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Novel Paired Starter Culture System for Sauerkraut, Consisting of a Nisin-Resistant *Leuconostoc mesenteroides* Strain and a Nisin-Producing *Lactococcus lactis* Strain

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Nisin-resistant *Leuconostoc mesenteroides* NCK293 and nisin-producing *Lactococcus lactis* subsp. *lactis* NCK401 were evaluated separately and in combination for growth and nisin production in a model sauerkraut fermentation. Strains were genetically marked and selectively enumerated by using antibiotic-containing media. The growth and survival of *L. mesenteroides* were similar in the presence and absence of *Lactococcus lactis* subsp. *lactis*. The growth of *Lactococcus lactis* subsp. *lactis* was not inhibited, although the maximum cell density was reduced and the population decline was more pronounced in the presence of *L. mesenteroides*. Nisin was detected within 24 h, and levels were relatively constant over the 12-day test period. The maximum cell populations and nisin levels achieved could be altered by changing the initial cell ratios of *L. mesenteroides* and *Lactococcus lactis* subsp. *lactis*. Isogenic nisin-producing and nisin-negative *Lactococcus lactis* subsp. *lactis* derivatives were used in combination with nisin-resistant *L. mesenteroides* to demonstrate that nisin levels produced in mixed culture were sufficient to retard the onset of the growth of nisin-sensitive, homofermentative *Lactobacillus plantarum* ATCC 14917.

Early predominance of heterofermentative lactic acid bacteria is thought to be essential in the production of high-quality sauerkraut (13). Heterofermentative *Leuconostoc mesenteroides* typically dominates the early fermentation since it is present in larger numbers (10, 11), initiates growth sooner, and has a shorter generation time than other lactic acid bacteria in cabbage juice (14). Although quick to predominate, *L. mesenteroides* rapidly dies off as the fermentation proceeds, as a result of its relative sensitivity to acidic conditions (9). In the final stage of the fermentation, homofermentative *Lactobacillus plantarum* forms large quantities of lactic acid from remaining carbohydrates, further lowering the pH. The correct sequence of organisms is essential in achieving a stable product with the typical flavor and aroma of sauerkraut.

Natural fermentations, such as that used in the production of sauerkraut, rely on microbial populations present on the raw material; therefore they are subject to wide variations in flavor and quality. The sequence of organisms is highly influenced by production conditions. Manufacturing practices currently used by the U.S. sauerkraut industry (dry salting, lack of temperature control, and bulk storage) have added to product inconsistency and have made it difficult to expand or even maintain market levels (3).

Use of a suitable *L. mesenteroides* starter culture to produce sauerkraut could potentially improve product quality and uniformity and might allow greater flexibility in processing parameters. Success of a sauerkraut starter culture will depend on its ability to dominate over and delay the onset of the indigenous, acid-tolerant microbiota. Rapid growth of starter culture under fermentation conditions is essential, and it is reasonable to assume that bacteriocin production would provide an organism with a selective advantage over competing but sensitive strains (1).

Initial investigations designed to isolate suitable bacteriocin-producing *L. mesenteroides* strains from fermenting vegetables were unsuccessful (5). Rather, nisin-producing (Nip^+) strains of *Lactococcus lactis* subsp. *lactis* were unexpectedly isolated from a commercial sauerkraut fermentation (6). In the current study, we designed a novel starter culture system by pairing a naturally nisin-resistant (Nis^r) *L. mesenteroides* strain, isolated from brined cabbage, with a nisin-producing *Lactococcus lactis* subsp. *lactis* strain isolated from sauerkraut.

Filter-sterilized cabbage juice broth (CJB), demonstrated previously to be a satisfactory experimental substitute for shredded cabbage (14), was chosen as our model system for evaluation of the paired starter culture. Nis^r *L. mesenteroides* NCK293 and Nip^+ *Lactococcus lactis* subsp. *lactis* NCK401, or its Nip^- derivative NCK402, were evaluated individually and in combination to demonstrate that nisin levels produced in the model system were sufficient to inhibit Nis^s *Lactobacillus plantarum* ATCC 14917.

MATERIALS AND METHODS

Bacteria and culture conditions. Nip^+ *Lactococcus lactis* subsp. *lactis* NCK400 was isolated from the early stages (day 5) of a commercial sauerkraut fermentation (6). Isolation of the streptomycin-resistant (1 mg/ml) and rifampin-resistant (100 μg/ml) mutant, NCK401, and subsequently its isogenic nisin-negative (Nip^-) derivative, NCK402, was described previously (6).

L. mesenteroides NCK293 was isolated from brined, shredded cabbage prepared in the laboratory (5). This strain is naturally resistant to more than 500 μg of vancomycin per

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ml (5), a trait typical of most *Leuconostoc* spp. (12). Other strains were obtained from the culture collection maintained by the Food Fermentation Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Raleigh, N.C. All cultures were stored at −70°C in MRS broth (Difco Laboratories, Detroit, Mich.) with 16% glycerol. Frozen stock cultures were streaked onto MRS agar; an isolated colony was transferred to broth and subcultured once before each experiment. Cultures were incubated at 30°C unless otherwise noted.

CJB was used as the transfer medium for fermentation experiments; MRS broth (*Lactobacillus* and *Leuconostoc* spp.) or M17 broth with 0.5% glucose (M17G; *Lactococcus* spp.) was used for MIC determinations. When MRS or M17G agar medium was required, 1.5% granulated agar was added to the broth. All media and ingredients except CJB were obtained from Difco.

Preparation of CJB. Preparation of CJB is detailed elsewhere (6). When required, NaCl was added before filter sterilization.

Model sauerkraut fermentations. Overnight cultures were centrifuged and resuspended in fresh CJB to an optical density at 600 nm of 0.5. Sterile CJB, tempered to 20°C, was inoculated (0.01%) with an appropriate dilution of the culture in CJB. Fermentation tubes were incubated in a circulating-water bath (Brinkmann Instruments, Inc., Westbury, N.Y.) maintained at 20°C. Samples were removed at intervals to determine plate counts, pH, and nisin titer. Each fermentation was performed in duplicate, and results were averaged.

The pH was measured by using a combination electrode (Orion Research, Inc., Cambridge, Mass.) and a pH meter (Fisher Scientific Co., Pittsburgh, Pa.).

Selective enumeration strategy. Bacterial strains were differentially enumerated at 30°C on M17G agar containing streptomycin (500 μg/ml) and rifampin (50 μg/ml) (for *Lactococcus lactis* subsp. *lactis*), at 25°C on M17G or sucrose agar (8) with vancomycin (5 μg/ml) (for *L. mesenteroides*), and at 37°C on *Lactobacillus* selective agar (LBS agar; BBL Microbiology Systems, Cockeysville, Md.) (for *Lactobacillus plantarum*). All antibiotics were purchased from Sigma Chemical Co., St. Louis, Mo. The number of CFU per milliliter was determined by spotting the surface of a prepoured agar plate with 25 μl of appropriate dilutions made in sterile saline (0.85% NaCl). Bacteria were incubated at the indicated temperature for 24 or 48 h.

Nisin assay. Nisin production was assayed as previously described (6). Culture supernatant (200 μl) was heated in microcentrifuge tubes (1.5 ml) in a boiling-water bath for 1 min and cooled rapidly on ice. Serial twofold dilutions of the heated supernatant were made in 0.02 N HCl, and 10 μl of each dilution was spotted onto fresh, duplicate indicator lawns of *Lactococcus lactis* subsp. *cremoris* ATCC 14365. Cultures were incubated overnight. The nisin titer was defined as the reciprocal of the highest dilution which completely inhibited the indicator lawn and was expressed as activity units (AU) per milliliter. A similar titer, determined for purified nisin (37×10^6 IU/g; Aplin and Barrett Ltd., Trowbridge, United Kingdom), was used to estimate the quantity of nisin produced in CJB.

MIC. The MIC of purified nisin was determined by using microtiter plates (Costar, Cambridge, Mass.). A stock solution of nisin (2 mg/ml) was prepared in 0.02 N HCl and stored at −20°C. Serial twofold dilutions of the stock solution were made in 0.02 N HCl, and 100 μl of each dilution was distributed per well of a microtiter plate. Overnight cultures of the test organism were diluted 100-fold in sterile saline solution (0.85% NaCl) and then a further 100-fold in double-strength MRS broth. Duplicate wells of each nisin concentration were inoculated with 100 μl of the diluted culture to give a final bacterial concentration of approximately 10^5 CFU/ml. The MIC was defined as the lowest nisin concentration which showed no evidence of growth after 48 h at 30°C.

TABLE 1. Growth of and nisin production by *Lactococcus lactis* subsp. *lactis* NCK401 in CJB[a,b]

NaCl (%)	Nisin production (AU/ml)	pH[c]	Cell population[d] (log CFU/ml)
0	800	4.2	9.08
1	700	4.2	9.20
2	700	4.2	9.04
3	200	5.4	8.50

[a] After incubation for 48 h at 20°C.
[b] Results are the average of duplicate determinations of replicate trials.
[c] Uninoculated CJB has a pH of 5.7.
[d] The initial inoculum was 10^6 CFU/ml.

RESULTS

Nisin production in CJB. *Lactococcus lactis* subsp. *lactis* NCK401 was evaluated for its ability to produce nisin in CJB at different NaCl concentrations (Table 1). Minor differences were observed in nisin titer and cell population, but no difference was observed in pH after 48 h of incubation at 0, 1, and 2% NaCl. At a concentration of 3% NaCl, the nisin titer and cell population were lower and the pH was only slightly below that of the uninoculated CJB (pH 5.7).

Selection of nisin-resistant *L. mesenteroides* strains. *L. mesenteroides* strains were evaluated for their sensitivity to nisin in order to identify a resistant culture that could grow in combination with a nisin-producing strain. The MIC of purified nisin varied widely from strain to strain. Although many *L. mesenteroides* strains were relatively sensitive to nisin, several (ATCC 10882, ATCC 13146, and NCK293) were naturally resistant to levels of greater than 10 μg/ml (Table 2). In comparison, Nip^+ *Lactococcus lactis* subsp. *lactis* NCK400 and its antibiotic-resistant derivative, NCK401, were immune to 10 μg of nisin per ml, whereas Nip^- NCK402 was sensitive to nisin concentrations of 2 μg/ml. *Lactococcus lactis* subsp. *cremoris* ATCC 14365, used as an indicator organism to assay nisin levels in CJB, was sensitive to approximately 0.08 μg of nisin per ml. Nis^r *L. mesenteroides* NCK293 was chosen for the paired starter culture because of its ability to grow well in CJB (strain C-1 [5]).

Compatibility of paired starter culture. *L. mesenteroides* NCK293 and *Lactococcus lactis* subsp. *lactis* NCK401 were grown alone and in combination at 20°C in sterile CJB containing 2% NaCl. Both strains were inoculated at a level of 10^5 to 10^6 CFU/ml. *L. mesenteroides* NCK293 (Str^s Rif^s Van^r) and *Lactococcus lactis* subsp. *lactis* NCK401 (Str^r Rif^r Van^r) and *Lactococcus lactis* subsp. *lactis* NCK401 (Str^r Rif^r Van^s) were differentially enumerated on M17G agar with the appropriate antibiotics. The growth and survival of *L. mesenteroides* were virtually the same in the presence and absence of *Lactococcus lactis* subsp. *lactis* over the 7-day trial period (Fig. 1). The decrease in pH during growth was similarly unaffected in the mixed culture (Fig. 1). The pH rapidly dropped from 5.7 to 4.5 within the first 24 h and then decreased more slowly to pH 3.8 within the next 24 h.

TABLE 2. MICs of purified nisin

Strain[a]	MIC of nisin (μg/ml)[b]
Lactococcus lactis subsp. *lactis*	
NCK400 (Nis$^+$)	>10
NCK401 (Nis$^+$ Str^r Rif^r)	>10
NCK402 (Nis$^-$ Str^r Rif^r)	2
Lactococcus lactis subsp. *cremoris*	
ATCC 14365 (Nis^s)	0.8
Leuconostoc mesenteroides	
ATCC 8293	0.6
ATCC 10830	1
ATCC 10880	0.3
ATCC 10882	>10
ATCC 13146	>10
ATCC 29258	0.2
HETA	0.7
NCK293	>10
3B	10
3C	10
A	1
A1A4a	2
B1M1b	3
C33	1
Lactobacillus plantarum	
ATCC 14917	0.9
NCDO 343	0.9
NCDO 354	0.5
NCDO 16	1
NCDO 340	>10
LA23	4
NC-8	5

[a] Abbreviations: Nis$^+$, nisin producing; Str^r, streptomycin resistant (1 mg/ml); Rif^r, rifampin resistant (100 μg/ml); Nis$^-$, nisin negative; Nis^s, nisin sensitive.

[b] Results are the average of duplicate determinations of one or more trials.

Growth of *Lactococcus lactis* subsp. *lactis* was not greatly inhibited, although the maximum cell density was slightly reduced and cell death was much more pronounced in the presence of *L. mesenteroides*. The decrease in cell numbers accelerated after 24 h and was attributable to lower pH levels achieved in the presence of *L. mesenteroides* (pH 3.8 versus pH 4.0).

In mixed culture, 100 to 200 AU of nisin per ml was observed after 24 h (data not shown). This level was estimated to be equivalent to 2 to 4 μg/ml on the basis of the specific activity determined for purified nisin. Higher nisin levels (500 to 700 AU/ml) were achieved when *L. mesenteroides* was absent (Table 1). Titers were constant for the 7-day experimental period.

Sensitivity of *Lactobacillus plantarum* to nisin. Although nisin was detected within 24 h in mixed culture, it was unclear whether these quantities were sufficient to inhibit a sensitive strain. MICs of purified nisin in MRS broth were determined for seven *Lactobacillus plantarum* strains (Table 2). These strains varied widely in sensitivity to nisin. *Lactobacillus plantarum* ATCC 14917 was selected to challenge the mixed-culture fermentation because it was moderately sensitive to nisin (MIC, 0.9 μg/ml) and was originally isolated from fermented cabbage.

Selective enumeration strategy. A selective enumeration strategy was developed so that the growth of each organism could be monitored in mixed culture. *Lactobacillus plantarum* was differentially enumerated on LBS agar incubated at 37°C. Incubation at 37°C was necessary since *L. mesenteroides* grows on LBS agar at 30°C but not at 37°C. *Lactococcus lactis* subsp. *lactis* was incapable of forming colonies on LBS agar at any temperature. Both Nis$^+$ and Nis$^-$ *Lactococcus lactis* subsp. *lactis* were resistant to streptomycin and rifampin and were enumerated on M17G agar containing these antibiotics. *L. mesenteroides* could be separated from *Lactococcus lactis* subsp. *lactis* on the basis of vancomycin resistance; however, *Lactobacillus plantarum* was resistant to high levels of vancomycin (>500 μg/ml). Therefore, *L. mesenteroides* was enumerated on sucrose agar with vancomycin, incubated at 20°C. *L. mesenteroides* grew rapidly on sucrose agar, giving rise within 18 h to large, viscous colonies that were easily distinguished from the pinpoint *Lactobacillus plantarum* colonies which formed after several days of incubation.

Mixed-culture fermentation. *Lactobacillus plantarum* ATCC 14917 was inoculated at approximately 10^2 CFU/ml

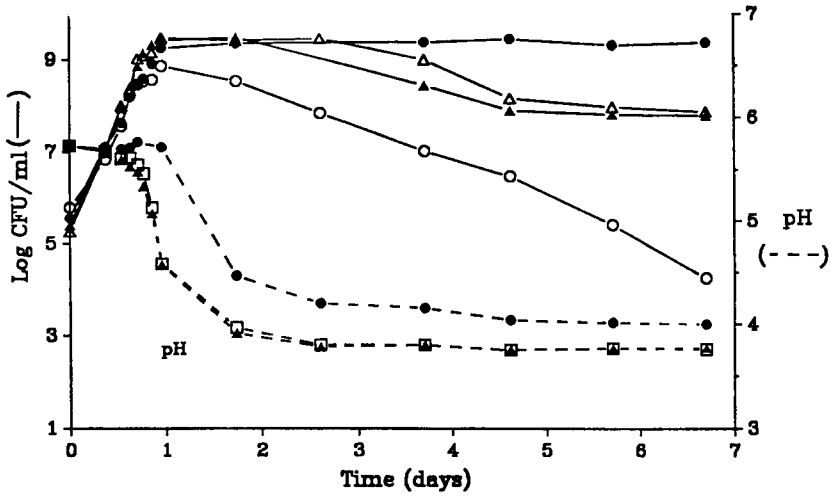

FIG. 1. Growth of pure and mixed cultures in CJB. *L. mesenteroides* NCK293 (▲, △) and *Lactococcus lactis* subsp. *lactis* NCK401 (●, ○), alone (solid symbols) and in combination (open symbols). Solid lines denote growth; broken lines denote pH. The mixed-culture pH is also shown (□).

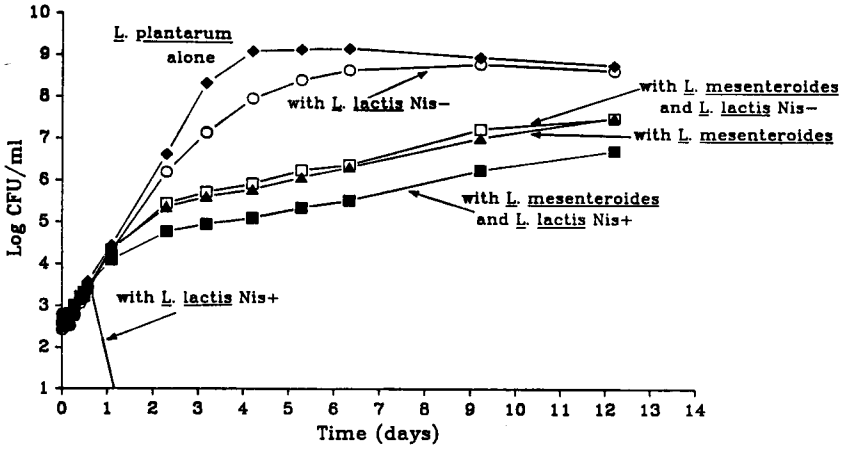

FIG. 2. Growth of *Lactobacillus plantarum* in a model sauerkraut fermentation. Growth of Nis^s *Lactobacillus plantarum* ATCC 14917 in pure culture and mixed culture with Nis^r *L. mesenteroides* NCK293, Nip$^+$ *Lactococcus lactis* subsp. *lactis* NCK401, or Nip$^-$ *Lactococcus lactis* supsp. *lactis* NCK402 in a model sauerkraut fermentation. Initial inoculum levels were 4×10^5 CFU/ml (*Lactococcus lactis* subsp. *lactis*), 2×10^3 CFU/ml (*L. mesenteroides*), and 3×10^2 CFU/ml (*Lactobacillus plantarum*).

alone or with one or both of the starter culture strains. To distinguish the effects of non-nisin inhibition, an isogenic, nisin-negative derivative of *Lactococcus lactis* subsp. *lactis* NCK401, NCK402, was also incorporated into experiments. The growth rates of Nis$^+$ NCK401 and Nis$^-$ NCK402, compared in CJB, did not differ (data not shown). The growth of both *Lactococcus lactis* subsp. *lactis* strains was quantitated from mixed cultures by plating on M17G agar with streptomycin and rifampin.

The growth of *Lactobacillus plantarum* in CJB at 20°C was slower than that of either *Lactococcus lactis* subsp. *lactis* or *L. mesenteroides*. *L. mesenteroides* and *Lactococcus lactis* subsp. *lactis* reached maximum cell densities within 24 h (Fig. 1). Even in the absence of other strains, *Lactobacillus plantarum* did not achieve maximum cell densities until 4 days after inoculation (Fig. 2). This was also true of several other *Lactobacillus plantarum* strains (data not shown).

Nip$^-$ *Lactococcus lactis* subsp. *lactis* NCK402 alone had only a slight inhibitory effect on the growth of *Lactobacillus plantarum* (Fig. 2). This effect was smaller than that observed for *L. mesenteroides* alone or in combination with the nisin-negative strain. In mixed culture with *L. mesenteroides*, Nip$^+$ *Lactococcus lactis* subsp. *lactis* NCK401 produced a maximum level of 100 AU of nisin per ml after 24 h. At this nisin concentration, the growth of *Lactobacillus plantarum* was slightly suppressed. When the activity of nisin was less than 100 AU/ml, inhibition of *Lactobacillus plantarum* was independent of nisin production (data not shown). In the absence of *L. mesenteroides*, a nisin titer of 800 AU/ml was reached after 24 h and the *Lactobacillus plantarum* population was reduced to below the limits of detection (<10 CFU/ml). *Lactobacillus plantarum* was not recovered from these tubes over the course of the experiment.

When *Lactobacillus plantarum* levels reached 10^9 CFU/ml after 4 days (Fig. 2), pH levels dropped to 3.4 to 3.3 (data not shown). In cases when *Lactobacillus plantarum* was inhibited, either through nisin production or strain competition, pH levels were closer to those of *L. mesenteroides* or *Lactococcus lactis* subsp. *lactis* grown alone (pH 3.8 and 4.0, respectively).

Nisin production in mixed culture. Since more nisin was produced when *Lactococcus lactis* subsp. *lactis* was grown alone, initial ratios of *Lactococcus lactis* subsp. *lactis* to *L. mesenteroides* were adjusted to determine whether nisin levels could be increased in mixed culture. *Lactococcus lactis* subsp. *lactis* populations and nisin levels, determined after incubation at 20°C for 48 h, increased in mixed culture as the initial population of *L. mesenteroides* decreased (Table 3).

To obtain a titer of approximately 500 AU/ml, the ratio of *Lactococcus lactis* subsp. *lactis* to *L. mesenteroides* was adjusted to 10^3: 1 in the final mixed-culture experiments (10^6 CFU of *Lactococcus lactis* subsp. lactis per ml and 10^3 CFU of *L. mesenteroides* per ml). A nisin titer of 600 to 700 AU/ml was detected after 24 h in mixed cultures of Nip$^+$ *Lactococcus lactis* subsp. *lactis* NCK401. This nisin level was sufficient to eliminate the *Lactobacillus plantarum* population which had developed in the first 13 h of incubation (Fig. 3).

DISCUSSION

A method was developed to simultaneously enumerate three bacterial genera in order to make a quantitative assessment of mixed-population dynamics in a model sauerkraut fermentation. Nip$^+$ *Lactococcus lactis* subsp. *lactis* was paired with Nis^r *L. mesenteroides* to successfully inhibit a nisin-sensitive *Lactobacillus plantarum* strain. The use of an isogenic nisin-negative derivative ensured that the effects

TABLE 3. Nisin production in CJB[a]

Amt of *L. mesenteroides* NCK293 (log initial CFU/ml)	Nisin production (AU/ml)[b]
5.30	200
4.48	400
3.30	500
2.60	700
1.48	800
0.48	1,000
0.0	800

[a] *Lactococcus lactis* subsp. *lactis* NCK401 was inoculated at 10^6 CFU/ml.
[b] Results are the average of duplicate determinations of replicate trials after incubation for 48 h at 20°C.

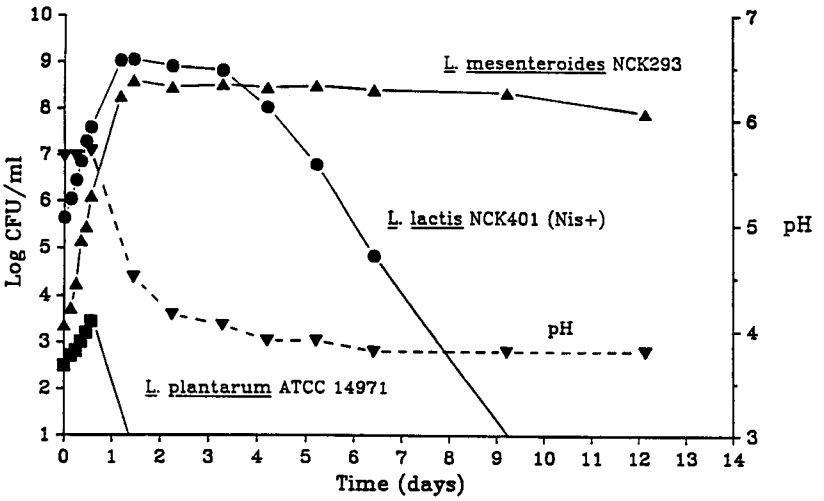

FIG. 3. Mixed-culture fermentation. Growth of *L. mesenteroides* NCK293, *Lactococcus lactis* subsp. *lactis* NCK401, and *Lactobacillus plantarum* ATCC 14917 in CJB. Solid lines denote growth, and broken lines denote pH.

observed were a direct result of nisin production and not a result of other inhibitory factors.

In mixed culture, a nisin level of 100 AU/ml (estimated to be 2 μg of nisin per ml) slightly suppressed the growth of *Lactobacillus plantarum* ATCC 14917. Higher nisin levels (600 AU/ml; 12 μg/ml) completely inhibited this strain. To obtain nisin levels sufficient to inhibit *Lactobacillus plantarum* in mixed culture, we adjusted the initial ratio of *Lactococcus lactis* subsp. *lactis* to *L. mesenteroides* ratio to 10^3: 1 (see Results). At higher *L. mesenteroides* levels, nisin production was suppressed, presumably as a result of increased competition and more rapid pH decline.

The temperature and the salt concentration both have a strong influence on the bacterial sequence in a sauerkraut fermentation, and both are difficult to control reliably by using current production practices (3). We chose 2% NaCl and 20°C for the model fermentation since these parameters are within the range recommended for production of high-quality sauerkraut (13). Slower growth of *Lactobacillus plantarum* at 20°C ensures that this organism does not predominate the early part of a sauerkraut fermentation under optimum fermentation conditions. Rapid growth of *L. mesenteroides* and the consequent pH drop to 4.5 after 24 h have an additional inhibitory effect on the growth of *Lactobacillus plantarum*.

Higher temperatures and salt concentrations allow other lactic acid bacteria to outcompete *L. mesenteroides*. Salt concentrations and fermentation temperatures are not easily controlled and vary significantly from tank to tank in commercial sauerkraut operations. Temperatures from roughly 10 to 30°C and salt concentrations between 1 and 3% NaCl can occur (5). Temperatures in excess of 20°C may increase the rate and amount of nisin production by the starter culture. Sufficient nisin production will suppress the growth of competing microbiota and maintain the desirable predominance of *L. mesenteroides* strains. On the other hand, salt concentrations greater than 2% will retard the growth of *Lactococcus lactis* subsp. *lactis* and, consequently, decrease the nisin production. Further investigations, by using the model fermentation system, are necessary to determine the effect of suboptimal process conditions on the production of nisin and subsequent inhibition of *Lactobacillus plantarum* ATCC 14917.

Development of a secondary, homofermentative population is considered necessary for completion of a sauerkraut fermentation (13). From a practical standpoint, a nisin level which suppresses but does not eliminate the growth of a mixed *Lactobacillus plantarum* population will be most desirable. The wide variations in nisin sensitivity among *L. mesenteroides* and *Lactobacillus plantarum* strains in this study are most probably representative of the indigenous microbiota on raw cabbage. The extent and type of nisin resistance within this biota will influence the outcome of an actual sauerkraut fermentation and the success of the paired starter culture strategy. Production of a nisinase(s) would have a detrimental effect since nisin levels would be reduced or eliminated (7).

Pilot-scale studies using the paired starter culture inoculated into fresh cabbage are necessary to determine whether the presence of nisin can actually promote and extend the dominance of heterofermentative *L. mesenteroides* in a natural fermentation. Nisin-resistant mutants of a *Leuconostoc oenos* starter culture were used in combination with purified nisin to successfully carry out a controlled malolactic fermentation in wine (2). This approach may also be applicable to a sauerkraut fermentation; however, it would require regulatory approval for the addition of nisin to sauerkraut and would increase production costs.

There has been much speculation, but there have been few actual studies on the in vivo role of bacteriocins in microbial ecology (4). Research in this area has been hindered by the lack of tools to accurately study the interactions between bacteriocin-producing and bacteriocin-sensitive strains in mixed culture fermentation. The availability of isogenic, antibiotic-resistant, bacteriocin-negative derivatives will enhance the interpretation of results generated from these types of studies.

From a microbiological standpoint, the sauerkraut fermentation is an excellent model system for the study of mixed-culture ecology. The lack of literature available on the application of starter cultures for sauerkraut production is, in part, due to the difficulties involved in studying a natural fermentation. The development of a marked starter culture system and selective enumeration strategy will greatly enhance our ability to further investigate the ecology of mixed-culture fermentations.

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