittsburgh, PA, USA) and a Corning electrode (model 476570, Corning Glass Works, Corning, NY, USA).

Results

NCK403-CM [(Fig. 1(a), does not produce nisin] and NCK400-CM [Fig. 1(b), nisin producer]

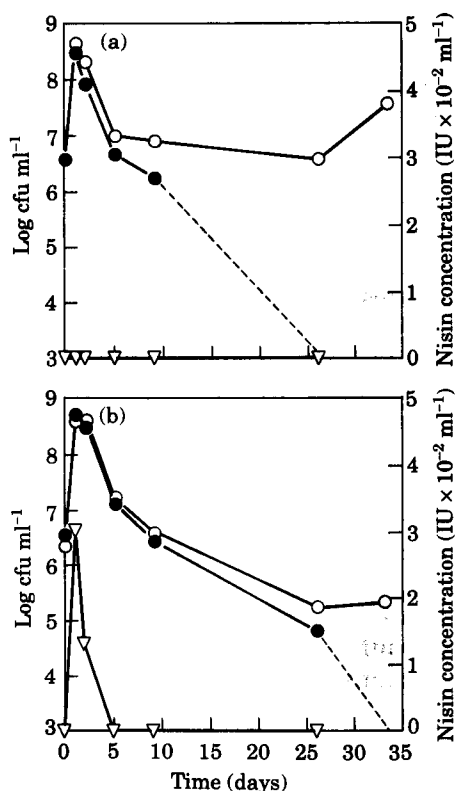


Figure 1. Fermentation of brined cabbage with nisin-producing and non-producing *Lactococcus lactis* strains. The brined cabbage was inoculated with either nisin-non-producing NCK403-CM (a) or with nisin-producing NCK400-CM (b). cfu ml⁻¹ were determined on GM 17 agar without (total) and with (NCK400-CM and NCK403-CM) chloramphenicol. The dotted lines in Fig. 1 represent estimated cell counts, i.e. below the limit of detection. ○, Total; ●, NCK403-CM; ▽, nisin.

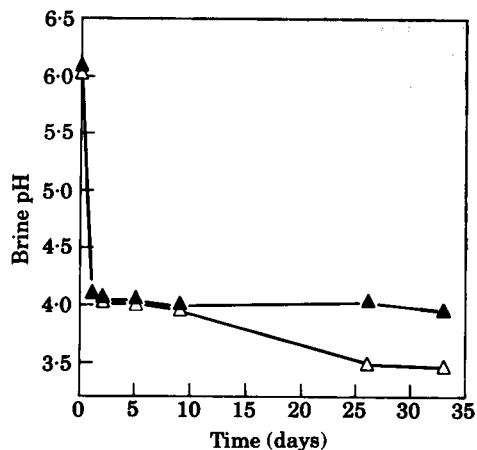


Figure 2. pH changes in brined cabbage inoculated with nisin-producing and non-producing *Lactococcus lactis* strains. ▲, NCK400-CM; △, NCK403-CM.

were inoculated into brined cabbage to determine the amount of nisin produced. In both fermentations the cell counts of the chloramphenicol-resistant *Lactococcus* strains approximated the total cell counts up to 5 days after inoculation. Approximately 300 IU ml⁻¹ of nisin produced by NCK400-CM was detectable in the brine after 1 day into the fermentation. However, no nisin was detectable in the fermentation brine after 5 days. This amount of nisin clearly affected the fermentation, as seen by the difference in pH (Fig. 2) between the fermentations with NCK400-CM and NCK403-CM after 10 days and the difference in the cell counts for the *L. lactis* strains after 5 days. The pH data indicated that a homolactic fermentation by the indigenous microflora did not occur within 60 days of fermentation with NCK400-CM [Fig. 1(b)] because the pH remained at 4.0 for up to 60 days (60 day data not shown), while the pH in the NCK403-CM [Fig. 1(a)] fermentations was below pH 3.5 at 26 days, and remained below 3.6 for at least 60 days. Fifty-six isolated colonies from the GM17 plates (without chloramphenicol selection) from the NCK400-CM and NCK403-CM fermentations at 33 days were inoculated into Durham tubes to determine the percentage of homolactic vs heterolactic fermenting strains. We found that 100% of the colonies from the

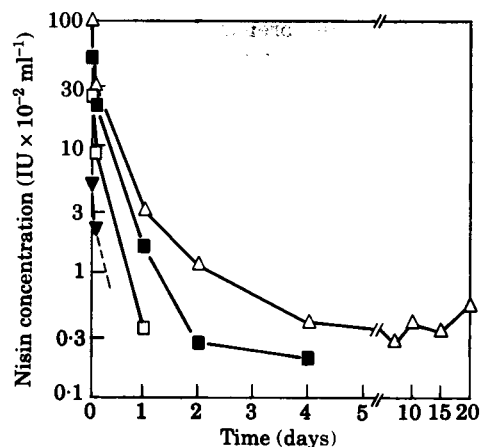


Figure 3. Depletion of purified nisin added to brined cabbage. The lines on the graph that terminate prior to 20 days indicate that the concentration of nisin fell below the limit of detection, 20 IU ml⁻¹, in the cabbage brine. Nisin added (IU × 10⁻²); △, 100; ■, 50; □, 25; ▼, 5.

fermentations with NCK403-CM were homolactic fermenters, while 60% of the colonies from the fermentations with NCK400-CM were homolactic fermenters and 40% were heterolactic fermenters.

To determine the fate of nisin in cabbage fermentations, we added purified nisin at different concentrations to brined cabbage (Fig. 3). By the first sampling time at 2 h after the addition of nisin, less than half of the nisin that was added to the brined cabbage was detected by bioassay. After 1 day, greater than 90% of the nisin was not detectable in all cases. Only with the addition of 10 000 IU ml⁻¹ was nisin detectable after 4 days, and with this treatment the residual concentration of nisin remained constant thereafter at around 50 IU ml⁻¹ for at least 20 days. We have observed similar results with purified nisin added to sterile cabbage juice (data not shown).

To determine the effect of nisin and a nisin-resistant *L. mesenteroides* strain on the indigenous microflora we carried out brined cabbage fermentations with the addition of 12 500 IU ml⁻¹ (250 µg ml⁻¹) of purified nisin. Four cabbage fermentation treatments were used, with the results for microbial growth shown in Fig. 4(a-d). Fig. 5 shows the pH

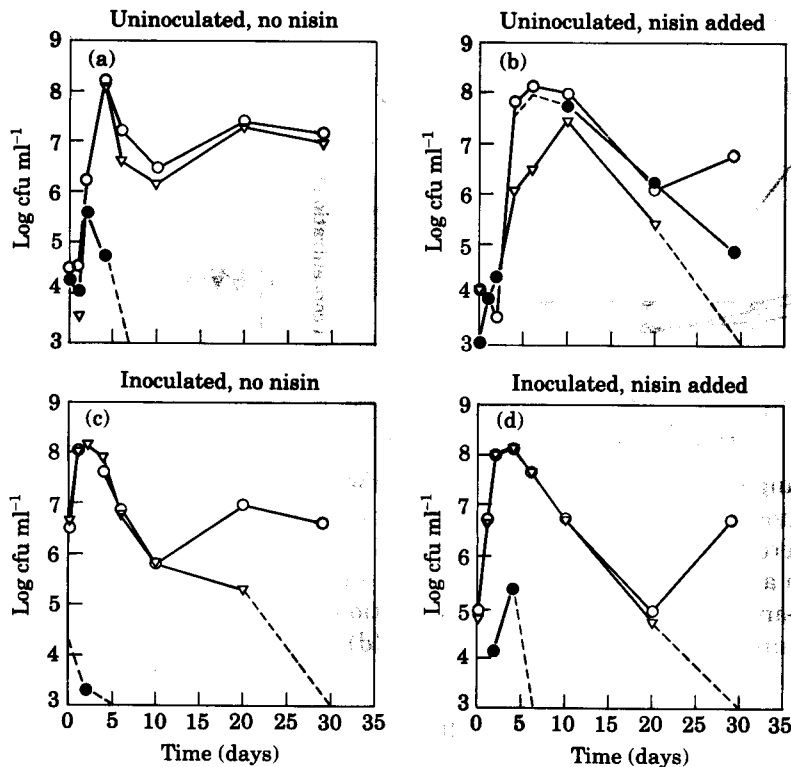


Figure 4. Effects of addition of nisin and nisin-resistant *Leuconostoc mesenteroides* on the microbiology of cabbage fermentations. The treatments included the following additions to the brined cabbage: (a) none (control), (b) nisin (12 000 IU ml⁻¹), (c) *L. mesenteroides* (LA113M3-CE), and (d) nisin + *L. mesenteroides*. The dotted lines in (a–d) represent estimated populations, i.e. outside of the countable range because of the dilution used, or were below the limits of detection. Total aerobic cell counts were determined on PCA. (a) and (b): ○, total aerobes; ●, *Enterobacteriaceae*; ▽, total lactic acid bacteria. (c) and (d); ○, total aerobes; ●, *Enterobacteriaceae*; ▽, LA 113M3-CE.

profile from the four fermentations. Fig. 6 shows the fate of nisin in the two fermentations to which nisin was added [fermentations (b) and (d) of Fig. 4].

The control fermentation [Fig. 4(a)] followed a pattern typical of sauerkraut fermentations (Pederson and Albury 1969). The initial heterolactic population of lactic acid bacteria (LAB; as determined by inoculation of Durham tubes) exceeded 10⁸ cfu ml⁻¹ within 5 days. This population was displaced by a homolactic-fermenting lactic acid bacteria (LAB) population by 20 days into the fermentation. The pH of these fermentations was below 3.5 by 20 days. With the addition of nisin to the fermentation [Fig. 4(b)], a significant change was observed in the *Enterobacteriaceae* popu-

lation, with these bacteria approximating the total aerobic cell count for the first 20 days of fermentation. The LAB counts never exceeded 5 × 10⁷ cfu ml⁻¹, and the brine pH of this fermentation remained above 4.8. This result was not surprising since nisin is primarily active against Gram-positive bacteria. The effect of adding the nisin-resistant LA113M3-CE culture to the cabbage brine is shown in [Fig. 4(c)]. These fermentations proceeded much the same as was observed in the control fermentations [Fig. 4(a)]. LA113M3-CE cell counts approximated the total cell counts for the first 10 days of the fermentation. The initial drop in brine pH of these fermentations occurred faster than the control fermentations. As the pH declined, so did the

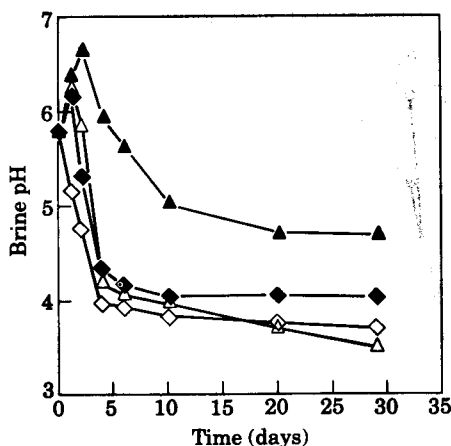


Figure 5. Changes in the brine pH of cabbage fermentations a–d. See Fig. 4 legend for comparable microbiological assays and Fig. 6 legend for nisin assays. Fermentation: Δ , control (a); \blacktriangle , nisin (b); \diamond , inoculated (c); \blacklozenge , inoculated + nisin (d).

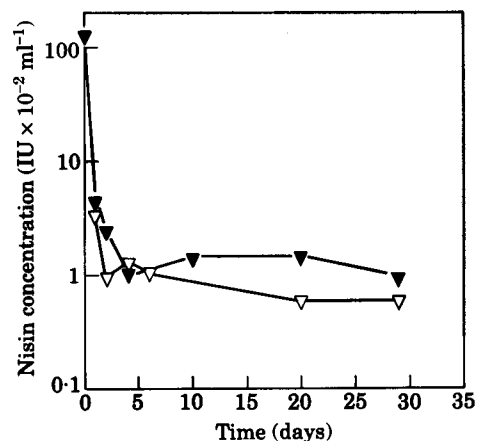


Figure 6. Nisin concentration in the brines of cabbage fermentations b and d. See Fig. 4 legend for comparable microbiological assays and Fig. 5 legend for pH assays. Fermentation: ∇ , nisin (b); \blacktriangledown , misin + LA113M3-CE (d).

Enterobacteriaceae cell counts. Fig. 4(d) shows the results of the addition of both nisin and the nisin-resistant *L. mesenteroides* LA113M3-CE. In these fermentations the LA113M3-CE cell counts approximated the total cfu ml⁻¹ for 20 days, dropping to 10⁵ cfu ml⁻¹ at that time. The *Enterobacteriaceae* cell counts were similar to the control fermentations. The pH of these fermentations remained above 4.0 for the entire 30 days of the experiment. The increase in total aerobic

cell counts at 30 days shown in both Fig. 4 (b) and (d) was due to a pink pigmented bacterium that appeared to be a Gram-positive rod microscopically. Subsequent attempts to cultivate this bacterium were not successful. Because the microflora from the fermentations shown in Fig. 4(b) were different than the microflora shown in Fig. 4(d), and the decrease in nisin was similar with both treatments, we suggest the possibility that the loss of nisin bioactivity may be due to the nisin

Table 1. HPLC data from cabbage fermentations a–d.

Fermentation ^a	Glucose	Fructose	Mannitol	Succinic	Lactic	Acetic
	Concentrations, mM ^b					
None	93.7	71.0	0.0	0.0	0.0	0.0
a1	72.7	11.3	26.4	3.1	49.1	23.5
a2	24.7	14.3	11.3	2.8	96.1	15.4
b1	29.2	19.0	0.0	11.8	28.4	9.5
b2	46.6	0.7	37.6	3.7	33.1	36.6
c1	32.1	2.4	39.5	1.7	63.1	30.3
c2	45.1	2.2	41.2	1.7	35.1	30.5
d1	52.2	6.3	38.4	1.6	32.9	29.5
d2	59.1	10.5	38.3	1.6	33.5	30.0

^aFermentations a–d were carried out in duplicate (1 and 2), as described in the text.

^bConcentrations of compounds in raw cabbage (no fermentation treatment) were adjusted (multiplied by 0.55) to allow a direct comparison with the brined samples. This correction is necessary because of the dilution due to brining.

binding to the cabbage. Further experiments will be needed to determine the cause of the decrease of nisin activity in the cabbage brine.

The concentrations of organic acids and sugars at 20 days for duplicate cabbage fermentations with treatments a–d, as described above, are shown in Table 1. a1 and a2 were control (no added culture or nisin) fermentations, b1 and b2 were fermentations carried out with the addition of 12 500 IU ml⁻¹ nisin, c1 and c2 were fermentations carried out with the addition of LA113M3-CE, and d1 and d2 were fermentations with both LA113M3-CE and nisin added to the fermentation brine. The fermentation results shown for b1 and b2 (predominantly Gram-negative bacteria grew) included unidentified end-products as determined by HPLC (data not shown). The variability in sugar utilization and acid production seen between duplicate fermentations for treatments a–c was not observed with treatment d. The fermentation products produced with treatment d are consistent with those expected for a heterolactic fermentation by LA113M3-CE (Breidt et al. 1993).

Discussion

We have shown that nisin can be used to control laboratory cabbage fermentations. The principal of this method, described originally by Harris et al. (1992), may be adopted for use with a variety of types of LAB fermentations. Daeschel et al. (1991) used a resistant *Leuconostoc oenos* in combination with nisin to control the malolactic fermentation of wine. Foegeding et al. (1992) used a bacteriocin-producing *Pediococcus acidilactici* strain to control the fermentation of dry sausage and prevent the growth of *Listeria monocytogenes*.

Currently the industrial fermentation of cabbage (as well as other vegetable brine fermentations) relies on the indigenous microflora. This confers a degree of variability on these fermentations, especially the cabbage fermentation, which proceeds with a complex series of LAB species. The addition of the nisin-producing *L. lactis* NCK400-CE

to brined cabbage resulted in a fermentation that showed no evidence of a homolactic fermentation after 30 days. That this effect was due to nisin was confirmed by the presence of homolactic LAB species in the fermentation inoculated with the nisin-non-producing isogenic *L. lactis* starter culture. This experiment demonstrated that nisin can have a long-term effect on the cabbage fermentation by influencing the initial LAB population, as nisin bioactivity was only observed during the first 5 days of the *L. lactis* NCK400-CE fermentation. The reason for the rapid loss of nisin in the cabbage brine, seen both with the addition of purified nisin and with the nisin produced by *L. lactis* NCK400-CE was not determined. We found, however, that with the initial addition of 10 000 IU ml⁻¹ of nisin or greater, 50 to 100 IU ml⁻¹ remained for up to 30 days. To insure the presence of nisin throughout the course of our nisin treated fermentations, we used 12 500 IU ml⁻¹.

The use of nisin and a nisin-resistant *L. mesenteroides* strain allowed us to produce a heterolactic fermentation in cabbage with the absence of competing LAB. Duplicate fermentations with this treatment resulted in typical heterolactic fermentation end-products at 20 days and did not show the variability observed with the inoculated fermentation in the absence of nisin, or in the control fermentations. Nisin has been used previously in the kimchi fermentation to delay the onset of a homolactic fermentation. Choi et al. (1990) used a relatively low concentration of nisin (100 IU ml⁻¹) to slow the rate of acid production by naturally occurring LAB in kimchi fermentation. Excessive acid production in kimchi results in a less desirable product, and nisin addition could extend the shelf-life of this product. One should be concerned, however, with the possible growth of undesirable, nisin-resistant bacteria such as Gram-negative bacteria if the natural LAB should be completely inhibited. Addition of a desirable, nisin-resistant lactic acid bacterium to assure a proper fermentation in the presence of nisin, as in the present study, could assure a proper fermentation. Preliminary results from our laboratory (Breidt,

Kyung and Fleming, unpublished data not shown) with Chinese cabbage prepared in the Korean kimchi style, but with 12 500 IU ml⁻¹ of nisin added, indicated that this product resulted in predominant growth by Gram-negative bacteria; results were similar to those shown in Fig. 4(b) for sauerkraut fermentation. The addition of a nisin-resistant *L. mesenteroides* to the nisin-treated Chinese cabbage resulted in a heterolactic fermentation, which gave a pH of 4.2 at 20 days, similar to results shown in Fig. 5 for fermentation treatment d.

We have not determined the optimum concentration of nisin to appropriately suppress naturally occurring LAB, including high acid-producing homofermentative species in sauerkraut or kimchi fermentations. This and other questions must be addressed before possible commercial application of nisin for control of vegetable fermentations.

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