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Effects of Nisin and Temperature on Survival, Growth, and Enterotoxin Production Characteristics of Psychrotrophic *Bacillus cereus* in Beef Gravy

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The presence of psychrotrophic enterotoxigenic *Bacillus cereus* in ready-to-serve meats and meat products that have not been subjected to sterilization treatment is a public health concern. A study was undertaken to determine the survival, growth, and diarrheal enterotoxin production characteristics of four strains of psychrotrophic *B. cereus* in brain heart infusion (BHI) broth and beef gravy as affected by temperature and supplementation with nisin. A portion of unheated vegetative cells from 24-h BHI broth cultures was sensitive to nisin as evidenced by an inability to form colonies on BHI agar containing 10 μg of nisin/ml. Heat-stressed cells exhibited increased sensitivity to nisin. At concentrations as low as 1 μg/ml, nisin was lethal to *B. cereus*, the effect being more pronounced in BHI broth than in beef gravy. The inhibitory effect of nisin (1 μg/ml) was greater on vegetative cells than on spores inoculated into beef gravy and was more pronounced at 8°C than at 15°C. Nisin, at a concentration of 5 or 50 μg/ml, inhibited growth in gravy inoculated with vegetative cells and stored at 8 or 15°C, respectively, for 14 days. Growth of vegetative cells and spores of *B. cereus* after an initial period of inhibition is attributed to loss of activity of nisin. One of two test strains produced diarrheal enterotoxin in gravy stored at 8 or 15°C within 9 or 3 days, respectively. Enterotoxin production was inhibited in gravy supplemented with 1 μg of nisin/ml and stored at 8°C for 14 days; 5 μg of nisin/ml was required for inhibition at 15°C. Enterotoxin was not detected in gravy in which less than 5.85 $\log_{10}$ CFU of *B. cereus*/ml had grown. Results indicate that as little as 1 μg of nisin/ml may be effective in inhibiting or retarding growth of and diarrheal enterotoxin production by vegetative cells and spores of psychrotrophic *B. cereus* in beef gravy at 8°C, a temperature exceeding that recommended for storage or for most unpasteurized, ready-to-serve meat products.

Bacillus cereus is an aerobic sporeformer commonly found in soil and groundwater and often found on plants and animals at the point of harvest or slaughter. Its presence on or in raw and processed grain, vegetable, meat, and dairy products has been documented by several researchers (10, 14, 19, 26, 29, 30, 40, 41). Dry dessert mixes (45), infant foods (2), meat spices and seasonings (29), and a wide range of ready-to-serve foods (44) have also been shown to contain *B. cereus*. Certain strains of the bacterium are capable of producing a heat-labile diarrheal enterotoxin and/or a heat-stable emetic enterotoxin, as well as other toxins (13). Observations that psychrotrophic strains have been implicated in outbreaks of food-borne illness (43) and are capable of producing enterotoxins (15, 16) have raised concern about their growth and toxin production characteristics in refrigerated ready-to-serve (18, 44) and sous vide food products.

Meats and meat products that have not received process treatments to assure inactivation of vegetative cells and spores of *B. cereus* are among the ready-to-serve foods that represent potential vehicles of intoxication. Konuma et al. (29) reported that 50 of 211 (23.7%) heated hamburger samples contained *B. cereus*, and Grant and Patterson (12) observed that *B. cereus* constituted a significant proportion of the microflora before and after chill storage of a roast beef stew. The ability of *B. cereus* to grow (11, 39, 43) and produce enterotoxin (11) in beef and poultry products held at refrigeration and ambient temperatures has been described. *B. cereus* has been reported to produce toxin in milk held at 8°C (5) and in ground meat at 4°C (44).

Attempts have been made to identify processes and chemicals, either alone or in combination, that kill or control the growth of *B. cereus* in raw and pasteurized foods. Sorbic acid (42), fatty acids and their salts (1), sodium chloride (33, 42), and garlic extracts (37) are inhibitory to the organism. Nisin is effective in controlling both the growth of *B. cereus* in crumpets (a high-moisture, baked, flour-based product [25]), liquid egg (9), and other foods (8) and the growth of gram-positive pathogenic bacteria on the surface of fresh beef (6). Whether inhibition of growth of *B. cereus* in vacuum-packed sausage (32) was due to the production of a bacteriocin(s) or increased acidity is not known.

The objectives of the study reported here were to determine the effects of nisin on the growth of four strains of psychrotrophic *B. cereus* in laboratory broth and on the growth of and enterotoxin production by two strains in beef gravy. The combined effects of heat and nisin on inactivation of *B. cereus* in broth and gravy were also studied.

MATERIALS AND METHODS

Strains used. Four strains of *B. cereus* were used in broth studies. Strain F3812/84 has been observed to grow at 5°C in brain heart infusion (BHI) broth (22). This atoxigenic strain, isolated from pasteurized milk, was obtained from John M. Kramer, Public Health, Laboratory Service, London, United Kingdom. Strain F3802A/84, also obtained from Kramer and reported to grow at 7°C (31), was isolated from pasteurized milk. This strain is capable of producing diarrheal enterotoxin but not emetic toxin. The third strain (1230-88), isolated from oriental stew, was obtained from Per Granum, Norwegian College of Veterinary

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Medicine, Oslo, Norway. Strain 038-2 was obtained from Stephanie Doores, Pennsylvania State University, University Park. This strain was isolated from infant formula and produces diarrheal and emetic enterotoxins (3, 4). In beef gravy studies, *B. cereus* F3802A/84 and 038-2 were used. We have observed strains F3802A/84, 1230-88, and 038-2 to grow in BHI broth at 8°C but not at 5°C (22).

Preparation of inocula. Stock cultures were activated in BHI broth (pH 7.4) (Difco, Detroit, Mich.) and incubated at 30°C. At least three successive loop transfers at 24-h intervals were made before using vegetative cells as inocula for various experiments.

Nutrient agar (NA) (pH 6.8) (Difco) supplemented with 0.05 g of manganese sulfate/liter (NAMS agar) was used as a sporulation medium. Vegetative cell cultures of each strain were grown for 24 h in BHI broth at 30°C, and 0.1-ml aliquots were surface spread (0.1 ml) onto NAMS agar in petri dishes and incubated for 72 to 76 h at 30°C, at which time at least 85% of cells had formed spores. Spores were harvested by depositing 5 ml of sterile distilled water on the surface of each plate and rubbing gently with a sterile bent glass rod. The suspension of spores and vegetative cells was filtered through sterile glass wool and collected in sterile tube; the washing and filtering procedure was repeated twice. Pooled suspensions were centrifuged at 2,600 × *g* (5°C) for 20 min, and the supernatant liquid was discarded. The resulting pellet was suspended in 100 ml of sterile distilled water and centrifuged at 6,000 × *g* for 10 min. This procedure was repeated twice; the final pellet was suspended in sterile deionized water, and aliquots of suspensions were stored at 1°C for use in subsequent experiments.

Spores were subjected to heat treatment before they were used as inocula. Stock spore suspensions were brought to room temperature, and 1.0 ml was deposited into 10 ml of sterile distilled water tempered at 80°C in a water bath. Suspensions were heated for 5 min and then serially diluted in sterile deionized water before being used as inocula.

Preparation of Nisaplin solution. Nisaplin (Alpin and Barrett Ltd., Beaminster, United Kingdom) contains 25,000 μg of nisin/g. A solution containing 2,000 μg of nisin/ml of 0.02 N HCl was used to supplement the BHI medium and beef gravy.

Combined effects of nisin and temperature on viability. Strains F3812/84, F3802A/84, 1230-88, and 038-2 were examined for their ability to survive in BHI broth at 30 and 56°C. Sterile BHI broth (100 ml per 250-ml Erlenmeyer flask) was adjusted to pH 6.50 by adding sterile glacial acetic acid. Broth was supplemented with nisin at concentrations of 0, 1.0, 5.0, 10, and 50 μg/ml and adjusted to 30 or 56°C in a water bath shaker. Cultures (1.0 ml) of vegetative cells (24 h, BHI broth, pH 7.4) of *B. cereus* test strains were added to broth which was then incubated under constant agitation for 15 or 30 min. Cells suspensions were serially diluted in sterile 0.1% peptone and surface spread (0.1 ml in duplicate) on BHI agar (pH 6.50) or BHI agar supplemented with 10 μg of nisin/ml (BHI+N agar). Plates were incubated at 30°C, and colonies were counted after 2 days.

Two strains (F3802A/84 and 038-2) of *B. cereus* were investigated for their ability to survive, grow, and produce diarrheal enterotoxin in beef gravy supplemented with nisin. The first series of experiments was designed to determine retention of viability of vegetative cells in gravy supplemented with nisin and held at 30 or 56°C. A commercially manufactured beef gravy containing beef pieces (pH 5.37) was diluted with sterile tap water (7:3 [wt/wt], gravy/water) and adjusted to pH 5.97 ± 0.02 by adding 1.0 N NaOH. Nisaplin solution was added to gravy to give nisin concentrations of 0, 1.0, 5.0, 10, and 50 μg/ml. After thorough mixing, gravy was dispensed (100 ml) into 250-ml Erlenmeyer flasks and adjusted to 30 or 56°C in a water bath. Tests to determine populations of *B. cereus* in gravy and recovery on BHI agar and BHI+N agar were carried out as described above for BHI broth studies.

The second series of experiments was done to determine the ability of *B. cereus* (strains F3802A/84 and 038-2) to grow and produce diarrheal enterotoxin in beef gravy supplemented with nisin and held at 8 or 15°C. Diluted (7:3) beef gravy (pH 5.97 ± 0.02) supplemented with nisin (0, 1.0, 5.0, 10, or 50 μg/ml) and adjusted to 8 or 15°C was inoculated with appropriately diluted 24-h BHI cultures of vegetative cells or heat-shocked spores of test strains to give populations of 10^3 to 10^4 CFU/ml. The gravy was again thoroughly mixed and then dispensed (10 ml) into sterile test tubes which were sealed and placed in incubators at 8 or 15°C. Triplicate samples of the control (0 μg of nisin/ml) and each treatment were analyzed for populations of *B. cereus* after 0, 1, 3, 6, 9, and 14 days. Since preparation of samples from commercially sterile gravy was done aseptically, the use of a selective medium for enumerating *B. cereus* was not necessary. Instead, BHI agar (pH 7.4) was used. Gravy was thoroughly mixed before withdrawing samples for analysis. Four 0.25-ml samples or duplicate 0.1-ml samples (either not diluted or serially diluted in sterile 0.1% peptone) were surface spread (in duplicate) on BHI agar. Plates were incubated at 30°C for 24 h before colonies of *B. cereus* were counted. The remaining portion of gravy was held at −18°C until being analyzed for enterotoxin.

Diarrheal enterotoxin analysis. Frozen gravy was thawed at room temperature for 1 h; 6.0 g was combined with 6 ml of chilled (5°C) 0.85% sodium chloride (J. T. Baker, Phillipsburg, N.J.) in a stomacher bag and pummeled for 1 min. The mixture was then poured into a 50-ml graduated conical tube (Falcon; Becton Dickinson Labware, Lincoln Park, N.J.) and centrifuged at 1,000 × *g* for 30 min. All samples were kept chilled (4 to 5°C) before and after centrifugation. The supernatant liquid was decanted and filtered through a 0.45-μm-pore-size small-protein-binding filter (Gelman Sciences, Inc., Ann Arbor, Mich.) into a clear, 5-ml glass vial. The filtrate was collected and assayed for the presence of *B. cereus* diarrheal enterotoxin.

BHI broth in which test strains were cultured for 16 h at 37°C was analyzed for the presence of diarrheal enterotoxin. Cultures were filtered and filtrates were collected as described above.

The *B. cereus* enterotoxin (diarrheal type) test kit (BCET-RPLA; Unipath-Oxoid, Columbia, Md.) was used to analyze gravy and BHI broth filtrates for the presence of enterotoxin. A 96-well, V-bottom cell culture cluster plate (Costar, Cambridge, Mass.) fitted with a lid was used; two columns of eight wells were labeled for each sample. Diluent (25 μl of phosphate-buffered saline containing bovine serum albumin) was placed in each well in rows B to H. Test samples (25 μl of filtrate) were placed in each well of rows A and B. The contents of wells in row B were mixed, and 25 μl was transferred to the corresponding well in row C. Double dilutions (1:1 in buffered saline) were successively deposited in rows C through G; row H contained diluent only. Latex sensitized with specific *B. cereus* antienterotoxin (rabbit immunoglobulin G, 25 μl) was added to the first column of each row of samples. A control reagent (25 μl) consisting of latex suspension sensitized with nonimmune rabbit globulins was added to the second column of each row. Plates were agitated by hand and left undisturbed at room temperature for 20 to 22 h. Agglutination in the first column indicated the presence of diarrheal enterotoxin; the second column served as a negative control. Relative amounts of enterotoxin in samples were estimated as described in the test kit instructions, based on a detection sensitivity level of 4 ng/g of gravy.

RESULTS AND DISCUSSION

Heating studies. Populations of *B. cereus* recovered after holding vegetative cells at 30 or 56°C in nisin-supplemented BHI broth for 15 or 30 min are listed in Table 1. The effects of nisin (10 μg/ml) in the BHI agar (BHI+N agar) used to enumerate viable cells are also shown. Without exception, a portion of cells from all four strains of 24-h cultures that had not been exposed to nisin did not form colonies on BHI+N agar. Reductions of as great as 4 $\log_{10}$ CFU/ml (strain F3802A/84) were observed on BHI+N agar compared to BHI agar.

The presence of as little as 1 μg of nisin/ml in BHI broth held at 30°C caused a reduction in the numbers of cells recovered on BHI agar. The magnitude of the reduction was increased as the concentration of nisin was increased to 5, 10, or 50 μg/ml and the holding time was increased to 30 min. The number of cells recovered was further decreased when BHI+N agar was used as an enumeration medium, indicating that a portion of cells were sublethally injured as a result of previous exposure to nisin.

Test strains exhibited differences in tolerance to heat treatment. However, for cells surviving treatment at 56°C for 15 or 30 min (strains 1230-88 and 038-2), the lethal effects of nisin in BHI holding broth and recovery agar were more pronounced than those observed for cells held at 30°C for the respective periods of time. Increased sensitivity of sublethally injured bacterial cells to nisin was also reported by Kalchayanand et al. (27, 28). Bacteriocin-resistant *Lactococcus lactis*, the producer of nisin, has been shown to have increased sensitivity to nisin after being frozen at −20°C for 2 h and then being rapidly thawed. Roberts and Hoover (35) reported that *Bacillus coagulans* spores became more sensitive to nisin after pressurization.

The primary action site of nisin against vegetative cells is considered to be the cytoplasmic membrane, with nisin acting as a voltage-dependent polarizer (8, 36). Henning et al. (21) showed that synthesis of *N*-acetylglucosamine by *Bacillus subtilis* and DNA, RNA, and protein synthesis by *Micrococcus luteus* were inhibited by nisin. They suggested that the antimicrobial effect of nisin is caused by its interaction with phospholipid components of the cytoplasmic membrane followed by interference with membrane function. Dissipation of the proton motive force of *Clostridium sporogenes* is known to be affected by nisin (34), and the rate of efflux of K^+ from *Listeria monocytogenes* is known to increase when cells are exposed to nisin (7). Exposure of heat-stressed microbial cells generally

TABLE 1. Combined effects of temperature and nisin on inactivation of vegetative cells of *B. cereus* in BHI broth

Strain	Holding temp (°C)	Holding time (min)	Recovery medium	Population (log_{10} CFU/ml) of *B. cereus* at nisin concn (μg/ml)[a]				
				0	1	5	10	50
F3812/84	30	0	BHI	5.14				
			BHI+N	4.13				
		15	BHI	5.25	3.85	2.13	1.51	—[b]
			BHI+N	4.18	3.00	1.81	0.94	—
		30	BHI	5.18	2.75	1.65	1.00	—
			BHI+N	4.17	2.41	1.15	1.20	—
	56	0	BHI	5.15				
			BHI+N	4.22				
		15	BHI	—	—	—	—	—
			BHI+N	—	—	—	—	—
		30	BHI	—	—	—	—	—
			BHI+N	—	—	—	—	—
1230-88	30	0	BHI	4.53				
			BHI+N	4.04				
		15	BHI	4.71	4.41	3.80	3.54	2.61
			BHI+N	4.56	4.15	3.47	3.08	2.54
		30	BHI	4.70	4.46	3.72	3.18	2.31
			BHI+N	4.45	4.37	3.44	3.19	2.25
	56	0	BHI	4.66				
			BHI+N	4.44				
		15	BHI	3.45	2.42	2.11	1.81	—
			BHI+N	1.09	—	—	—	—
		30	BHI	2.64	2.44	2.19	1.98	—
			BHI+N	—	—	—	—	—
F3802A/84	30	0	BHI	4.38				
			BHI+N	0.69				
		15	BHI	4.45	1.27	0.43	—	—
			BHI+N	0.87	—	—	—	—
		30	BHI	4.36	—	—	—	—
			BHI+N	0.39	—	—	—	—
	56	0	BHI	4.27				
			BHI+N	—				
		15	BHI	—	—	—	—	—
			BHI+N	—	—	—	—	—
		30	BHI	—	—	—	—	—
			BHI+N	—	—	—	—	—
038-2	30	0	BHI	4.78				
			BHI+N	4.16				
		15	BHI	4.82	4.32	3.68	2.91	1.93
			BHI+N	4.37	4.24	3.35	2.56	1.39
		30	BHI	4.74	4.32	3.34	2.87	1.27
			BHI+N	4.37	4.27	3.02	2.36	—
	56	0	BHI	4.57				
			BHI+N	4.36				
		15	BHI	2.25	1.20	1.00	—	—
			BHI+N	—	—	—	—	—
		30	BHI	1.37	1.20	—	—	—
			BHI+N	—	—	—	—	—

[a] Values are average of three replicate trials.
[b] Less than 1 CFU/ml.

results in cytoplasmic membrane disfunction characterized by a release of nucleic acids, amino acids, ATP, and K^+. Resuscitation occurs only if the integrity of the cytoplasmic membrane can be restored. Observations in our studies on heat-injured cells of *B. cereus* suggest that concentrations of nisin as low as 10 μg/ml prevented this repair process from occurring.

The level of activity of antimicrobial agents can be influenced by the pH and composition of foods. Emulsifiers, for example, diminish the activity of nisin against *Bacillus coagulans* (20). Studies using BHI broth were therefore extended to beef gravy. Two diarrheal enterotoxin-producing strains (F3802A/84 and 038-2) with different sensitivities to nisin were examined. Results of experiments to determine the combined effects of nisin and heat on viability of *B. cereus* in beef gravy, as well as the influence of nisin in BHI recovery agar, are shown in Table 2. As with BHI broth studies (Table 1), strain F3802A/84 was less resistant than strain 038-2 to heat treatment and to nisin in beef gravy and recovery agar. However, both strains tolerated higher concentrations of nisin in gravy compared to BHI broth, and both were less adversely affected by nisin in recovery agar. Our observations on the combined effects of nisin and heat on survival of vegetative cells of *B. cereus* in BHI and gravy are contrary to observations on *B. cereus* spores reported by Dufrenne et al. (10). These researchers found that nisin (250 μg/ml) did not substantially influence the thermal destruction of *B. cereus* spores in phosphate buffer (pH 7.0). The stability and activity of nisin increase with decreasing pH. The pH of beef gravy used in our study was 5.37. Differences in effectiveness may have been due to differences in cell type, pH, or constituents in the heating menstruum.

Growth and enterotoxin production in gravy. Growth characteristics of vegetative cells and spores of *B. cereus* (strains F3802A/84 and 038-2) in beef gravy as affected by temperature and nisin are shown in Table 3. Initial inoculum populations of 6.17 to 7.08 log_{10} CFU/ml (0 day) in gravy not supplemented with nisin were markedly reduced within 1 day. The magnitude of reduction was greater at 8°C than at 15°C and with vegetative cells compared with spores. Strain F3802A/84 grew at both temperatures to populations exceeding the initial inoculum during subsequent storage for up to 14 days. Strain 038-2 gradually lost viability at 8°C, but within 3 days at 15°C, it grew to populations exceeding those at day 0.

Reductions in populations of *B. cereus* within the first day of

TABLE 2. Combined effects of temperature and nisin on inactivation of vegetative cells of *B. cereus* in beef gravy

Strain	Holding temp (°C)	Holding time (min)	Recovery medium	Population (log_{10} CFU/ml) of *B. cereus* at nisin concn (μg/ml)[a]				
				0	1	5	10	50
F3802A/84	30	0	BHI	4.79				
			BHI+N	2.69				
		15	BHI	4.63	3.31	1.31	1.10	—[b]
			BHI+N	2.64	1.96	1.04	—	—
		30	BHI	4.67	2.69	1.15	—	—
			BHI+N	1.56	2.08	1.00	—	—
	56	0	BHI	4.44				
			BHI+N	1.57				
		15	BHI	—	—	—	—	—
			BHI+N	—	—	—	—	—
		30	BHI	—	—	—	—	—
			BHI+N	—	—	—	—	—
038-2	30	0	BHI	4.88				
			BHI+N	4.67				
		15	BHI	4.88	4.72	4.78	4.52	3.61
			BHI+N	4.71	4.57	4.54	4.39	3.37
		30	BHI	4.92	4.74	4.59	4.49	3.42
			BHI+N	4.75	4.68	4.56	4.41	3.43
	56	0	BHI	4.90				
			BHI+N	4.70				
		15	BHI	1.95	2.37	2.05	1.93	1.25
			BHI+N	—	—	—	—	—
		30	BHI	1.91	2.16	1.65	—	—
			BHI+N	—	—	—	—	—

[a] Values are averages of three replicate trials.
[b] Less than 1 CFU/ml.

TABLE 3. Population of *B. cereus* in gravy supplemented with nisin, inoculated with vegetative cells or spores, and held for up to 14 days at 8 or 15°C

Strain	Cell type	Incubation temp (°C)	Nisin concn (μg/ml)	Population (log_{10} CFU/ml) of *B. cereus* at incubation time (days)[a]					
				0	1	3	6	9	14
F3802A/84	Vegetative	8	0	6.81	1.82	2.43	4.84	6.67	7.37
			1		—[b]	—	0.70	3.72	4.12
			5		—	—	—	—	—
			10		—	—	—	—	—
			50		—	—	—	—	—
		15	0	7.05	4.42	6.78	7.80	7.77	7.10
			1		1.75	5.85	7.37	7.85	7.52
			5		—	3.49	5.22	6.48	—
			10		—	3.67	4.91	5.50	—
			50		—	—	—	—	—
	Spore	8	0	6.17	3.70	4.12	6.27	7.46	8.17
			1		2.45	2.33	2.31	3.83	4.03
			5		1.85	1.78	1.71	1.81	2.37
			10		1.56	1.38	1.34	1.07	1.72
			50		0.52	0.70	0.92	—	—
		15	0	7.08	5.88	7.78	7.61	7.16	6.82
			1		2.98	6.58	7.84	7.91	7.13
			5		2.00	1.97	0.96	4.11	6.23
			10		1.91	1.81	—	3.60	6.67
			50		1.03	1.20	—	—	2.92
038-2	Vegetative	8	0	6.76	2.27	2.13	1.77	1.73	1.41
			1		—	—	—	—	—
			5		—	—	—	—	—
			10		—	—	—	—	—
			50		—	—	—	—	—
		15	0	6.90	3.77	7.42	7.46	7.27	7.80
			1		2.78	5.32	7.45	7.45	8.00
			5		1.37	4.80	5.52	6.70	7.24
			10		—	—	4.90	5.08	4.38
			50		—	—	—	—	—
	Spore	8	0	6.16	2.67	2.10	1.60	—	—
			1		1.30	1.14	0.94	—	—
			5		0.69	1.18	1.20	—	—
			10		0.94	1.24	0.79	—	—
			50		—	—	—	—	—
		15	0	6.93	4.91	7.28	7.88	7.35	7.70
			1		1.35	3.77	4.20	5.42	7.55
			5		1.24	0.70	—	—	—
			10		0.57	0.58	—	—	—
			50		0.94	—	—	—	—

[a] Values are averages of three replicate trials.
[b] Less than 1 CFU/ml.

storage were enhanced in gravy containing 1 μg of nisin/ml. The inhibitory effect of nisin was greater on vegetative cells of strain F3802A/84 than on spores and was more pronounced at 8°C than at 15°C. At concentrations of 5 and 50 μg/ml, nisin inhibited growth in gravy inoculated with vegetative cells and stored for 14 days at 8 and 15°C, respectively. Nisin at a concentration of 10 μg/ml was sporostatic through at least 9 days at 8°C and through 6 days at 15°C. Outgrowth of spores at 15°C after 9 or 14 days in gravy containing 10 or 50 μg of nisin/ml, respectively, suggests that nisin may undergo degradation.

Vegetative cells of *B. cereus* 038-2 were likewise more sensitive than spores to nisin (Table 3). Within 1 day at 8°C, the number of vegetative cells was reduced from 6.76 log_{10} CFU/ml to less than 1 CFU/ml in gravy supplemented with 1 μg of nisin/ml; 50 μg of nisin/ml was required to achieve the same reduction in number of viable spores within 1 day at 15°C. In gravy containing 1 μg of nisin/ml which was held at 8 or 15°C, inactivation of spores was enhanced within 1 day. Control of outgrowth of spores at 15°C was achieved by supplementing gravy with 5 μg of nisin/ml.

Compared to observations in a previous study using BHI broth (22), similar concentrations of nisin in beef gravy were not as effective in controlling growth of *B. cereus*. Scott and Taylor (38) observed that nisin was less effective in inhibiting the outgrowth of *Clostridium botulinum* spores in a cooked meat medium (pH 7) than in BHI broth. The explanation for this phenomenon was that nisin binds to meat particles, its effectiveness thereby being limited. Other factors may also have influenced the inhibitory activity of nisin against *B. cereus* in gravy. Type and level of fatty acids (1) and sugars (17), as well as sodium chloride (33, 42) and starch (20), all of which are components of beef gravy, are known to influence the growth of *B. cereus*. Conditions imposed by one or more of these or other factors may have altered the effectiveness of nisin.

Growth of vegetative cells and spores of *B. cereus* in gravy containing nisin after a period of inhibition during storage may be due to loss of activity of nisin. Losses are known to be more rapid as temperature and pH are increased (8). Also, proteolytic enzymes produced by *B. cereus* are known to inactivate

TABLE 4. Titers of diarrheal enterotoxin produced in beef gravy inoculated with vegetative cells or spores of *B. cereus* F3802A/84 and incubated for up to 14 days at 8 or 15°C

Temp (°C)	Cell type	Nisin concn (μg/ml)	Titers (ng/ml) of diarrheal enterotoxin at incubation time (days)[a]				
			1	3	6	9	14
8	Vegetative	0	—[b]	—	—	32	96
		1	—	—	—	—	—
	Spore	0	—	—	—	—	27
		1	—	—	—	—	—
15	Vegetative	0	—	213	192	192	107
		1	—	16	256	256	171
		5	—	—	—	—	—
	Spore	0	—	43	171	213	128
		1	—	3	65	131	96
		5	—	—	—	—	—

[a] Values are averages of three replicate trials.
[b] None detected.

nisin (23, 24). Production of such enzymes by the two strains of *B. cereus* used in our studies may have contributed to reducing the concentration of nisin to levels that no longer inhibited growth.

The production of diarrheal enterotoxin by *B. cereus* in beef gravy as affected by temperature and nisin was determined. Enterotoxin was not detected in BHI broth or any of the gravy samples inoculated with strain 038-2. This strain is apparently unable to produce enterotoxin in gravy at 15°C or may, in fact, have lost its ability to produce enterotoxin, regardless of the substrate. Its ability to produce toxin was not confirmed in our laboratory. Vegetative cells of strain F3802A/84 inoculated into gravy produced enterotoxin at 8 and 15°C within 9 and 3 days, respectively; gravy inoculated with spores was positive for enterotoxin after 14 days at 8°C or after 3 days at 15°C (Table 4). Enterotoxin production was inhibited at 8°C, regardless of the type of cell in the inoculum, by adding nisin at a concentration of 1 μg/ml to gravy; 5 μg of nisin/ml was required for inhibition at 15°C. In gravy stored at 15°C, maximum levels of enterotoxin were followed by decreases as storage approached 14 days. Reasons for this decrease are not clear. Degradation of *B. cereus* enterotoxin in milk (13) was also without explanation. Reaction of enterotoxin with protein components of beef or milk, or degradative enzymatic activity by living or lysed cells of *B. cereus* at some stage following enterotoxin production, is possible.

After an initial decline in population in inoculated gravy, enterotoxin was not detected until *B. cereus* populations reached at least 5.85 $\log_{10}$ CFU/ml (vegetative cell inoculum, 1 μg of nisin/ml; 15°C, 3 days) (Table 3). In an outbreak of *B. cereus* gastroenteritis linked to the consumption of stew, the estimated infective dose was 10^4 to 10^5 CFU/g (13). Kramer and Gilbert (30), however, stated that at least 10^5 CFU/g (ml) are necessary to cause illness. The beef gravy analyzed in the present study, as well as foods analyzed by others (15), has been shown to contain enterotoxin when populations of *B. cereus* are in the range of 10^5 to 10^6 CFU/g (ml). Whether beef gravy which supported growth of *B. cereus* to a population of 5.85 to 7.78 $\log_{10}$ CFU/ml within 3 days at 15°C would cause illness is not known. Results do indicate, however, that growth of *B. cereus* at 15°C with consequent production of enterotoxin and higher risk of illness can be avoided by supplementing gravy with 5 μg of nisin/ml. A concentration of 1 μg of nisin/ml was effective at 8°C.

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