

Prevalence and Fate of *Bacillus cereus* in African Traditional Cereal-Based Foods Used as Infant Foods

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ABSTRACT

The objective of the present work was to estimate the prevalence of *Bacillus cereus* group species in traditional cereal-based lactic acid-fermented slurries and nonfermented flours used to prepare infant foods in an African context. High counts on mannitol–egg yolk–polymixin agar medium were determined for the fermented slurries (median, 4.5×10^4 CFU/ml of slurry) compared with the nonfermented flours, most of whose counts were lower than 10^{-1} CFU/g. Virulence genes were characterized in 60 isolates from 26 traditional cereal-based foods in Ouagadougou (Burkina Faso). Seventy-two and 38% of isolates were positive for the complete set of genes coding for hemolysin BL and nonhemolytic enterotoxin, respectively, suggesting a high enterotoxigenic potential for these foodborne isolates. No potentially emetic toxin-producing strains were detected. Because of the high counts found for fermented slurries, survival tests with vegetative cells inoculated in fermented slurries were performed, which showed that growth of *B. cereus* was inhibited. This result suggests that fermentation in traditional production units is presumably not adequately controlled, enabling growth during any unit operations before fermentation, or even during the fermentation step, when the process was poorly controlled. However, adding nisin (0.1 mg/ml) enabled a 5-log reduction in the *B. cereus* population in 5 h, suggesting that the use of nisin could be a way to upgrade the hygienic quality of this type of food.

In sub-Saharan Africa, cereals are considered one of the most important sources of nutrients (8), and cereal-based gruels are often given to young children younger than 5 years as complementary foods to breast feeding (12). These foods are produced in traditional production units under poor sanitary conditions (11). In addition, traditional gruels made from fermented pearl millet slurries are retailed as ready-to-eat street foods and consumed by the population, including young children, in Ouagadougou (Burkina Faso) (18). Considering traditional processing, retailing, and storage conditions, *Bacillus cereus* contamination by soil and dust can therefore be expected (1, 11), and thus represents a potential health hazard that must be evaluated, particularly when the health of infants and young children is at stake.

Diarrhea is a leading cause of death (25%) among children aged 1 to 59 months in sub-Saharan Africa (9) and is also linked to weight loss and reduced growth in children (16). Compared with other pathogens and other types of foods, there is a paucity of data in Africa regarding the prevalence of *B. cereus* in infant foods in relation to diarrheic syndrome in young children. Despite reports indicating the presence of *B. cereus* in some African foods

in South Africa and Nigeria (10, 13), the prevalence of *B. cereus* food poisoning is probably underestimated in infant foods for a number of reasons (4), including misdiagnosis of the illness, which is symptomatically similar to other types of food poisoning. Indeed, two distinct types of foodborne diseases, emetic and diarrheal, are associated with *B. cereus*. The symptoms of the diarrheal type of the disease mimic those of *Clostridium perfringens* food poisoning, while the symptoms of the emetic type mimic those caused by *Staphylococcus aureus* foodborne intoxication (5). The emetic toxin is a small, ring-formed dodecadepsipeptide, whereas the diarrheal disease is caused by one or more enterotoxins (5). The main enterotoxins that have been implicated as etiological agents of the diarrheal disease are pore-forming cytotoxins hemolysin BL (HBL), nonhemolytic enterotoxin (NHE), and cytotoxin K (CytK) (1).

The objective of the present work was to evaluate the prevalence of *B. cereus* in traditional cereal flours or fermented pearl millet slurries used to prepare infant foods in Ouagadougou, and the use of nisin as a further barrier to inactivate *B. cereus* vegetative cells.

MATERIALS AND METHODS

Isolation of the strains and identification. Two groups of samples were purchased in Ouagadougou (Table 1). The first

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TABLE 1. Isolates of *Bacillus cereus* from fermented pearl millet slurries and maize or pearl millet flours used to prepare infant gruels in Ouagadougou (Burkina Faso)

Food type	Samples (n)	<i>B. cereus</i> isolates (n = 60)
Pearl millet slurries (fermented)	17	AMYP 2,2; AMYP 2,3; AMYP 7,4; AMYP 8,5; ABP 6,1; BMYP 7,1; BMYP 7,3; BMYP 7,7; BMYP 7,9; BMYP 7,10; BMYP 8,6; BBP 5,1; BBP 5,2; BBP 7,3; BBP 8,2; BMC 1,2; CMYP 2,1; DMYP 9,4; 4PL1; 4PL3; 11PL1; 11PL2; 11PL3; 11PL4; 11PL5; 11PL6; 12PL1; 12PL2; 12PL3; 12PL4; 12PL5; 12PL6
Pearl millet flours (unfermented)	8	AM1; AM2; AM3; AM4; AM5; AF1; AF2; AF3; BF1; BF2; BF3; BF4; BF5; BF6; BF7; DF1; DG1; EF1; EF2; GF1; GF2; GF3; GF4; GF1b; GF2b; GF3b; GG1
Maize flour (unfermented)	1	FS1

corresponded to fermented pearl millet slurries purchased in traditional household producing units, and the second to dry products, i.e., nonfermented flours of pearl millet, and one sample of maize flour produced in small-scale traditional production units and purchased from street vendors. These products are used locally to prepare gruels consumed as complementary food to breastfeeding for young children.

For isolation and numeration, serial dilutions of each sample were plated on the *B. cereus* selective medium mannitol–egg yolk–polymixin agar (MYPA; Difco, BD, Sparks, MD). The isolates were characterized for their Gram stain coloration (Color Gram 2 kit, bioMérieux, Inc., Marcy l'Etoile, France) and production of catalase (ID Color Catalase, bioMérieux, Inc.) and identified according to their biochemical characteristics by using the API 50 CHB gallery (bioMérieux, Inc.) and by sequencing of 16S rRNA gene. For the 16S rRNA gene-based typing, DNA was extracted from the pellet of overnight cultures by using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI), with an additional lyses step with an amalgamator with zirconium beads (VWR, Fontenay-sous-Bois, France). Primers W001 (2, 3) and 23S1 were used to amplify the 16S rRNA-coding gene including the intergenic region located between 16S and 23S rRNA. PCR product was sequenced by Eurofins MWG, GmbH (Ebersberg, Germany). Each sequence was identified by comparison with the Ribosomal Database Project II (<http://rdp.cme.msu.edu>).

The genomic diversity of the isolates was analyzed by using REP-PCR with primers Bc-REP-1 (5'-ATTAAAGTTTCACTT-TAT-3') and Bc-REP-2 (5'-TTTAATCAGTGGGG-3'), as previously described (15, 19).

Detection of genes coding for the toxins. Genes *hblC*, *hblD*, and *hblA*, coding for HBL; genes *nheA*, *nheB*, and *nheC*, coding for NHE; and gene *cytK*, coding for CytK were detected with PCR, under conditions as described by Guinebreteire et al. (7). Emetic toxin-producing *B. cereus* strains were detected by the molecular assay described by Ehling-Schulz et al. (5). Primers EM1F (5'-GACAA-GAGAAATTTCTACGAGCAAGTACAAT-3') and EM1R (5'-GC-AGCCTTCCAATTACTCCTTCTGCCACAGT-3') were used to enable amplification and detection of putative nonribosomal peptide synthetase gene fragments in *B. cereus* (5). The type strain *B. cereus* ATCC 14579^T, which contains all the genes coding for HBL, NHE, and CytK, and the emetic reference strain F4810/73 (LMG 12334) were included as positive controls.

All PCR amplifications were made with the Applied Biosystems Veriti thermocycler (VWR, Strasbourg, France), and tests were performed in triplicate.

Survival of isolates in pearl millet slurries. Tests were performed to estimate the survival of *B. cereus* isolates in pearl millet slurries under different inoculation conditions (contamination): inoculation in sterile pearl millet slurries (unfermented),

inoculation at the beginning of the natural fermentation step, and inoculation at the end of the fermentation step. An additional test was performed with nisin (Sigma-Aldrich, St. Louis, MO) added at a final concentration of 0.1 mg/ml to the fermented slurries and then inoculation with *B. cereus*. Pearl millet slurries were prepared at the laboratory. Spontaneous lactic acid fermentation occurred, as described in previous works (18). Tests were performed in duplicate and incubated at 30°C. Overnight cultures of *B. cereus* isolates cultivated in medium MYPA at 30°C were washed twice in 0.9% NaCl and inoculated at 6% (vol/vol). Samples were taken immediately after inoculation, and then every hour for 6 h. Serial dilutions were plated in duplicate on MYPA for numeration. Before spores were counted in the slurries, samples were first heated in a water bath at 80°C for 10 min.

RESULTS

Prevalence and characterization of *B. cereus* group isolates in cereal-based foods used to make complementary foods for young children. A total of 26 cereal-based foods sampled in Ouagadougou were investigated for the occurrence of *B. cereus* group strains. For the fermented pearl millet slurries, the median of count on MYPA medium was 4.5×10^4 CFU/ml of slurry (minimum of 2×10^1 , maximum of 1×10^7). The majority of dry flours had counts lower than 10^1 CFU/g, except for three samples whose counts were as follows: 10^1 , 2.2×10^2 , and 1.5×10^3 CFU/g. Sixty isolates were obtained from the food samples (Table 1); all were gram positive, catalase positive, and identified as belonging to the *B. cereus* group by using the API 50 CHB galleries. Identification by sequencing of the 16S rRNA gene was at 99% similarity or higher. REP-PCR showed large diversity among isolates that were divided into two clusters, with one cluster grouping 83.6% of the isolates. Regarding the prevalence of isolates harboring genes coding for HBL, NHE, and CytK, 72% of the collection was positive for the complete set of genes (*hblC*, *hblD*, and *hblA*) coding for HBL. Only 38% of the isolates carried the three genes coding for the NHE enterotoxin. For CytK, 83% of the collection was shown to have the potential for its synthesis. The PCR test to detect cereulide-producing *B. cereus* was negative for all 60 isolates.

Survival of *B. cereus* group isolates in pearl millet slurries. Since a relatively high number of *B. cereus* group isolates were identified in fermented pearl millet slurries, their survival was evaluated when pearl millet slurries were contaminated either at the beginning or at the end of the

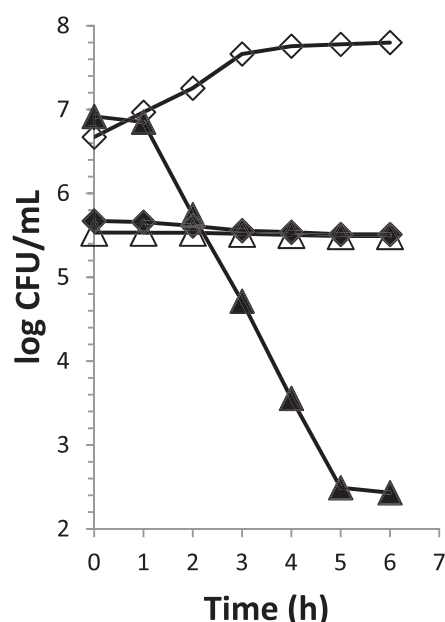


FIGURE 1. Survival tests of the isolate BMYP7,3 inoculated in pearl millet slurries (PMS): inoculation in sterile PMS (unfermented) (◇); inoculation at the beginning of natural fermentation of PMS (◆); inoculation at the end of the fermentation without nisin added (△) or with nisin added (▲).

fermentation step. Tests performed with the isolate BMYP7,3, belonging to the cluster that groups a majority of isolates, showed that it was able to grow in a sterile pearl millet (unfermented) slurry, whereas no growth was observed when the strain was inoculated at the beginning of the fermentation or after 6 h of incubation in pearl millet slurries inoculated at the end of fermentation (Fig. 1). However, in either condition, the *B. cereus* population remained stable from the beginning to the end of the incubation period (Fig. 1). The same tests were performed with other isolates (AMYP7,4, AMYP2,3, ABP6,1, BBP5,1, BBP5,2, and BBP7,3), which showed the same capacity to survive fermentation (data not shown), and the same results were obtained when gruels (cooked fermented slurries) were inoculated with BMYP7,3. In order to investigate a possible and convenient way to reduce the level of contamination, nisin was added to the fermented slurries before inoculation with strain BMYP7,3. The result was a sharp decrease in *B. cereus* counts, corresponding to about 5 log in 5 h (Fig. 1). At the end of the incubation period of the tests, spore counts after heating the slurries at 80°C for 10 min were lower than total counts without

heating (Table 2). In contrast, in the experiment with added nisin, these counts were of the same order of magnitude, suggesting that nisin eliminated nearly all vegetative cells.

DISCUSSION

Prevalence of *B. cereus* in foods of industrialized countries and related foodborne poisoning outbreaks is much more documented (6) than in African countries, for which reports are scant. Nineteen and 8 *B. cereus* group strains were isolated and characterized from fermented African locust bean-based condiments in Benin (17) and in Burkina Faso (14), respectively; however, data on prevalence of *B. cereus* in these condiments are not known. In South Africa, Kunene et al. (10) reported that 100% of sorghum flour samples ($n = 15$) and 40% of sorghum-based fermented porridges ($n = 15$) were positive for the presence of *B. cereus*. However, data on the prevalence of *B. cereus* in complementary foods to breastfeeding consumed by young children between 6 and 24 months in Africa are still needed. Surprisingly, in the case of cereal-based foods in Ouagadougou, we found a high prevalence of *B. cereus* group species in fermented pearl millet slurries, with counts in the range of reported infective doses, i.e., 10^3 to 10^8 CFU/g of food (1, 6). Considering that good correlation was found between PCR for the detection of *hbl* and *nhe* genes and commercial test kits for enterotoxin detection in 88 *B. cereus* strains (7), the percentage of isolates from Ouagadougou harboring the corresponding complete set of genes suggests high potential to synthesize these enterotoxins.

Soil is a natural reservoir of *B. cereus* and can contain between 10^3 and 10^5 spores of *B. cereus* per g (1, 6). Throughout the processing conditions of pearl millet in traditional production units (18), contaminations could have occurred at any step, but high levels of *B. cereus* in the fermented slurries cannot be explained only by soil contamination, and suggest growth during pearl millet processing. However, the inoculation experiments of fermented slurry showed that growth was inhibited, suggesting that in traditional production units, fermentation is presumably not adequately controlled, resulting in conditions that would permit growth in some instance of pathogenic bacteria such as *B. cereus*. Indeed, previous surveys in traditional processing units ($n = 24$) of pearl millet into fermented slurries showed a series of unit operations with variable durations and acidification kinetics for the longer steps (18). The operations were mainly soaking (mean duration of 16 h, minimum of 6 h, and

TABLE 2. Total counts and spore counts after 6 h of incubation of *Bacillus cereus* BMYP7,3, inoculated in pearl millet slurries at the beginning or at the end of lactic acid fermentation^a

Inoculation	Count (CFU/ml)		pH _i	pH _f
	Total	Spore		
Beginning of PMS fermentation	3.3×10^5	3.4×10^4	4.75	4.20
End of PMS fermentation	3.1×10^5	3.3×10^3	3.95	3.95
End of PMS fermentation, with nisin (0.1 mg/ml)	2.7×10^2	3.8×10^2	4.00	4.01

^a pH_i, pH at the beginning of the incubation period; pH_f, pH at the end of the incubation period (6 h); PMS, pearl millet slurries.

maximum of 31 h), grinding, kneading, sieving, and settling (lactic acid fermentation step, with a mean duration of 11 h, minimum of 2 h, and maximum of 20 h) (18). The average pH at the beginning of the fermentation step (settling) was 5.5 ± 0.5 (standard deviation), with extreme values between 4.10 and 6.53. Taking into consideration variations in processes and conditions, growth could have occurred during any steps before fermentation, or even during the fermentation step, when the process was poorly controlled. Therefore, implementation of measures aiming at improving control of the traditional processes and hygienic conditions is necessary. Among possible measures, the addition of nisin, which substantially reduced *B. cereus* counts in these cereal-based fermented slurries, provides a possible way to improve the hygienic quality of the food, without requiring major technological changes in processing.

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