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Influence of Type of Food on the Kinetics and Overall Production of *Bacillus cereus* Emetic Toxin

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ABSTRACT

Potato puree and penne pasta were inoculated with cereulide producing *B. cereus* 5964a and *B. cereus* NS117. Static incubation at 28°C proved these two foods to be a better substrate for higher cereulide production (4,080 ng/g in puree and 3,200 ng/g in penne were produced by *B. cereus* 5964a during 48 h of incubation) compared with boiled rice (2,000 ng/g). This difference occurred despite *B. cereus* counts of more than 10^8 CFU/g in all three products. Aeration of cultures had a negative effect on cereulide production, causing concentrations more than 10-fold lower than in some statically incubated samples. Cereulide production remained undetectable in shaken milk, whereas it reached 1,140 ng/ml in statically incubated milk. At 12 and 22°C, presence of background flora was also a determinative factor. A total *B. cereus* count of more than 10^6 CFU/ml did not necessarily lead to uniform cereulide production and was also dependent on the *B. cereus* strain involved. In this study, we confirm that a number of factors play a crucial role in the determination of the extent to which, if at all, cereulide will be produced. Among those, type of the food, temperature, pH, and whether additional aeration (via incubation on an orbital shaker) is induced had an important role. An important effect was also induced by the cereulide-producing strain involved.

Bacillus cereus is a versatile foodborne pathogen that can exhibit a variety of pathogenic mechanisms, most of which are related to extracellular peptide or proteinlike substances. A small (1.2 kDa) dodecadepsipeptide named cereulide is an emesis causing exotoxin produced by some strains of *B. cereus* and represents potentially the most dangerous of its virulence factors. It has been recently confirmed that it is produced by nonribosomal peptide synthesis *(14, 23).*

Cereulide acts as an ionophor for potassium ions, transporting them via an ion carrier system into the mitochondria downstream of the electrical and concentration gradients. The damaged mitochondria will fail in their oxidoreductive functions *(4, 5).* Cereulide's heat stability (126°C over 90 min) and resistance to extreme pH (pH 2 to 11) and resistance to proteolytic enzymes *(11)* render it difficult to eradicate or inactivate in foods. Therefore, the major threat is posed by cereulide preformed in foods. The clinical dose, suggested to be ca. 10 μg/kg of body weight *(20),* is higher than those of some other known toxins (e.g., *S. aureus* intoxication that resembles symptoms of cereulide intoxication induces mild effects at concentrations as low as 100 ng/kg of body weight *(6)*). Certain rice-containing bakery products were found to hold cereulide in concentrations of 5 to 8 μg/g *(16).* Even lower concentrations of cereulide, ranging from 0.01 to 1.280 μg/g, were reported in foods implicated in emetic-type food poisoning *(3).*

Although intoxication has in most cases been self-limiting, fatal outcomes have been reported *(7, 8, 18).* Statistical data on incidences of cereulide intoxication most likely underestimate the actual numbers because they are biased by the usually mild nature of the illness, remaining unreported to medical institutions *(24).*

For all of the foods reported by Agata et al. *(3)* high starch content was a common feature, even though cereulide strains are known to be unable to hydrolyze starch *(1, 21).* Epidemiology has established well-known connections between cereulide-related foodborne poisonings and farinaceous foods *(3, 10, 13, 17).* However, pure starch might not have the needed nutrients for cereulide production and three amino acids, valine, leucine, and threonine, were found essential for growth and toxin production by *B. cereus (2).*

At 10^5 CFU/g, an emetic-producing strain can generate a substantial amount of toxin that could cause illness 0.5 to 6 h after ingestion. The literature, however, suggests that actual cereulide production can be initiated by lower numbers of *B. cereus,* ca. 10^3 CFU/g *(17).*

Cereulide is most often detected by its biological activity. Loss of boar sperm motility in the presence of cereulide has been shown to be a useful detection device, both in laboratory media and foods *(4, 5).* Computer-aided semen analyses (CASA) of the boar semen and high-performance liquid chromatography with mass spectometry (HPLC-MS) were used to study cereulide production under different incubation conditions and with different cereulide-

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producing strains involved. Attention was given to starch rich products to understand the potential threats of ready-to-eat meals containing sauces, pasta, rice, and potato, all rich in starch content. Milk, which is frequently associated with *B. cereus (19)*, was also included in the study.

MATERIALS AND METHODS

***B. cereus* strains and preparation of inoculation.** Two cereulide-producing strains used in the experimental setup. *B. cereus* 5964a, isolated from a cold meal implicated in fatal foodborne outbreak *(7, 8)* (provided by K. Dierick, Belgian Institute of Public Health) and NS117 (culture collection of the research group of M. Salkinoja, University of Helsinki). As a non–cereulide-producing strain, *B. cereus* F528 (culture collection of the research group of M. Salkinoja) was used as a negative control. The strains were grown in brain heart infusion (BHI; Oxoid, Basingstoke, UK) broth for 24 h at 37°C. From the appropriate dilution in peptone saline solution (8.5% NaCl [Vel, Leuven, Belgium] and 1% peptone [Oxoid]), 0.1 ml of culture was taken and used as inoculum for foods, providing an inoculation level of ca. 3.5 log CFU/g (ml) of food in the first two experiments and ca. 6 log CFU/g in the third experiment.

Food samples and incubation conditions. Five different groups of commercial ready-to-eat food products—potato puree, pasta (penne and farfalle), rice, béchamel sauce, and milk—were obtained from a local producer on the day of production. Experimental setup comprised 224 samples tested in three separate experiments. Whereas in the first experiment the effect of background flora was included in the test, in the second and the third experiments, a heating step (80°C for 10 min) was introduced before inoculation to eliminate background flora. In the first and second experiments 25-g portions of food (penne and farfalle in the first experiment; penne, potato puree, and béchamel sauce in the second experiment) were inoculated in duplicate with *B. cereus* 5964a or F528 strain, packaged in stomacher bags (180 by 300 mm; Novolab, Geraardsbergen, Belgium), and further incubated at either 12°C (representing severe temperature abuse) or 22°C (representing room temperature). For every analysis, a blank (uninoculated) sample of each food also was included. In the third experiment 50 g (ml) of foods (penne, potato puree, rice, and milk) was mixed with peptone saline solution in 500-ml SCOTT bottles at a ratio of 1:5 to enhance the effect of aeration induced on a horizontal orbital shaker. Following heat treatment of 10 min at 80°C, food was inoculated with *B. cereus* 5964a and F528, and SCOTT bottles with inoculated foods were incubated statically or on a 150 rpm orbital shaker at 28°C. In addition, potato puree and milk were inoculated with *B. cereus* NS117 strain to gauge the effect on cereulide production when all other conditions are kept identical. For all foods, pH and water activity (a_w) were determined by a pH meter (type 763, Knick, Berlin, Germany) with the use of an electrode (Ingold 104063123, Urdorf, Switzerland) and a cryometer (AW-Kryometer, type AWK-20, NAGY Messsysteme GmbH, Gäufelden, Germany), respectively.

Monitoring of *B. cereus* growth in foods. Food samples from the first two experiments were diluted 10-fold and homogenized in peptone saline solution with a stomacher (Lab-Blender 400, Led Techno, Eksel, Belgium). A 10-fold serial dilution was made, and from the appropriate dilution, 100 μl was spread plated on a *B. cereus* agar medium (Lab M, Bury, Lancashire, UK), establishing a detection limit of 100 CFU/g. After incubation for 24 h at 30°C, typical *B. cereus* colonies were enumerated. For every food product and every strain involved, duplicates were made and blank samples were included. Samples incubated at 12°C were analyzed once a day for 6 days, whereas samples incubated at 22°C were analyzed after 18, 24, 42, and 48 h of incubation. Mesophilic aerobic counts on plate count agar (Oxoid; 24 to 72 h, 30°C), yeast and mold counts on yeast glucose chloramphenicol agar (Bio-Rad, Marnes-la Coquette, France; 3 to 5 days, 30°C), and lactic acid bacteria count on deMan Rogosa Sharpe agar (Oxoid; 72 h, 30°C) also were determined at every analysis for foods containing background flora (experiment 1).

Food samples that were in SCOTT bottles incubated at 28°C (static and shaken) were sampled every 6 h for *B. cereus* enumeration, either directly from the food suspension (detection limit of 10 CFU/g) or from an appropriate dilution step following the procedure described above. This protocol is standard in the laboratory; therefore, no replicated analyses were made.

Boar semen motility assay and HPLC-MS analysis of cereulide production. A boar semen motility assay was performed with minor modifications *(22)* to the original protocol *(4, 5)*. Computer-aided semen analyses by a Hamilton-Thorne computer-aided semen analyzer (HTR Ceros 12.1, Hamilton Thorne Research, Beverly, Calif.), consisting of an Olympus phase contrast microscope, a camera, a MiniTherm stage warmer providing a temperature of 37°C, an image digitizer, and a computer to save and analyze data, was used for relative quantitative and semiquantitative determination of cereulide presence in samples by measuring change in the percentage of progressive motility (PMOT%) of semen as a function of time. In short, 195 μl of sperm (Belgian Piétrain extra muscled standardized to a concentration of ca. 30 million cells per ml) was mixed with 5 μl of solution (blank or with cereulide or valinomycin) in a microtiter plate (96-well format) and immediately transferred into a Leja slide (standard count two-chamber, 20-μm slide, Leja, Nieuw-Vennep, The Netherlands). If only a relative quantitative toxicity evaluation was required, undiluted sample was subjected to the test, and results were dependent on the amount of cereulide obtained within 10 to 300 s. When quantitative estimation was required, the sample was serially diluted with dimethyl sulfoxide (Sigma-Aldrich, Steinheim, Germany) in a twofold serial dilution *(22)*. Two consecutive dilution steps giving PMOT% change in a semilinear range of a valinomycin standard curve were used to calculate cereulide concentration. The valinomycin standard curve was plotted with known concentrations of valinomycin on the *x* axis against the ratio of PMOT% change over the time period ($\Delta PMOT\%/\Delta\tau$) on the *y* axis. The standard curve application was limited to a concentration range of 20 to 400 ng/ml. Above 400 ng/ml, the curve remained parallel with the concentration axis, indicating that a further increase in valinomycin concentration would not result in measurable changes in progressive motility, thus approaching an asymptote at about $\Delta PMOT\%/\Delta\tau = -6.00$).

HPLC-MS analyses were performed as described elsewhere *(12, 15, 16)*.

Samples for cereulide determination were taken in parallel with those intended for *B. cereus* enumeration. Sampling consisted of taking 3 g (3 ml) of a food product into an open 10-ml conical flask, mixing with a double volume of methanol, and extracting at 100°C for 20 min (or until the liquid phase was evaporated). When all liquid was evaporated, a new 3 ml of methanol was added and mixed well for ca. 2 min; then, the whole volume of methanol was transferred into a 4-ml screw neck glass vial. Open vials were subjected to evaporation under N_2 either immediately or after partial evaporation under the air stream. The dry residue was suspended in 200 to 500 μl of dimethyl sulfoxide and kept at −20°C until analysis.

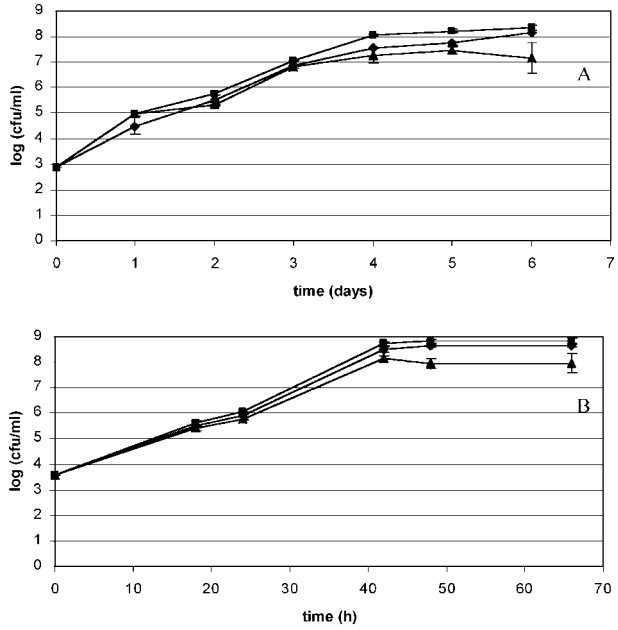

FIGURE 1. *Growth of* B. cereus *5964a in thermally treated potato puree (◆), sauce (▲), and penne (■) at 12°C (A) and 22°C (B) with* y*-axis error bars indicating standard deviation (*n *= 2).*

RESULTS

Bacterial enumeration and physical parameters. Counts of *B. cereus* 5964a in potato puree, penne, and sauce at 12 and 22°C (Fig. 1) showed unhampered growth when no background flora was present to compete with *B. cereus.* At both incubation temperatures and in all three food products, cereulide-producing *B. cereus* 5964a was able to reach counts of more than 10^7 CFU/g. Although 13 h was sufficient to cross the safety limit of 10^5 CFU/g at 22°C, it required ca. 24 h in penne and sauce and ca. 36 h in potato puree to cross this limit at 12°C.

In thermally nontreated samples, *B. cereus* counts at 12°C remained below the detection limit in penne and reached ca. 6 log CFU/g in farfalle after 96 h of incubation. Counts of total mesophilic flora and lactic acid bacteria at the end of the incubation at 12°C were 9.7 and 6.9 log CFU/g, respectively, for penne and 9.05 and 5.4 log CFU/g, respectively, for farfalle. At 22°C, *B. cereus* counts reached ca. 7.4 log CFU/g in penne and 8.1 log CFU/g in farfalle after 48 h of incubation. At the same time, total mesophilic count and lactic acid bacteria counted 8.9 and 7 log CFU/g, respectively, for penne and 8 and less than 5 log CFU/g, respectively, for farfalle.

Figure 2 shows growth of *B. cereus* 5964a in semiliquid potato puree, penne, rice, and liquid milk at 28°C. *B. cereus* NS117 (data not shown) grew to lower counts (maximum difference being 1 log unit after 48 h of incubation at 28°C) in the tested potato puree. In milk, the growth of NS117 was at every sampling point ca. 0.5 log CFU/ml higher than the growth of 5964a.

Although pH value of thermally nontreated penne dropped during incubation from ca. 6.5 to ca. 5.7, both at 12 and 22°C, pH of farfalle remained stable at ca. 6.5. Water activity of both penne and farfalle remained at ca. 0.996.

In thermally treated foods incubated at 12 and 22°C, the initial pH value of sauce was lowest (5.8). Sauce also had the lowest a_w of ca. 0.994. Potato puree and penne had an a_w of ca. 0.997 and pH of ca. 5.9 and 7.4, respectively. Potato puree incubated at 22°C had initial pH 6.4. These values remained stable during incubation.

Detection of cereulide in food samples. No cereulide was detected in thermally nontreated samples incubated at 12°C. At 22°C, only farfalle induced loss of motility in semen. Sample of penne did not show presence of cereulide.

High *B. cereus* 5964a counts in penne and potato puree resulted in cereulide production at both 12°C (after 4 and 5 days, respectively) and 22°C (after 42 and 24 h, respectively). Results of cereulide detection by loss of boar semen motility when exposed to the food extracts of potato puree, penne, and béchamel sauce inoculated with *B. cereus* 5964a and incubated at 12 and 22°C are shown in Figures 3 and 4 for respective incubation temperatures. No detectable cereulide concentrations were found in sauce at any of the incubation temperatures. Boar semen motility inhibition was not observed with negative controls (samples inoculated with nonemetic strain, *B. cereus* F528).

Inoculation of thermally treated semiliquid (1:5 dilution) potato puree, penne, and rice, as well as of commercially obtained ultrahigh-temperature sterilized milk, with 10^6 CFU/ml of cereulide producing *B. cereus* 5964a resulted in cereulide production that was largely dependent on the food product and incubation manner (150 rpm versus static, both at 28°C). The highest cereulide production as determined by HPLC-MS (Fig. 2) was for every food product observed in the statically incubated samples, ranging from below the detection limit (10 ng/g) up to 4,080 ng/g for potato puree, 1,000 to 3,300 ng/g for penne, 120 to 2,010 ng/g for rice, and 55 to 1,140 ng/ml for milk. For strain 5964a, potato puree and penne were the better substrates for growth and cereulide production, although rice has been the most reported food. Amount of cereulide found in rice after 48 h of static incubation was on the order of 2,000 ng/g (concentration detected with boar semen bioassay). Also, in statically incubated milk, large amounts of cereulide were detected (1,140 ng/ml).

Additionally, a strain of *B. cereus* NS117 showed a somewhat lower extent of cereulide production in tested potato puree. In milk after 48 h of incubation, *B. cereus* NS117 produced cereulide at 260 ng/ml.

DISCUSSION

It was shown in this study that growth of *B. cereus* to 5 log CFU/g will not necessarily result in cereulide production. However, when extrinsic, intrinsic, and implicit factors create a favorable environment, resulting cereulide production can accumulate to toxic levels in foods. These results remain in agreement with the findings of others *(3, 16).* Of all tested foods by Agata et al. *(3),* rice was reported as the food containing the most cereulide when artificially inoculated with the cereulide-producing *B. cereus* NC7401 strain. The amount of cereulide found

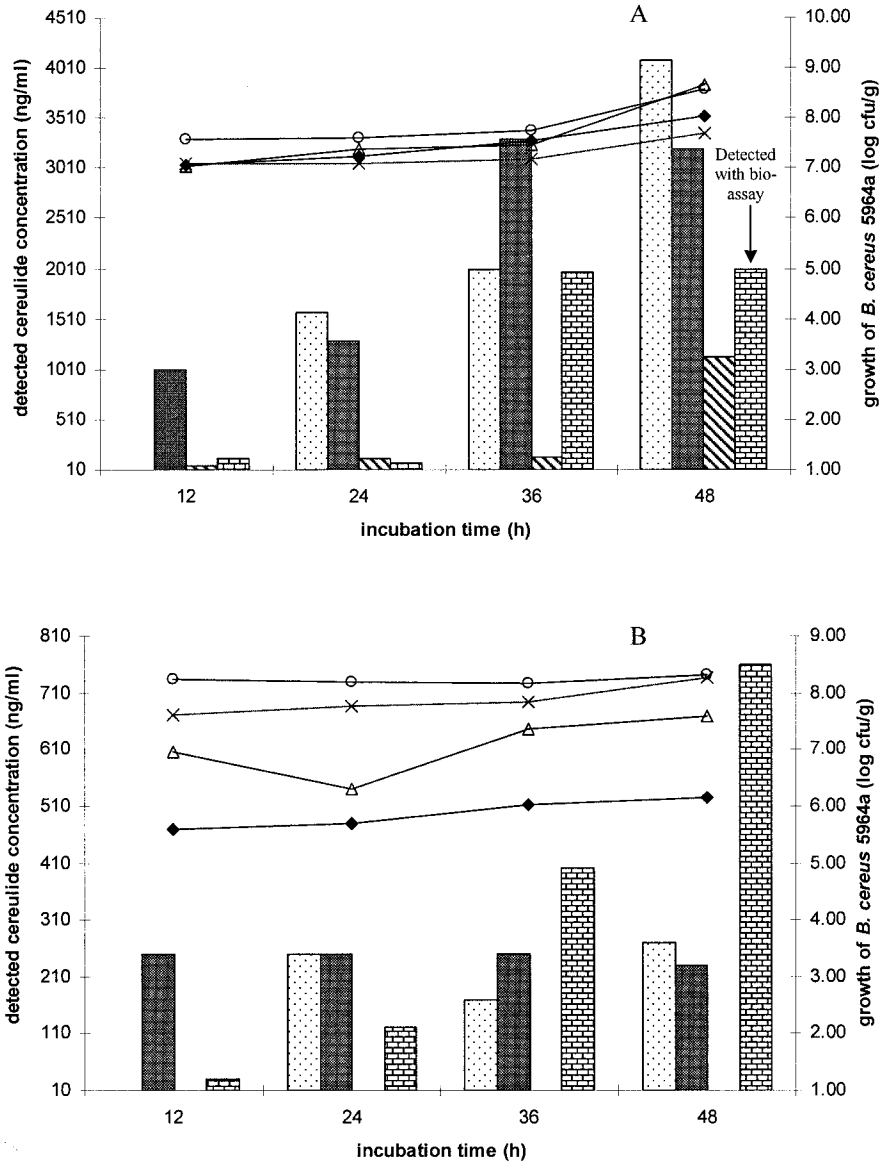

FIGURE 2. *Production of cereulide by* B. cereus *5964a incubated at 28°C in (A) statically and (B) shaken potato puree (dots), penne (shaded), rice (bricks), and milk (stripes) with* B. cereus *5964a growth in potato puree (△), penne (♦), rice (○), and milk (×) enumerated. Unless otherwise indicated, cereulide was detected with HPLC-MS (detection limit 10 ng/ml); enumeration was done in duplicate with a classical spread plate technique and detection limit of 10 CFU/ml.*

in potato puree and penne in this study stresses that rice does not necessarily support the best cereulide production. *B. cereus* 5964a grew to the highest counts and produced the highest amount of cereulide at the highest pace in penne. This was to be expected because the strain was originally isolated from a cold pasta salad implicated in a fatal foodborne outbreak in Kinrooi, Belgium *(7, 8).* These results remained consistent at both 12 and 22°C. Also, at 28°C, strain 5964a produced very high amounts in penne, which after the first 12 h of incubation, were higher than in potato puree and rice. However, the final amount after 48 h of incubation at 28°C was found to be higher in potato puree. Here, not only temperature played a role, but also pH of the potato puree at 28°C, which was 6.40 instead of 5.89 as in the experiments at 12 and 22°C. The lack of a detectable amount of cereulide in sauce can be partially explained by the lowest pH and a_w values of all tested foods. Although not too low, this pH could contribute to the slower rate of cereulide production *(4).* These findings contribute to the current understanding of the type of foods implicated in cereulide poisonings.

In 13 of 14 food samples implicated in cereulide-induced foodborne poisonings in Japan, the amounts of cereulide found ranged from 0.01 to 1.28 μg/g *(3).* This is in agreement with the findings of this study, in which 1 to 3 μg/g was found in tested foods. Results of this work also agree with the low levels of cereulide found in milk, with the difference that Agata et al. *(3)* found more cereulide in shaken samples and no cereulide in curdled milk.

The same authors found that at tested temperatures (30 and 35°C) *B. cereus* in boiled rice rapidly increased to 10^7 to 10^8 CFU/g and produced emetic toxin. The data of Jaaskelainen et al. *(16)* reported rice-containing pastries as a reservoir of high cereulide concentrations (0.3 to 5.5 μg/g of wet weight) when stored at room temperature. Finlay et al. *(9)* reported detectable amounts of cereulide after 48 h of incubation at 15°C compared with 24 h of incubation at 20 and 30°C. Current data indicated penne and potato puree as foods that were able to support cereulide production even at 12°C when no background flora was present.

Although the literature reports that production of cer-

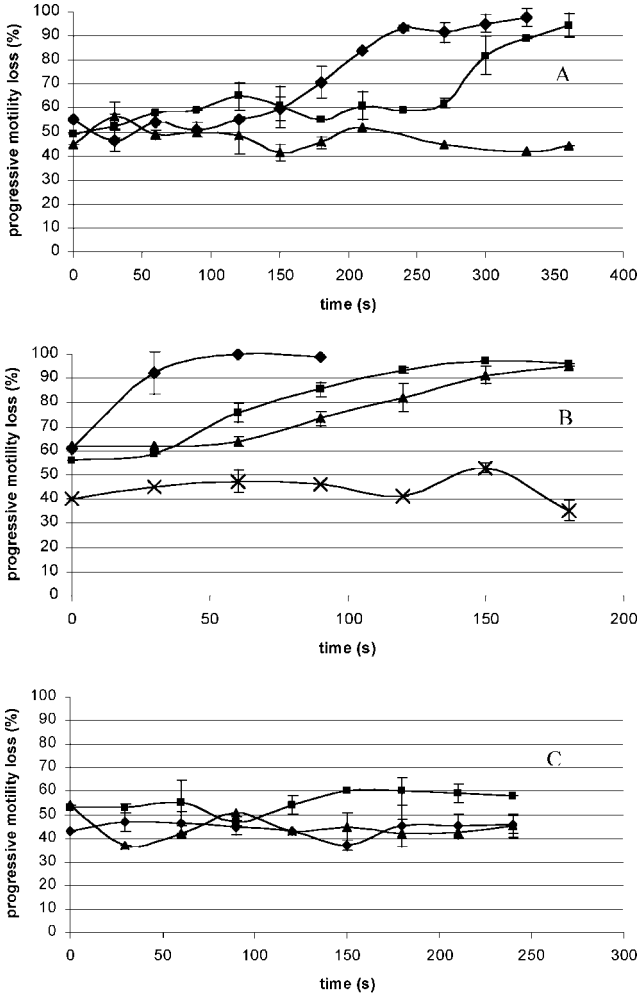

FIGURE 3. *Toxin production by* B. cereus *5964a: evolution of boar semen motility when exposed to the extract of potato puree (A), penne (B), and sauce (C) after incubation at 12°C for 6 (♦), 5 (■), 4 (▲), and 3 (×) days. The* y*-axis error bars indicate the 95% confidence interval (*n *= 6 to 10).*

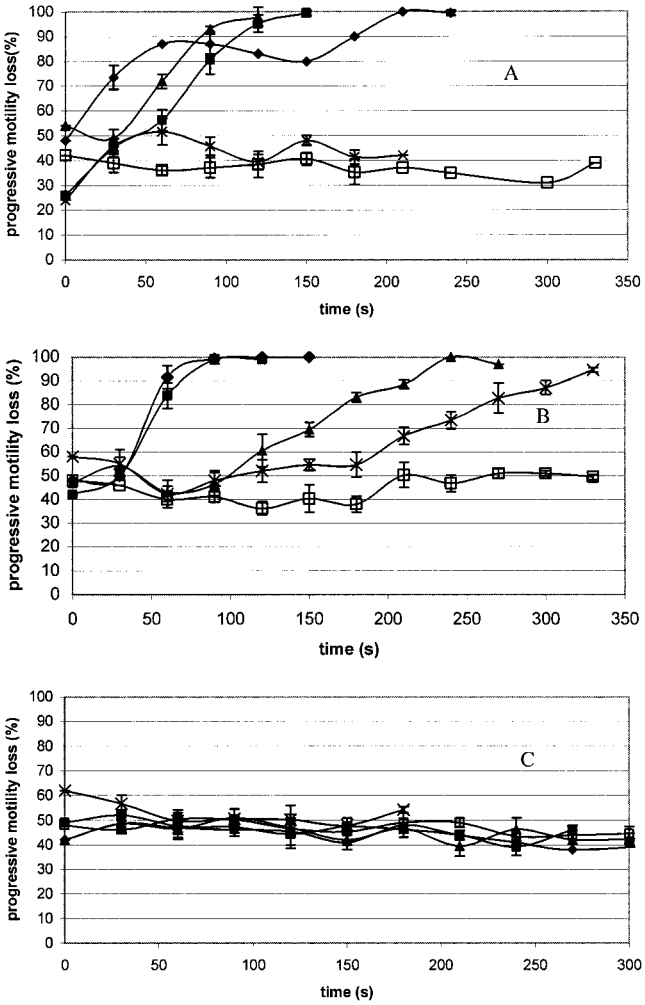

FIGURE 4. *Toxin production by* B. cereus *5964a: evolution of boar semen motility when exposed to the extract of potato puree (A), penne (B), and sauce (C) after incubation at 22°C for 66 (♦), 48 (■), 42 (▲), 24 (□), and 18 h (*). The* y*-axis error bars indicate the 95% confidence interval (*n *= 6 to 10).*

eulide can be enhanced by incubation with shaking in contrast with static conditions *(9, 12)*, results presented here showed, as seen in all food products and both tested strains, that cereulide concentrations found in statically incubated samples were 14 to 15 times higher than in shaken samples after 48 h of incubation. Data reported elsewhere *(22)* support current findings. Although the effect of shaking is of no relevance for solid foods, for liquid or semiliquid foods, some shaking, although limited, can still occur during the production process (e.g., reconstitution of a milk powder for standardization of a milk batch). Here, reported findings bring another insight into the possible effects on cereulide production of shaking. Although we report that shaking at 150 rpm had a negative effect on cereulide production, it remains unclear where the differences with the findings of other authors lie.

Bacillus cereus counts at which cereulide was detected were different in different foods and at different temperatures. At 12°C, the counts had to be higher than 7 log CFU/g in all foods. At 22°C, the required counts were dependent on the food but were ca. 6 log CFU/g. Because stationary phase can only begin at counts higher than 8 log CFU/g, it is apparent that cereulide production can begin in the early stages of exponential phase. Although detected concentrations of cereulide were always related to *B. cereus* counts of 6 log units and above, no exclusive connection between higher counts and higher cereulide concentrations was noticed. This was especially the case in shaken foods, in which aeration and physical dispersion of cells induced lower cereulide production. Therefore, no clear evidence can be given that even when a cereulide-producing strain attains a level of 6 to 7 log CFU/g will there be enough cereulide produced to be toxic.

It is clear that the regulatory mechanism of cereulide production is not straightforward and that the role played by the type of culture medium, type of food, environmental conditions, and presence of background flora is of utmost importance for cereulide production.

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