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Survival of *Listeria monocytogenes* and *Escherichia coli* O157:H7 during Sauerkraut Fermentation

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ABSTRACT

Sauerkraut was produced from shredded cabbage, as is typical in the United States, and from whole head cabbages, which is a traditional process in parts of Eastern Europe. The sauerkraut was inoculated with five strain mixtures of *Escherichia coli* O157:H7 and *Listeria monocytogenes*, and the populations of these bacteria, as well as lactic acid bacteria, pH, and titratable acidity, were monitored over the course of fermentation. Fermentation variables were temperature (18 and 22°C) and salt concentration (1.8, 2.25, and 3%). For most of the analyses, the type of cabbage processing was a significant factor, although within cabbage type, neither salt nor fermentation temperature had significant effects. The final pH of the whole-head sauerkraut was lower than the shredded sauerkraut, but the titratable acidity was significantly higher in the shredded sauerkraut. *E. coli* O157:H7 and *L. monocytogenes* persisted in the brines for most of the fermentation, although at the end of the fermentations (15 days for shredded, 28 days for whole head), neither pathogen had detectable populations. *E. coli* populations decreased more rapidly in the shredded sauerkraut even though the pH was higher because of the higher total acidity in the shredded sauerkraut. Acid-tolerant strains of *E. coli* and *L. monocytogenes* were isolated from both shredded and whole-head sauerkraut at different salt concentrations and temperatures after 15 days of fermentation and could be detected at 35 days in the whole-head sauerkraut.

Sauerkraut fermentation is spontaneous and relies on a small, naturally occurring lactic acid bacteria (LAB) present on the raw cabbage. A succession of various bacteria and their metabolic activities are responsible for the quality, and especially the safety, of these products *(7).* Several LAB species (mainly *Leuconostoc mesenteroides, Leuconostoc fallax, Lactobacillus brevis, Pediococcus pentosaceus,* and *Lactobacillus plantarum*) are known to contribute to the complex sauerkraut fermentation process *(2). L. mesenteroides* is thought to be the dominant species in the early heterofermentative stage of this fermentation, and *L. plantarum* strains complete the fermentation in the homofermentative stage with a final pH of approximately 3.5 *(5, 11).*

Commercial cabbage fermentations in the United States, but also in Europe, are typically carried out by epiphytic LAB, without the benefit of an added starter culture *(4).* The vegetable fermentation industry needs to reduce waste chloride production, so low-salt fermentation procedures are currently being developed *(12, 13).*

The traditional methods of homemade fermentation in Europe, the Balkans, and the United States do not include pasteurization of the final product. Another traditional type of sauerkraut fermentation, commonly found in the Balkans, uses whole heads of cabbage as starting material for fermentation. This results in a longer fermentation, which can last up to several months. This longer fermentation could expose people to greater risk; pregnant woman and people who are checking the taste of the product during fermentation could be exposed to foodborne pathogens. These potential pathogens can be found on raw cabbage and other vegetables contaminated with cattle manure, used to fertilize soil, or through the use of contaminated water for irrigation. As an example, washing cole slaw produced from cabbage contaminated with sheep manure resulted in an outbreak of *Listeria monocytogenes* in Canada in 1983 *(3).*

In the United States and other developed nations, *L. monocytogenes* and *Escherichia coli* O157:H7 are recognized as significant causes of foodborne diseases. The ability of *L. monocytogenes* to tolerate low-pH environments is of particular importance because the pathogen encounters such environments in vivo, both during passage through the stomach and within the macrophage phagosome. *L. monocytogenes* was shown to exhibit a significant adaptive acid tolerance response following a 1-h exposure to mild acid (pH 5.5), which is capable of protecting cells from severe acid stress (pH 3.5). It was shown that susceptibility to pH 3.5 acid is dependent on the growth phase. Stationary-phase *Listeria* cultures are naturally resistant to the challenge pH (pH 3.5), whereas exponential-phase cultures require adaptation at pH 5.5 to induce acid tolerance *(6).* Also, it was

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demonstrated that it is possible to isolate mutants that constitutively demonstrate increased acid tolerance and increased virulence at all stages of the growth cycle *(9)*.

Escherichia coli O157:H7 can contaminate cabbage and is exceptionally tolerant of acid pH. It could be detected in apple cider with a pH of 3.6 stored at a low temperature of 8°C and was shown not to be destroyed within the shelf life of apple cider at pH 2 to 4 *(8)*. Results also showed that this pathogen was recoverable from different fruit concentrates through 12 weeks of storage at −23°C *(10)*. Populations of *E. coli* O157:H7 were also shown to decrease noticeably on chopped cabbage stored at 22°C after 24 h, up to a total of 3 log units within 72 h. During the first 24 h, *E. coli* incubated at 0, 6, and 12°C showed an increase in number; nevertheless, at 22°C, it presented a significant decrease in numbers, probably because of competition and the important pH change (decreasing from pH 7.5 to 5.3). The decrease in pH can be attributed to the fermentative capability of this microorganism and to competition, including lactic bacteria *(1)*.

Usually, LAB control the sauerkraut fermentation, but it was shown recently that phages active against LAB, including *L. mesenteroides*, *L. plantarum*, and *P. pentosaceus*, are present in sauerkraut fermentations *(13)*. *Leuconostoc* phages have also been identified as factors responsible for the failure of fermentations *(2)*. When some LAB that produce nisin are present or when nisin is added to cabbage at the beginning of sauerkraut fermentation, *E. coli* O157:H7 can survive much longer—14 days—probably because growth and lactic acid production were inhibited *(14)*.

Our objectives were to investigate survival of *L. monocytogenes* and *E. coli* O157:H7 during and after lactic acid fermentation of sauerkraut produced from both whole-head and shredded cabbage.

MATERIALS AND METHODS

Preparation of bacteria. Composites of five strains were prepared for each pathogen. The *E. coli* O157:H7 composite included the following American Type Culture Collection strains: ATCC 35150, ATCC 43894, and ATCC 43895 and two strains isolated from foodborne outbreaks in Washington State (WS 3062 and WS 3331). The *L. monocytogenes* composites included Scott A, serotypes 1/2a and 1/2b, and two different serotype 4b isolates (strains H7762, H7769, 1/2a FSIS, and H7764 from the culture collection of the Food Safety Research Laboratory, Ames, Iowa), all isolated from foodborne outbreaks. All *E. coli* strains were grown individually from frozen (−80°C) laboratory stock cultures (Food Safety Research Laboratory) in tryptic soy broth (TSB; Becton Dickinson, Sparks, Md.) and *L. monocytogenes* in TSB with 0.6% yeast extract (TSBYE) for 24 h at 35°C and served as inocula. From these cultures, 4 ml of each strain was added to a single flask containing either 150 ml of TSB (*E. coli*) or TSBYE (*L. monocytogenes*) and incubated with shaking (120 ± 2 rpm) for 20 h at 35°C. Broth was centrifuged at 5,000 × *g* for 10 min, after which the supernatant was decanted and cells were resuspended in physiological saline solution (0.85% NaCl) to final concentration. Cells were composited to obtain approximately equal numbers of each strain (1×10^6 CFU/g). Each strain and composite was enumerated by serial dilution in 0.1% peptone water and spread plating on tryptic soy agar. Plates were incubated at 35°C for 24 h.

Sauerkraut fermentation. Cabbage was obtained from a commercial source as both whole cabbage heads and as coarsely shredded cabbage (pieces of ~1.5 × 1.5 cm).

Preparation of whole-head sauerkraut. Four to five heads (5 kg total) were randomly selected from the original shipment of good, mature cabbage heads (washed well with the external leaves removed; part of the hearts removed) and mixed. Cabbage was placed in 20-liter plastic containers, and different concentrations of salt in 10 liters of tap water (1.8, 2.25, and 3% by weight of cabbage) were added. The salt used in brine production was free of flow agents and iodine. The inocula with 100 ml of a composite of the 10 strains of bacteria were added, then cheese cloths with 2.5 kg weights were placed on top of the cabbage. Additional containers of cabbage were prepared in an identical manner for use as controls but were not inoculated with the pathogenic bacteria. Containers were incubated at 18 and 22°C. Composite samples were taken from the bottom and surface of the container.

Preparation of shredded sauerkraut. Commercially precut shredded cabbage was weighed (3 × 4 kg) and added into a ''Boy butcher'' mixer (Butcher Boy, Montebello, Calif.) with 1.8, 2.25, and 3% (wt/wt) salt and 150 ml of composite inocula and mixed for 5 min. Each plastic 9-liter container was filled with 4 kg of cabbage by alternately pressing layer upon layer of cabbage until the container was filled to the top. Cheese cloths were placed and weighted with 2.0 kg on the top, and the sauerkraut was incubated at 18 and 22°C. Additional containers of cabbage were prepared in an identical manner for use as controls but were not inoculated with the pathogenic bacteria. Both tanks for shredded and whole heads were fitted with a drainage tube with a protective net on the bottom of the container to take representative samples through pipes from the bottom and surface.

Sample collection and bacteriological analysis. Composite brine samples from the bottom and surface of the containers were collected on days 0, 1, 3, 4, 6, 10, and 15 for shredded and days 0, 4, 7, 9, 14, 19, 27, and 35 for whole heads. The cabbage juice (20 ml) was placed into a sterile plastic tube and frozen at −20°C for later chemical analysis.

***Escherichia coli* O157:H7.** Immediately after each incubation period, 25-g samples were taken and combined with 225 ml of sterile 0.1% peptone water in a sterile polyethylene bag and pummeled with a stomacher for 2 min. The homogenized sample was serially (1:10) diluted and surface plated (0.05 ml) on duplicate sorbitol McConkey agar (SMAC; Oxoid, Nepean, Ontario, Canada) with a WASP automatic spiral plater (Don Whitley Scientific, Shipley, West Yorkshire, UK). The SMAC plates were incubated at 35°C for 20 to 24 h before colonies of *E. coli* O157:H7 were enumerated with an automatic colony counter (ProtoCOL 60000, Synoptics Ltd., Fredrick, Md.). The same procedures for plating and enumeration of colonies were used with other bacteria and the controls.

Listeria monocytogenes. *L. monocytogenes* populations were enumerated on Oxford formulation Listeria selective agar–modified Oxford (MOX; Oxoid CM856, including selective supplement Oxoid SR140) and incubated at 37°C for 24 to 48 h. The growth of *L. monocytogenes* was routinely confirmed with assay VIP for *Listeria* (BioControl Systems, Inc., Bellevue, Wash.).

Lactic acid bacteria. LAB isolates were recovered from brine samples on the same days as for pathogens from different sauerkraut fermentation batches. The brine samples were plated on MRS agar (Difco Laboratories, Becton Dickinson, Sparks,

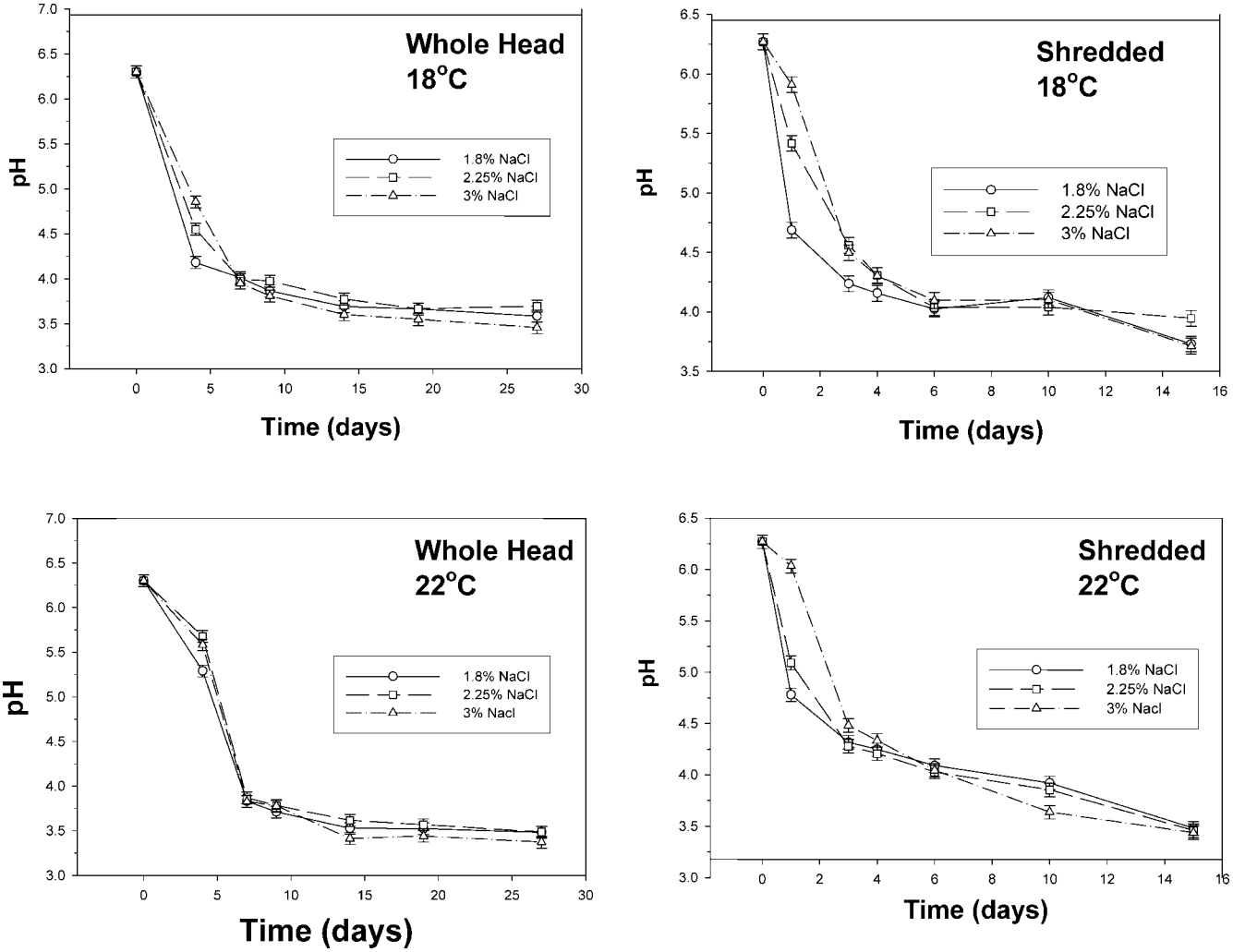

FIGURE 1. *pH of whole-head and shredded cabbage sauerkraut fermentations as affected by fermentation temperature and brine concentration.*

Md.) and incubated anaerobically in an incubator (ThermoForma, Marietta, Ohio). In the later phase of fermentation, a modified MRS agar was prepared by adding 0.02 M sodium azide before pouring of the plates to prevent the growth of yeasts and molds and to ensure selection for LAB. All cultures were incubated at 30°C for 48 h.

Identification of acid-resistant isolates. Bacterial populations were enumerated on selective media. If the target organism was not recovered directly on selective media in the later phases of fermentation, brine samples (15 ml) for the recovery of acid-resistant strains were centrifuged at 4,200 × *g* for 10 min, after which the supernatant was decanted, and pelleted cells were resuspended in physiological saline solution and surface plated (0.1 ml) on tryptic soy agar and incubated at 35°C for 3 h for recovery of cells. After that, SMAC and MOX were overlaid and plates were incubated at 35°C for 20 to 24 h.

Characterization of acid-resistant isolates. The pathogenic bacteria that grew on the overlay plates, but not on the direct plating, were considered to be acid tolerant and possibly injured. Presumptive isolates were selected from the respective SMAC or MOX overlay plates and transferred to tryptic soy agar slants for confirmation. The isolates were analyzed to confirm that they were in fact the pathogenic bacteria and were transferred to blood agar and incubated at 37°C overnight. DNA extraction was performed with a Roche MagNA Pure instrument and the Roche DNA Isolation Kit III (Roche Diagnostics, Penzberg, Germany). The MagNa Pure instrument is a robotic workstation for fully automated nucleic acid preparation and extraction. The instrument performs all procedural steps, including sample uptake, lysis, binding of DNA to magnetic glass particles, wash steps, and elution. The extracted DNA was amplified with primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′), 515F (5′-PGTGCCAGCAGCCGCGGTAA-3′), 27F (5′-AGAGTTTGATCMTGGCTCAG-3′), RD1 (5′-AGGAGCGTGAAGAAGACGAC-3′), and 1492R (5′-AGATAGAAACCAACCTGG-3′). Reactions were first incubated for 3 min at 95°C followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, 1 min 30 s at 72°C, and a final incubation of 5 min at 72°C. The resulting PCR products were then purified with the QIAGEN Kit for Purification of PCR Products (QIAGEN, Valencia, Calif.). Cycle sequencing was performed with a Big Dye terminator cycle sequencing kit (Applied BioSystems, Foster City, Calif.). The extension products were then purified by QIAGEN DyeEX column purification (QIAGEN) and analyzed with an Applied BioSystems model 3100 automated DNA sequencing system. The sequences of the acid-resistant isolates were compared with the sequences obtained from the original cultures in the inoculum to determine if the isolates matched the original inocula.

pH and acidity determinations. pH of the undiluted brine juice sample was measured with a pH meter (Accumet 15, Fisher

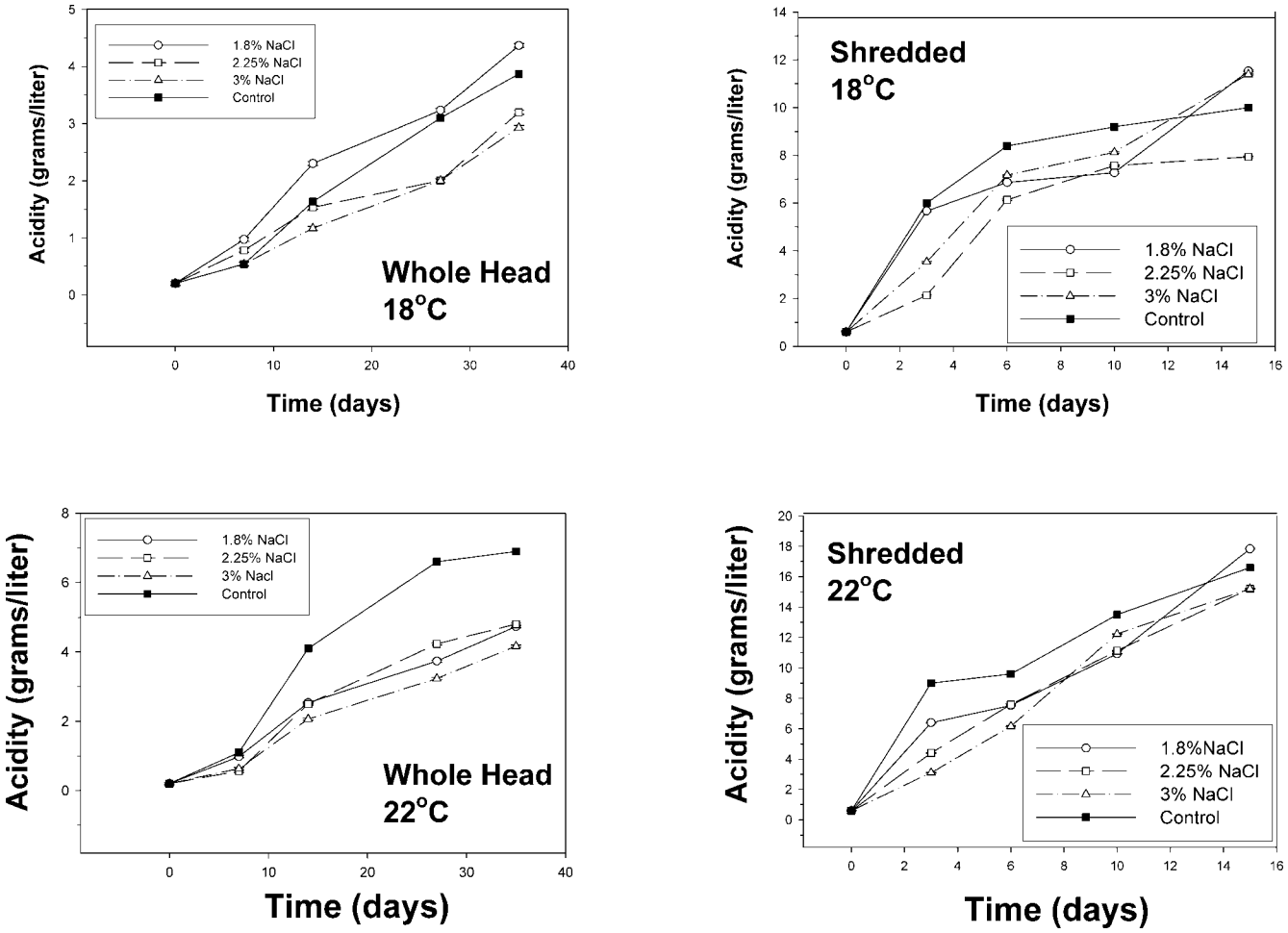

FIGURE 2. *Titratable acidity of whole-head and shredded cabbage sauerkraut fermentations as affected by fermentation temperature and brine concentration.*

Scientific, Kansas City, Mo.) at appropriate time intervals. Total acidity was determined by titration with 0.1 M NaOH and 1.0% phenolphthalein. Juice samples were diluted with 10 ml of distilled water, boiled for 1 min to drive off the dissolved carbon dioxide, and titrated until a light pink color persisted. With the formula % total acid = [(ml of 0.1 M NaOH) × (0.9)]/(sample volume), the percentage of total acids were calculated. Total acidity was expressed as grams per liter of sauerkraut brine.

Data analysis. The populations of bacteria were converted to log CFU per milliliter of brine. The detection limit of the assay was 20 CFU/ml (1.3 log CFU/ml) on the basis of plating 100 μl of the undiluted brine. The value ''0'' was entered when no colonies were recovered from a sample. In some cases, the least squares means appear below the detection limit on the graphs. This is because one or more of the three replications had a population below the detection limit (reported as 0), whereas the remaining replications had populations above the detection limit. The least squares mean in those cases would numerically be below the detection limit. All data were analyzed by JMP statistical software (SAS Institute, Cary, N.C.) Least squares mean values were determined for data with apparent differences, and these values were analyzed by a one-way analysis of variance to determine significant variance among the treatments.

RESULTS AND DISCUSSION

pH and titratable acidity. There was a significant difference ($P < 0.05$) observed between the pH values of the shredded and whole-head sauerkraut (Figs. 1 and 2). The final pH of the whole-head sauerkraut was lower than that of the shredded sauerkraut (especially at 18°C), although this might have been the result of the longer fermentation time for the whole-head product. Within cabbage types (whole head versus shredded), there was no significant effect ($P > 0.10$) of either salt concentration or temperature.

The titratable acidity of the sauerkraut was affected by the type of cabbage and the temperature within cabbage types. It is estimated that approximately 85% of the acidity represented by this analysis is from lactic acid, with the remainder being other organic acids (primarily acetic). The acidity was significantly higher ($P < 0.05$) in the shredded sauerkraut than in the whole-head variety, and within cabbage types, the higher fermentation temperature generally resulted in higher acidity. Significantly more acid was produced in significantly less time in the shredded cabbage fermentations (Fig. 2).

The marked difference between pH and titratable acidity is important for further interpretation of the data. Although the actual pH of the sauerkraut manufactured from the whole-head cabbage was lower than that of the shredded cabbage, the titratable acidity was significantly higher with the shredded cabbage. This suggests that shredding the cabbage releases cellular components from the cabbage that

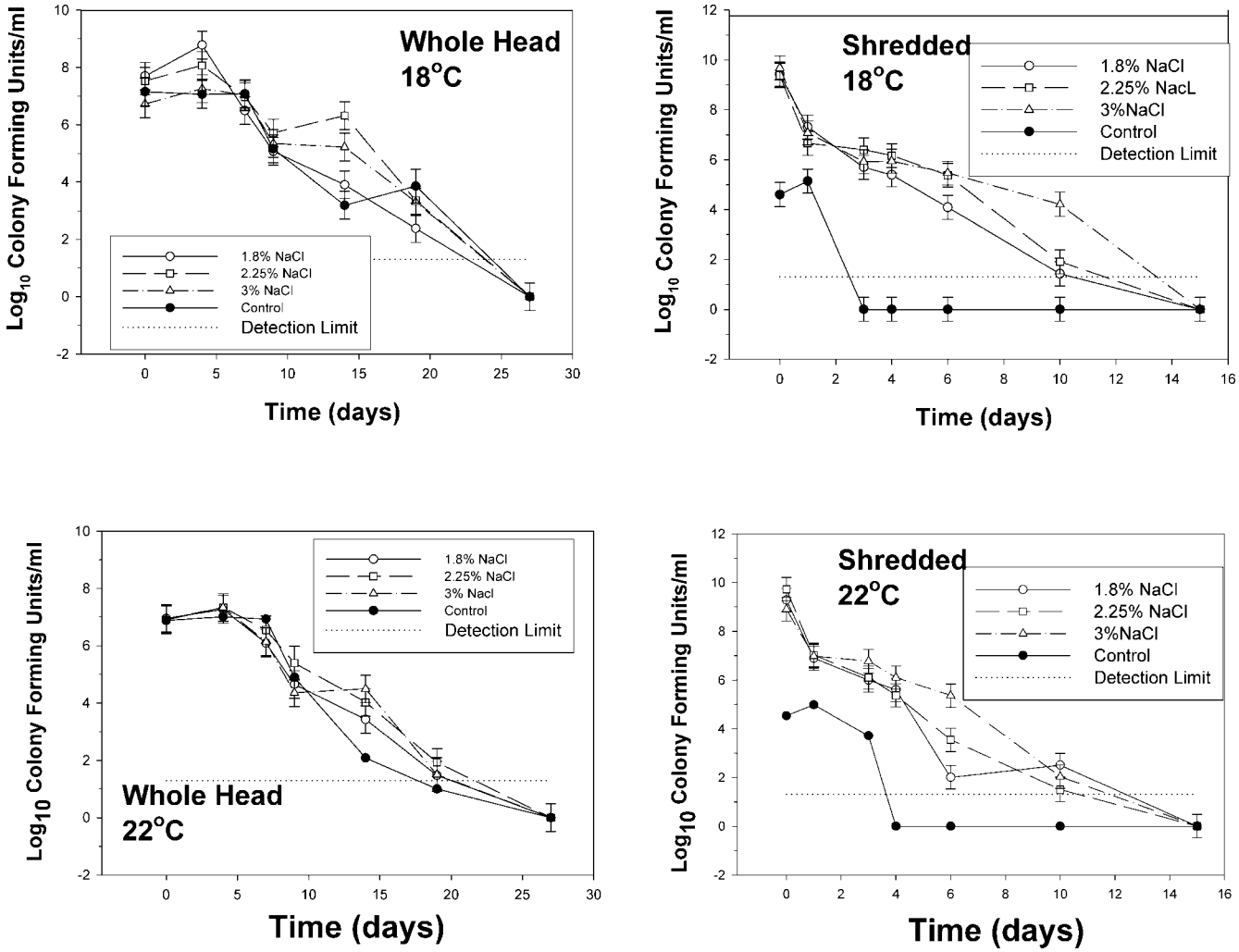

FIGURE 3. *Survival of* Escherichia coli *O157:H7 in whole-head and shredded cabbage sauerkraut fermentations as affected by fermentation temperature and brine concentration.*

establishes a buffering system within the fermentation and results in a slightly higher pH. However, shredding also releases carbohydrates from the cabbage cells, which results in a more rapid fermentation.

***Escherichia coli* O157:H7.** No significant differences were observed in the survival of *E. coli* O157:H7 between whole-head or shredded cabbage or between temperature or salt concentration within cabbage type ($P > 0.10$; Fig. 3). There was, however, a significant interaction between the type of cabbage (head versus shredded) and the sampling day, with the whole-head sauerkraut consistently having higher populations over time compared with the shredded cabbage. The observed differences in titratable acidity would, in all probability, account for these observed differences in survival. The decrease in *E. coli* O157:H7 populations is associated with the increase in titratable acidity, and the more rapid decrease in populations in the shredded sauerkraut is associated with the significantly higher levels of acidity observed with this product. This is consistent with the results reported by Arias et al. *(1)* and Yu et al. *(14),* who showed that the populations of *E. coli* O157:H7 declined during cabbage fermentation.

Populations of bacteria capable of growth on sorbitol MacConkey agar, which were sorbitol-negative, were enumerated in the control (noninoculated) sauerkraut fermentations. Although the naturally occurring populations were low in the shredded cabbage, higher populations were found in the whole-head sauerkraut. This is attributable to outer leaves being left on whole-head cabbage; therefore, higher populations of soil bacteria were present in this fermentation. The outer leaves were removed from the cabbage heads that were shredded, and this removal resulted in a significant reduction in the level of naturally occurring microorganisms capable of growth on sorbitol MacConkey agar. Random colonies were selected from the control samples and tested with a rapid *E. coli* O157 lateral flow enzyme-linked immunosorbent assay (Reveal, Neogen, Lansing, Mich.), and all of the randomly selected colonies were determined to be other bacteria, and not *E. coli* O157:H7.

Listeria monocytogenes. There was a significant difference ($P < 0.05$) in the survival of *L. monocytogenes* in sauerkraut made from the two types of cabbage, with higher populations of *L. monocytogenes* persisting for longer periods of time in the sauerkraut made from the whole-head cabbage (Fig. 4). There were no significant effects of salt concentration or temperature within the cabbage type. Con-

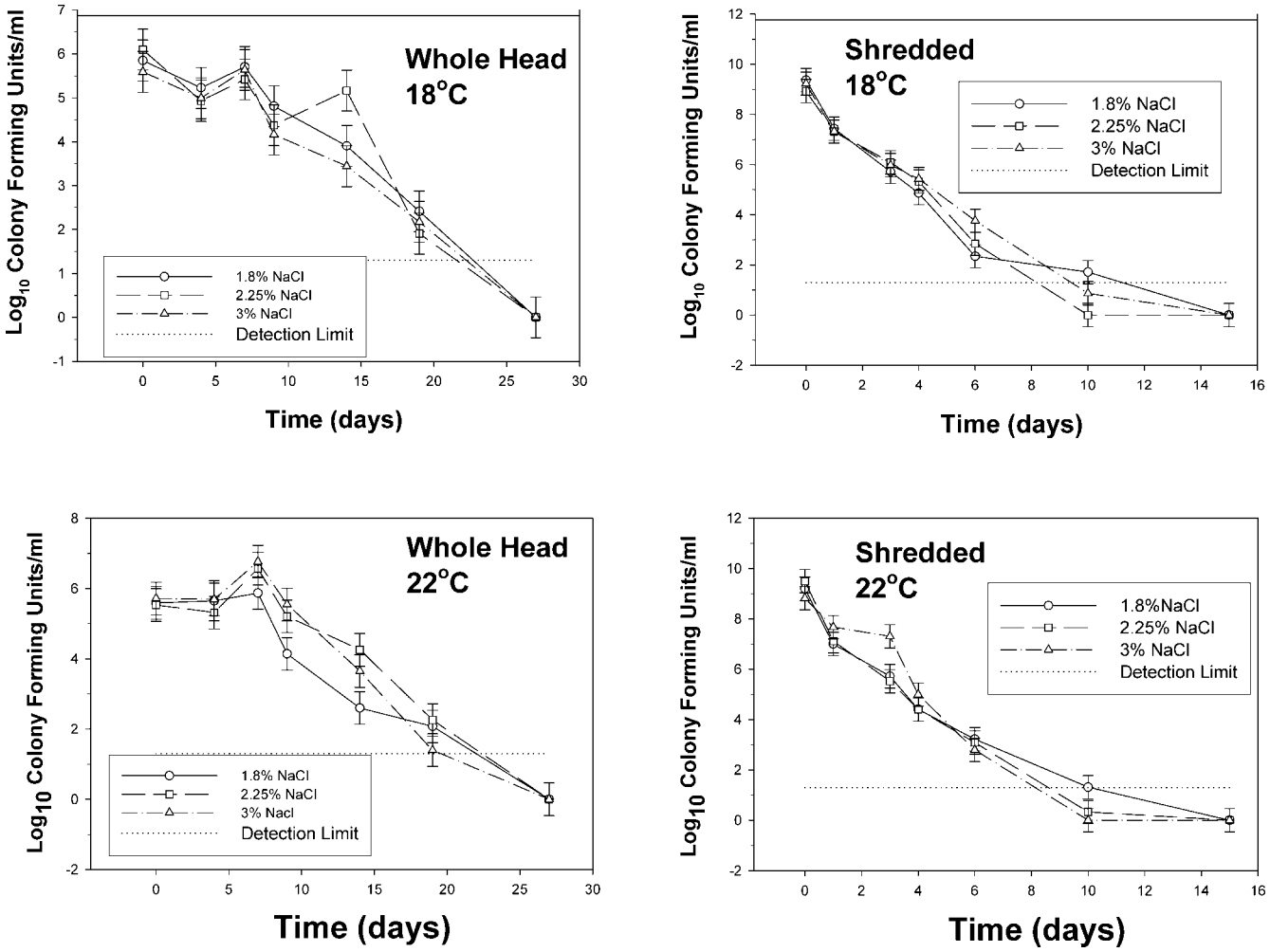

FIGURE 4. *Survival of* Listeria monocytogenes *in whole-head and shredded cabbage sauerkraut fermentations as affected by fermentation temperature and brine concentration.*

ner et al. *(3)* reported on the growth of *L. monocytogenes* as affected by temperature and salt concentration in cabbage juice. These authors noted, at similar NaCl concentrations in cabbage juice, a continual decrease in population over time. They also reported that *L. monocytogenes* populations in cabbage juice declined more rapidly at 30°C than at 5°C, irrespective of NaCl concentration *(3).* These results are consistent with the results presented in this report.

As with *E. coli* O157:H7, the persistence of the bacterial populations was consistent with the level of acid present, as determined by titratable acidity. The sauerkraut made from the whole-head cabbage had consistently lower acidity over time, with a resulting higher survival of the bacteria. The control samples were also enumerated, but no colonies capable of growth on MOX agar were present, even in the initial (day 0) samples.

Lactic acid bacteria. The population trends for LAB were similar in some respects to those observed with *L. monocytogenes.* There was a significant difference ($P < 0.05$) in the populations in sauerkraut made from the two types of cabbage, with higher populations for longer periods of time in the sauerkraut made from the whole-head cabbage (Fig. 5). There were no significant effects of salt concentration or temperature within cabbage type. This is primarily attributable to the availability of nutrients in the two types of sauerkraut. With the sauerkraut made from shredded cabbage, the shredding process released many of the carbohydrates from the cabbage cells. As a result, a high concentration of nutrients were initially available for rapid consumption by the bacteria. After the initial population growth, the high acidity resulted in a decrease in the populations. In contrast, the nutrients in the whole-head cabbage diffused out of the cabbage slowly, and the population was sustained by a continual source of nutrients. This resulted in a slower production of acid and was associated with a longer survival of the bacterial populations. As with the other bacteria, the high titratable acidity in the shredded sauerkraut resulted in a more rapid decline in the populations.

Acid-adapted and acid-tolerant bacteria. Fermentation temperature affected the recovery of acid-tolerant *E. coli* O157:H7, with most isolates being recovered from the 18°C fermentation (Table 1). There was also an association between salt concentration and the isolation of acid-tolerant *E. coli* O157:H7 in the whole-head sauerkraut, with more

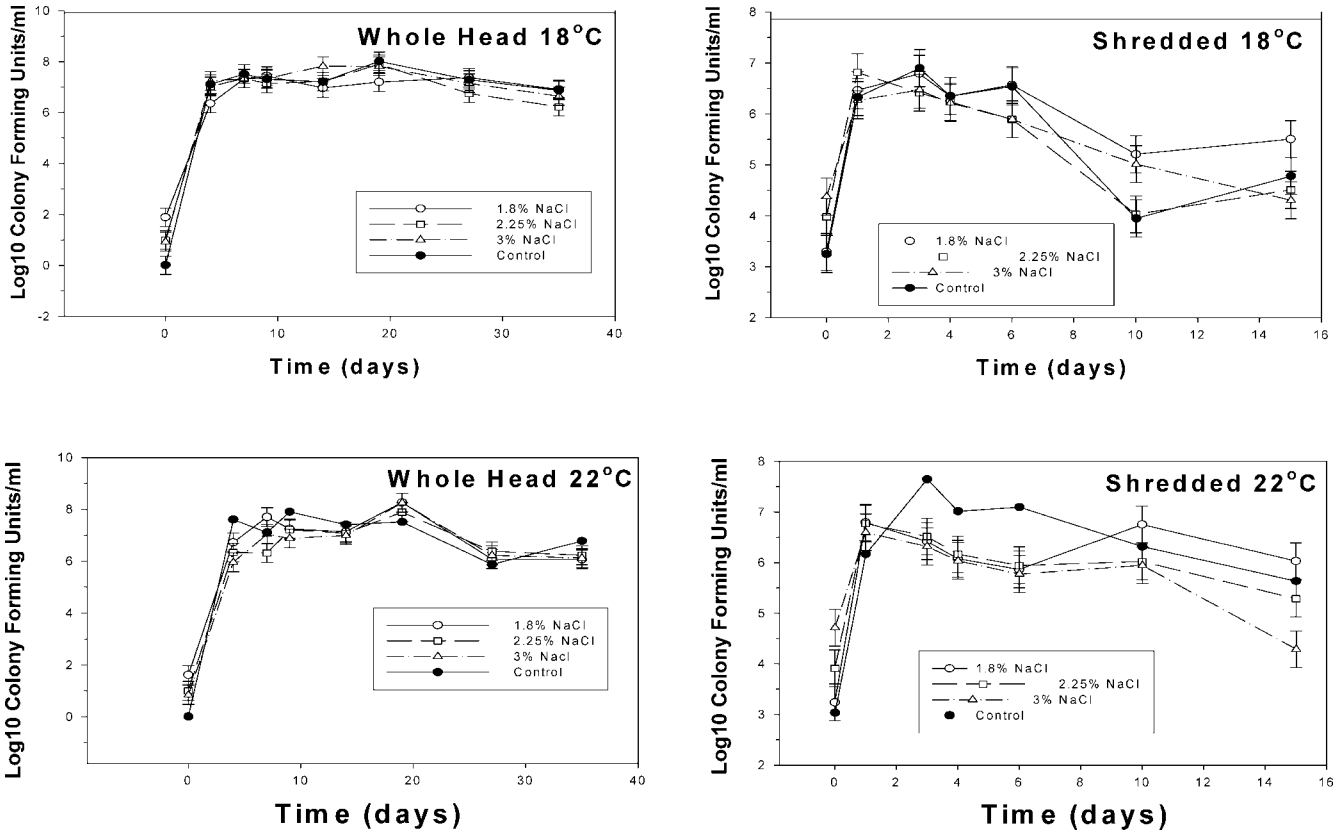

FIGURE 5. *Growth of lactic acid bacteria in whole-head and shredded cabbage sauerkraut fermentations as affected by fermentation temperature and brine concentration.*

isolates coming from 2.25% than from 1.8 or 3% salt concentration at 18°C. This association could have public health significance because the 2.25% salt concentration is considered the optimum level for flavor and quality. Reasonable explanations for these results are that suboptimal temperature (18°C) allowed for the survival of bacteria, and normal fermentation temperatures (22°C) allowed fermentation to inhibit the bacteria. Seventeen of the 18 acid-tolerant *E. coli* O157:H7 isolates were recovered from the 18°C fermentations. The salt concentration might have affected the survival in whole-head sauerkraut by allowing

TABLE 1. *Sauerkraut brine samples containing* Escherichia coli *O157:H7 bacteria that were not recoverable by enumeration methods but were recoverable by sample concentration and nonselective recovery methods*

Cabbage type	Temperature (°C)	Brine concentration %	No. of isolates recovered[a]	
			Day 19	Day 27
Whole	18	1.8	1/3	1/3
		2.25	2/3	3/3
		3.0	1/3	0/3
	22	1.8	0/3	0/3
		2.25	1/3	0/3
		3.0	0/3	0/3
			Day 15	
Shredded	18	1.8	3/3	
		2.25	2/3	
		3.0	3/3	
	22	1.8	0/3	
		2.25	0/3	
		3.0	0/3	

[a] Fermentation day; see Fig. 3. Values are number of positive samples/number of replications.

TABLE 2. *Sauerkraut brine samples containing* Listeria monocytogenes *bacteria that were not recoverable by enumeration methods but were recoverable by sample concentration and nonselective recovery methods*

Cabbage type	Temperature (°C)	Brine concentration (%)	No. of isolates recovered[a]		
			Day 19	Day 27	Day 35
Whole	18	1.8	3/3	0/3	0/3
		2.25	1/3	0/3	0/3
		3.0	2/3	0/3	1/3
	22	1.8	1/3	1/3	0/3
		2.25	2/3	0/3	0/3
		3.0	1/3	0/3	0/3
Shredded	18	1.8	0/3	0/3	0/3
		2.25	0/3	0/3	0/3
		3.0	0/3	0/3	0/3
	22	1.8	0/3	0/3	0/3
		2.25	0/3	0/3	0/3
		3.0	0/3	0/3	0/3

[a] Fermentation day; see Fig. 4. Values are number of positive samples/number of replications.

competitive bacteria to inhibit the bacteria at lower salt concentrations (1.8%), whereas the higher salt concentration (3%) might simply have inhibited the bacteria after 19 days.

No acid-tolerant *L. monocytogenes* were recovered from the sauerkraut made from shredded cabbage (Table 2). However, acid-tolerant *Listeria* were isolated from both the 18°C fermentation (days 19 and 35) and the 22°C fermentation (days 19 and 27). Acid adaptation has been reported to enhance the survival of *L. monocytogenes* in fermented and acidic foods *(9).* There was no obvious association between the isolation of the acid-tolerant organisms and either the fermentation temperature or the concentration of salt in the brine. Seven of the isolates were from the 18°C fermentation, and the remaining five were from the 22°C fermentation. Five of the 12 isolates were isolated from 1.8%, three from the 2.25%, and the remaining four from the 3% brine. The lack of isolates from the shredded cabbage fermentations are most likely attributable to the rapid increase in titratable acidity seen in these fermentations.

The traditional method of sauerkraut fermentation in the Balkans, with the use of whole-head cabbage, results in a product that eliminates *E. coli* O157:H7 and *L. monocytogenes*, if the fermentation is performed properly. Variations in salt content and fermentation temperature can affect the survival of these pathogens during fermentation, but neither survived in significant numbers after the fermentation was complete. However, shredded cabbage resulted in a higher level of titratable acidity, which greatly reduced the risk associated with survival of these bacteria.

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