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# EMERGENCE OF *BACILLUS CEREUS* AS A DOMINANT ORGANISM IN IRISH RETAILED POWDERED INFANT FORMULAE (PIF) WHEN RECONSTITUTED AND STORED UNDER ABUSE CONDITIONS

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## ABSTRACT

*One hundred powdered infant formulae (PIF), representative of the 10 leading brands available in Ireland, were subjected to a variety of preparation and storage conditions. All PIF analyzed immediately after reconstitution were of satisfactory bacteriological quality, exhibiting a total aerobic mesophilic count of* $<10^4$ *cfu/g (mean* $3.8 \times 10^2$ *cfu/g) and a* Bacillus cereus *count of* $<10^3$ *cfu/g powder (mean* $1.9 \times 10^2$ *cfu/g).* Enterobacter sakazakii *was not detected in PIF. While 24 of all PIF examined contained* B. cereus, *subsequent reconstitution and storage over a 24-h period at* $\geq$*20C resulted in this organism being detected in a further 35 PIF at levels in excess of* $10^3$ *cfu/g. The bacteriological quality of PIF depended on the type and number of organisms initially present and on the product temperature and duration of storage. While PIF predominantly consisted of members of the* Bacillus subtilis *group, subsequent reconstitution and storage at* $\geq$*20C for 14 h resulted in the emergence of* B. cereus *as the dominant organism. Co-culture studies revealed that* B. cereus *inhibited the growth of members of the* B. subtilis *group and* Listeria monocytogenes. *Not all diarrheagenic and emetic strains of* B. cereus *exhibited antagonistic activity, and there was also evidence of intraspecies antagonism among* B. cereus *isolated from PIF.*

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## PRACTICAL APPLICATION

The high biocidal temperatures that occur during spray drying of powdered infant formulae (PIF) combined with good manufacturing practice compliance ensure that these high-risk foods are of satisfactory bacteriological quality. However, acceptably low numbers of recalcitrant bacterial endospores with toxigenic potential do frequently survive this process. Thus, identifying interrelated preparation and storage conditions (including abuse scenarios) that foster growth of bacterial contaminants in reconstituted PIF to potentially hazardous levels is of paramount importance, as this will inform and safeguard consumer health. This study reports on the occurrence, interaction, emergence and predominance of *Bacillus cereus* in reconstituted PIF under various storage regimes. The tests described in this study are practical and will benefit food technologists in the profiling of spoilage and potentially pathogenic bacteria that may contaminate these high-risk products. Guidance is also provided to consumers on conditions for safe storage of reconstituted PIF prior to consumption.

## INTRODUCTION

Reconstituted powdered infant formulae (PIF) are considered to be a food class of high risk because of the susceptibility of the infant population to enteric bacterial pathogens such as *Enterobacter sakazakii* (*Cronobacter* spp.), severe response to toxins and increased mortality (Iversen and Forsythe 2003; Townsend *et al.* 2007). That said, the occurrence of infections associated with consumption of contaminated PIF is rare, where cares are often low-birth weight premature neonates who are generally more susceptible to gram-negative bacterial sepsis and endotoxemia (Simmons *et al.* 1989; Stoll *et al.* 2004). The vulnerability of infants to low numbers of pathogenic organisms may be because of the host's underdeveloped immunity (Anderton 1993). But despite the elevated temperatures employed in the manufacture of PIF, there have been a number of food-related illnesses where PIF has been implicated as the vehicle of infection (Rowe 1987; Louie 1993; Gericke and Thurn 1994; van Acker *et al.* 2001; Himelright *et al.* 2002; Caubilla-Barron *et al.* 2007).

Generally, PIF are known to be predominantly contaminated with aerobic spore formers of the genus *Bacillus* (Rowan *et al.* 1997; Shaheen *et al.* 2006). Of particular concern is the occurrence of toxigenic *Bacillus cereus* in these products as this pathogen has been previously implicated as the cause of several clinically significant systemic infections in infants (Becker *et al.* 1994; Hilliard *et al.* 2003; Usama *et al.* 2007). Contamination and growth of *B. cereus* in infant food are common, where Becker *et al.* (1994) reported previously that 54% of 261 samples of infant food distributed in 17 countries were contaminated with *B. cereus* at a level of up to $6 \times 10^2$ cells/g. The emetic type of illness is attributed to *B. cereus* strains that produce the heat-stable peptide toxin called cereulide, whereas diarrheagenic strains of *B. cereus* produce heat-labile enterotoxins (Stenfors Arnesen *et al.* 2008). Previous reports have also implicated other members of the genus *Bacillus* as etiological agents in proven foodborne illness outbreaks (Granum 1994; Jackson *et al.* 1995). Shaheen *et al.* (2006) recently reported that mishandling and temperature abuse of infant foods may cause food poisoning when emetic *B. cereus* is present. Despite evidence of the predominace of *Bacillus* spp. in PIF with enterotoxin production potential, no previous study has investigated the possible dynamic interaction of bacterial contaminants in reconstituted PIF under abuse conditions.

Therefore, the aim of the present work was to examine the bacteriological quality of PIF commercially available in Ireland under various preparation and storage abuse regimes, and to investigate interactions between these bacteria in reconstituted PIF and on agar surfaces.

## MATERIALS AND METHODS

### Preparation and Storage of Reconstituted PIF Samples

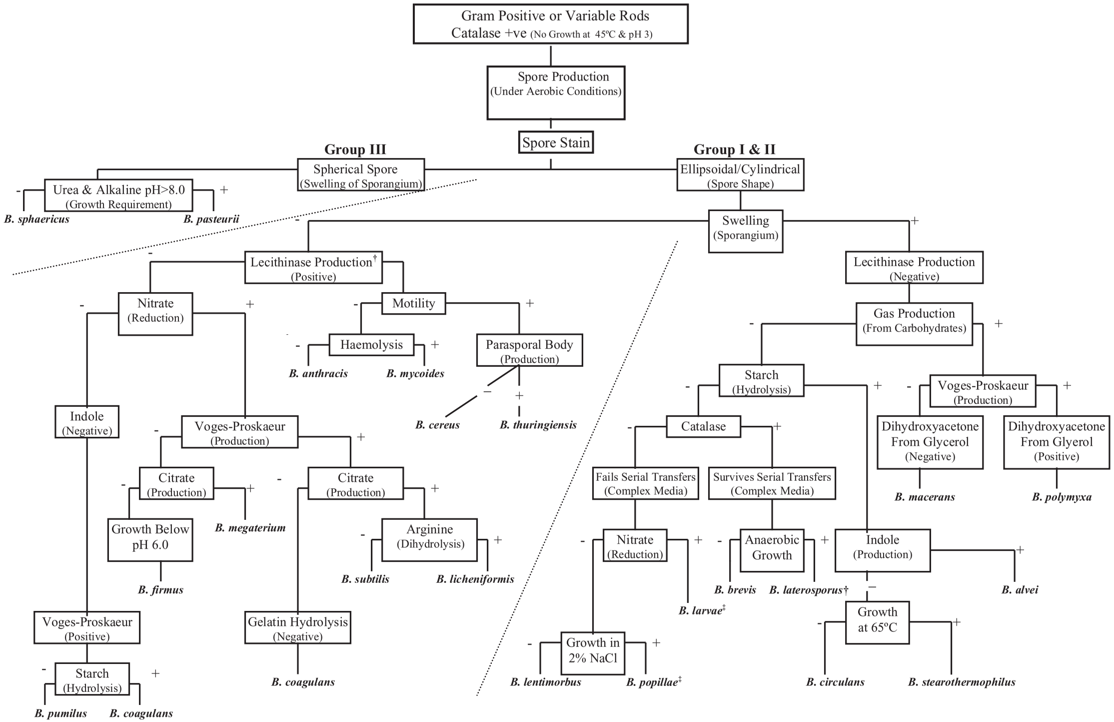
The survey was composed of 100 PIF representative of the leading brands available in Ireland and were prepared as previously described (Rowan *et al.* 1997) with slight modifications. Briefly, 25 g of PIF was reconstituted in 225-mL sterile distilled water at a water temperature of either 45C and/or 70C (±0.2C) by shaking 25 times through an excursion of 30 cm. These temperatures were achieved by equilibrating the Duran bottles containing the sterile water in preheated water baths (Techne Tempette Junior TE-8J, Bibby Scientific Ltd., Staffordshire, U.K.) prior to reconstitution. Following a 30-min cooling period, triplicate aliquots of 1 mL were removed for total aerobic mesophilic counts and for other bacteriological enumerations as outlined below. The reconstituted PIF were then incubated at either 4, 10, 20, 25, 30 and/or 35C for periods up to, and including, 24 h in order to simulate conditions of storage abuse.

### Bacteriological Analysis

Total aerobic mesophilic bacteria in PIF were enumerated and identified at 0, 8, 14 and 24-h sample time intervals by pour, spread and spiral plating (Spiral plater model B, Spiral Systems Inc., Nesbit, MS) decimal diluted samples in buffered peptone water (BPW) and plating on tryptone soya agar supplemented with 0.6% (w/v) yeast extract (Cruinn Diagnostics, Dublin, Ireland) followed by aerobic incubation of plates at 37C for 48 h. This procedure was repeated in duplicate for three separate samples analyzed from the same PIF. *Bacillus* spp. present in these PIF samples was identified as per methods described previously (Rowan *et al.* 1997) with slight modifications. Cultures obtained after growth on Blood Agar No. 2 supplemented with 7% (v/v) defibrinated horse blood (BA) and *B. cereus* Selective Agar (Oxoid, Cambridge, U.K.) were examined for key morphological and/or biochemical properties as outlined in Fig. 1. The identity of each *Bacillus* isolate was confirmed using the API 50CHB and API 20E galleries (bioMérieux Ltd., Basingstoke, Hampshire, U.K.). PIF were also examined for the presence of *E. sakazakii* (*Cronobacter* spp.) using conventional isolation method according to Iversen and Forsythe (2004). Briefly, 10 mL of the BPW pre-enrichment was added to 90-mL Enterobacteriaceae enrichment (EE) broth (Oxoid) and incubated for 24 h. Thereafter, EE broth was streaked on violet red bile glucose agar (Lab M, Cruinn Diagnostics) and colony morphology observed after incubation for 24 h at 37C. Colonies that produced yellow pigments after incubation at 25C for 48–72 h were termed presumptive *E. sakazakii*, and their identities were confirmed using API 20E. PIF were also enriched for heat-sensitive *Salmonella* spp. by enrichment in Rappaport-Vassiliadis soya peptone broth for 24 h at 37C followed by streaking of samples on xylose lysine deoxycholate (Oxoid) and brilliant green agar (Oxoid), and incubated for 24 h at 37C as per methods described by Iversen and Forsythe (2004). Presumptive *Salmonella* isolates were confirmed using API 20E. Staphylococcal isolates were identified based on Gram reaction, ability to produce catalase, and oxidase and coagulase activity, with subsequent use of API Staph (bioMérieux) to confirm identity. Efficacy of bacterial detection was evaluated using PIF samples that were artificially inoculated with low concentrations of lyophilized positive control strains comprising *E. sakazakii* (NCTC 8155), *Listeria monocytogenes* (NCTC 11994), *Salmonella enteritidis* (NCTC 3046), *Escherichia coli* (ATCC 29522), *Staphylococcus aureus* (ATCC 29523) and *B. cereus* (NCTC 11145).

### Detection of Toxin Production From *Bacillus* spp. Isolated From PIF

Detection of diarrheagenic enterotoxin in *Bacillus* spp. was carried out via the *B. cereus* enterotoxin reverse-phase latex agglutination test system (Oxoid) as previously described (Rowan *et al.* 1997). Emetic toxin producers were identified from methanol extracts heated at 100C for 10 min using the rapid sperm microassay as described by Andersson *et al.* (2004). *B. cereus* NCTC 11145 and NCTC 11143 were used as positive test controls for diarrheagenic and emetic toxin production, respectively.



‡ ***B. larvae*** and ***B. popillae*** are catalase negative.
† ***B. laterosporus*** is lecithinase (lecithovitellin) positive even though the ellipsoidal (oval) spores distend the sporangium in Group II

FIG. 1. FLOWSCHEME OF CHARACTERISTICS MORPHOLOGICAL AND PHYSIOLOGICAL TESTS FOR IDENTIFICATION OF MEMBERS OF *BACILLUS* SPP.

### *In Vitro* Growth Inhibition of Agar Surface Inoculated Indicator Test Bacteria

Growth suppression (or antagonism assay) was determined on plates essentially as described by Dorenbos *et al.* (2002). Briefly, indicator strains and strains to be tested for inhibitory substance(s) were grown overnight in Luria-Bertani broth (Cruinn Diagnostics); thereafter, 100-μL aliquots of $10^{-2}$ dilution of indicator strain (comprising ca. $10^{6}$ organisms/mL) was lawned on LB plates. After drying of plates, 2-μL aliquots of undiluted overnight cultures to be tested for inhibitory substance(s) were spotted in triplicate onto the aforementioned plates. The plates were then incubated overnight at 37C, and growth inhibition of the indicator strain was determined the next day by measuring zones of growth inhibition (mm) or clearing around the test organism.

### Statistical Analysis

The Fisher's exact test was used to compare the bacteriological quality of 10 leading brands of infant powder. The effects of PIF preparation and storage temperature on microbial numbers (where total aerobic counts for 100 PIF were pooled and compared as a unit under these conditions), were examined using two-way analysis of variance (Minitab version 13.1, Minitab Ltd., State College, PA). All significant differences were reported at the 95% level of confidence ($P < 0.05$). Standard errors of means have been given as bars in Figs. 2 and 3.

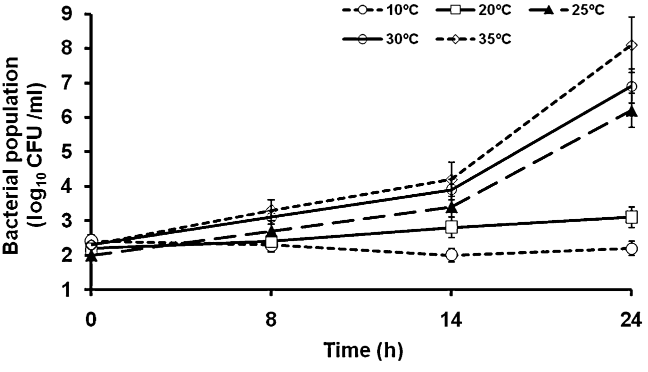


FIG. 2. INFLUENCE OF TEMPERATURE AND DURATION OF STORAGE ON BACTERIAL POPULATIONS PRESENT IN RECONSTITUTED PIF (MEAN ± SD FROM TRIPLICATE SAMPLES)

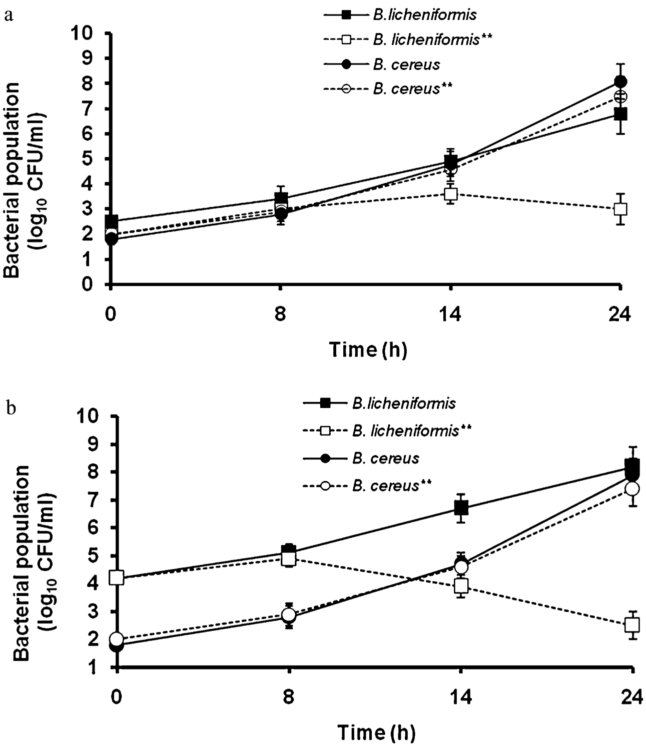


FIG. 3. ANTAGONISTIC ACTIVITY OF *B. CEREUS* AIT24 TOWARD GROWTH OF *B. LICHENIFORMIS* AIT07 WHEN CO-CULTURED IN RECONSTITUTED PIF AT A STORAGE TEMPERATURE OF 30C OVER 24 H (a) SHOWS DATA FOR GROWTH INTERACTIONS WHEN BOTH BACTERIA ARE PRESENTED AT EQUAL STARTING POPULATIONS, (b) *B. LICHENIFORMIS* PRESENT AT SUPERIOR A STARTING LEVEL TO THAT OF *B. CEREUS* (■) Population of *B. licheniformis* in reconstituted PIF when sole culture; (□) population of *B. licheniformis* when co-cultured with *B. cereus*; (●) population of *B. cereus* when sole culture; (○) population of *B. cereus* when co-cultured with *B. licheniformis*.

# RESULTS

## Bacteriological Quality of PIF Examined Immediately After Reconstitution

All 100 PIF examined immediately after reconstitution were of satisfactory bacteriological quality as per new guidelines recommended by the Codex code of hygienic practice for powdered formulae for infants and young children (Codex Alimentarius Commission 2009). All PIF had total aerobic mesophilic counts less than the $5 \times 10^4$ bacteria per gram (Table 1). Two members of Enterobacteriaceae were detected without enrichment, namely *Enterobacter*

TABLE 1.
VARIATION IN AEROBIC PLATE COUNTS FOR NAMED BACTERIAL SPECIES IN 100 PIF ENUMERATED IMMEDIATELY AFTER RECONSTITUTION AT A WATER TEMPERATURE OF 45C OR 70C

| *Bacillus* spp. | Number of PIF where named bacterial spp had total aerobic count ($\log_{10}$ cfu/g) in the below ranges after reconstitution at 45C or 70C | | | | | | | | Total aerobic count ($\log_{10}$ cfu/g) mean* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | <2 | | 2 | | 2–3 | | 3–4 | | | |
| | 45C | 70C | 45C | 70C | 45C | 70C | 45C | 70C | 45C | 70C |
| *B. licheniformis* | 13 | 14 | 19 | 16 | 18 | 18 | 2 | 4 | 2.8 ± 1.1 | 2.7 ± 1.4 |
| *B. subtilis* | 12 | 10 | 10 | 11 | 16 | 16 | 0 | 1 | 2.6 ± 0.9 | 2.6 ± 0.8 |
| *B. pumilus* | 2 | 2 | 5 | 6 | 3 | 3 | 2 | 1 | 2.3 ± 1.0 | 2.4 ± 0.7 |
| *B. cereus* | 8 | 8 | 7 | 10 | 8 | 6 | 1 | 0 | 2.2 ± 0.6 | 2.2 ± 0.4 |
| *B. mycoides* | 0 | 1 | 1 | 0 | 3 | 2 | 0 | 0 | 2.1 ± 0.7 | 2.0 ± 0.6 |
| *B. megaterium* | 2 | 3 | 2 | 1 | 1 | 1 | 0 | 0 | 2.0 ± 0.7 | 2.0 ± 0.5 |
| *B. polymyxa* | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 1.6 ± 0.3 | 1.8 ± 0.3 |
| *B. sphaericus* | 1 | 2 | 4 | 5 | 2 | 0 | 0 | 0 | 2.0 ± 0.5 | 2.0±0.5 |
| *B. circulans* | 1 | 1 | 2 | 2 | 0 | 0 | 0 | 0 | 2.0 ± 0.4 | 2.0 ± 0.4 |
| *Non-Bacillus* spp. | | | | | | | | | | |
| *Enterobacteriaceae* | 1† | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1.5 ± 0.3 | 0 |
| *Staphylococcus* spp. | 2 | 4 | 5 | 3 | 2 | 0 | 0 | 0 | 2.1 ± 0.6 | 2.0 ± 0.3 |
| *Listeria* spp. | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| TAMC‡ | 27 | 46 | 38 | 23 | 30 | 25 | 5 | 6 | 2.7 ± 1.3 | 2.6 ± 1.1 |

* Mean ± standard deviation refer to variation in total aerobic counts ($\log_{10}$ cfu/g) among PIF in each brand prepared at either 45C or 70C ($n = 100$). No significant difference in microbial numbers was observed between brands of PIF.

† A further four strains of Enterobacteriaceae were detected in PIF after enrichment in EE.

‡ Number of PIF exhibiting TAMC in named ranges ($n = 100$).

EE, Enterobacteriaceae enrichment; PIF, powdered infant formulae; TAMC, total aerobic mesophilic counts.

*agglomerans* and *Serratia ficaria*. A further four strains of Enterobacteriaceae were recovered from PIF after enrichment in EE broth that comprised *Enterobacter cloacae* (1), *Pantoea* spp. (2) and *Klebsiella ozaenae* (1). However, *E. sakazakii* (*Cronobacter* spp.) was not detected in any PIF examined. While the latter Codex does not recommend an action limit for *B. cereus*, all PIF were shown to have a *B. cereus* count less than $10^3$ cfu/g that is below the safety limit threshold recommended by the Association of Dietetic Food Industries of the European Community (IDAEC). The PIF examined, which were representative of 10 leading brands currently available in Ireland, were of similar bacteriological quality ($P < 0.05$). While the temperature of water used to reconstitute the powdered formula did not significantly affect the the type or number of *Bacillus* spp. in PIF ($P < 0.05$), samples contaminated with non-aerobic spore formers that were rehydrated at 45C exhibited significantly higher total aerobic mesophilic counts (comprising mainly of staphylococci) compared with the same products prepared at 70C, respectively (Table 1). However, these products were still below the $5 \times 10^4$ cfu/g threshold recommended by the Codex Alimentarius Commission (2009). The largest concentration of organisms present in any PIF product was $8.4 \times 10^3$ cfu/g (consisting solely of *Bacillus subtilis*), while the mean total aerobic plate count for all infant foods analyzed was $3.8 \times 10^2$ cfu/g (Table 1). Although *B. cereus* was present in 59 of PIF examined, only 11 (18%) and 13 (22%) of *B. cereus* isolates were shown to be capable of producing diarrheagenic and emetic toxins, respectively. The largest number of *B. cereus* recovered from any formulation was $5.7 \times 10^2$ cfu/g, while the mean *B. cereus* count for PIF shown to contain this organism was $1.9 \times 10^2$ cfu/g (Table 1). The microbial flora of PIF before reconstitution consisted mainly of aerobic spore formers of the genus *Bacillus*, with the most prominent species isolated belonging to members of the subgroup *B. subtilis*.

## Bacteriological Quality of Reconstituted PIF After Periods of Storage Abuse

Improper storage of reconstituted PIF at ≥20C for 24 h (or ≥25C for ≥14 h) resulted in the microbial population present in a number of formulae increasing to potentially hazardous levels, where all 52 formulae containing *B. cereus* (five samples contained both toxigenic forms of *B. cereus*) being above the reconstitution IDAEC safety limit of $10^3$ cells/g (Fig. 2). Products held at these higher storage temperatures achieved greater microbial numbers sooner, e.g., 34% of foods exceeded the satisfactory reconstitution limit of $10^3$ *B. cereus* cells/g after only 8 h at 35C. While incubation of PIF at ≤10C for 24 h did not alter the bacteriological quality of these formulations ($P < 0.05$), the quality of each PIF was shown to depend on the number of organisms initially

TABLE 2.
NUMBER OF POWDERED INFANT FORMULAE (PIF) ($n = 100$) THAT WERE RECONSTITUTED AND SAMPLED FOR PRESENCE OF *BACILLUS* SPECIES OVER A 24-H STORAGE PERIOD AT ≥25C

| *Bacillus* species | Number of PIF that the named *Bacillus* species were detected over 24-h storage period at ≥25C | | | |
|---|---|---|---|---|
| | 0 h | 8 h | 14 h | 24 h |
| *B. licheniformis* | 52 | 52 | 53 | 44 |
| *B. subtilis* | 38 | 40 | 40 | 32 |
| *B. pumilus* | 10 | 15 | 15 | 8 |
| *B. cereus* (non-toxic) | 13 | 14 | 35 | 35 |
| *B. cereus* (diarrhoeagenic) | 6 | 10 | 11 | 11 |
| *B. cereus* (emetic) | 5 | 13 | 13 | 13 |
| *B. mycoides* | 3 | 10 | 11 | 11 |
| *B. megaterium* | 5 | 14 | 16 | 16 |
| *B. polymyxa* | 2 | 4 | 4 | 5 |
| *B. sphaericus* | 7 | 24 | 25 | 25 |
| *B. circulans* | 3 | 3 | 3 | 3 |
| *B. laterosporus* | 0 | 2 | 2 | 2 |
| *B. coagulans* | 3 | 5 | 5 | 5 |
| No *Bacillus* spp. detected | 8 | 5 | 4 | 4 |

present and on the product temperature and duration of storage. Storage of reconstituted PIF at ≥20C for ≥8 h frequently resulted in an increase in the number of foods containing different types of *Bacillus* spp., such as the emergence of *Bacillus laterosporus* that had not been recovered from similar powdered samples (Table 2).

## Antagonistic Activity of *B. cereus* toward Other *Bacillus* spp. in PIF and Toward Other Unrelated Bacterial Species

While *Bacillus licheniformis*, *B. subtilis* and *Bacillus pumilus* (i.e., *B. subtilis* group) were initially predominant in PIF examined immediately after reconstitution, additional storage of PIF resulted in the emergence of *B. cereus* as a dominant organism often growing to the exclusion of the former *Bacillus* species (Fig. 3). Figure 3b revealed that irrespective of the fact that *B. subtilis* was present in PIF in higher cell numbers compared with *B. cereus*, the latter outgrew and eventually suppressed the growth of the former when co-cultured in these feeds. Findings from co-culturing *B. cereus* with other *Bacillus* spp. on agar plates revealed varying levels of antagonistic activity by the former as demonstrated by zones of clearing around the latter indicator strains (Table 3). This antagonism was also observed when *B. cereus* was co-cultured with *L. monocytogenes* on agar plates (Table 3). However, gram-negative bacteria

TABLE 3.
RELATIONSHIP BETWEEN GROWTH OF *BACILLUS* SPECIES AND ASSOCIATED INHIBITION (mm) OF VARIOUS MEMBERS OF *BACILLUS SUBTILIS* GROUP (AND OTHER FOODBORNE BACTERIAL PATHOGENS) AS DETERMINED BY DIRECT STAB-INOCULATION ASSAY, WHERE LATTER INDICATOR ORGANISMS WERE SEEDED ON TSYEA PLATES AT ca. $10^5$ cfu/cm$^2$ AND INCUBATED FOR 24 H AT 37C BEFORE ENUMERATION

| Test *Bacillus* spp. | | Zone of growth inhibition (mm)* for different indicator organisms | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Description | Code | A | B | C | D | E | F | G | H | I | J |
| *B. cereus* | AIT08 | 1.1 | 2.3 | 3.0 | 2.5 | 3.5 | 2.6 | – | – | – | – |
| *B. cereus (d)* | AIT24 | 1.2 | – | 3.6 | 3.0 | – | 1.5 | – | – | – | – |
| *B. cereus (e)* | AIT28 | 4.3 | 3.8 | 3.5 | 4.1 | 3.9 | 4.1 | – | – | – | – |
| *B. cereus* | AIT35 | 0.9 | – | 3 | 0.5 | 3.0 | 2.3 | – | – | – | – |
| *B. cereus* | AIT59 | 3.1 | – | 2.6 | – | – | 3.5 | – | – | – | – |
| *B. cereus (e)* | AIT13 | 1.5 | 2.3 | 3.0 | 1.5 | 0.9 | 1.6 | – | – | – | – |
| *B. cereus (d)* | AIT45 | 2.0 | 0.5 | 0.9 | 2.8 | 2.3 | 2.1 | – | – | – | – |
| *B. mycoides* | SU58 | 3.3 | 3.1 | 2.8 | 1.9 | 4.1 | 1.9 | – | – | – | – |
| *B. mycoides* | AIT82 | 2.1 | 1.8 | 0.6 | 1.7 | 2.8 | 3.2 | – | – | – | – |
| *B. licheniformis* | AIT87 | – | – | – | – | 1.3 | – | – | – | – | – |
| *B. subtilis* | AIT93 | – | – | – | – | 1.6 | 3.0 | – | – | – | – |
| *B. pumilus* | AIT104 | – | – | – | – | 3.1 | 2.1 | – | – | – | – |
| *B. megaterium* | AIT81 | 1.4 | 0.6 | – | 1.8 | 1.1 | 1.9 | – | – | – | – |

* Mean of triplicate measurements. (A) *B. licheniformis* AIT07; (B) *B. cereus* AIT33; (C) *B. subtilis* AIT38; (D) *B. pumilus* AIT21; (E) *B. licheniformis* NCTC 10341; (F) *Listeria monocytogenes* NCTC 11994; (G) *Enterobacter sakazakii* (*Cronobacter* spp.) NCTC 8155; (H) *E. sakazakii* ATCC 29004; (I) *Escherichia coli* NCTC 8623; (J) *Salmonella enteritidis* NCTC 3046.

(d), diarrheagenic; (e), emetic; TSYEA, tryptone soya agar supplemented with 0.6% (w/v) yeast extract.

appeared not to be affected by co-culture with *B. cereus* (Table 3). Some intraspecies antagonism was also observed among *B. cereus* strains, but this could not be attributed to strains that exclusively produced diarrheagenic or emetic toxins. Of the 11 *B. cereus* that produced diarrheagenic enterotoxin, only 7 demonstrated antagonism toward members of the *B. subtilis* group, whereas 10 of the 13 strains of *B. cereus* that produced emetic toxin also inhibited the growth of the latter.

## DISCUSSION

While *E. sakazakii* (*Cronobacter* spp.) was not isolated from 100 reconstituted PIF in this study, six samples from different products were shown to be positive for members of the genus Enterobacteriaceae after enrichment. The findings from this study are congruent with the previous work of Iversen and Forsythe (2004) who identified nine members of Enterobacteriaceae in 82 powdered infant milk formulae purchased from retailers in the U.K. However, unlike the present study, the latter did isolate two strains of *E. sakazakii* from these formulae. Muytjens *et al.* (1988) also isolated *E. agglomerans* at a much higher frequency ($n = 35$) (25%) in 141 different powdered formulae obtained from 35 countries. These researchers cultured *E. cloacae* and *E. sakazakii* from 30 (21%) and 20 (14%) formulae, respectively, and attributed their high prevalence in PIF to the high thermal resistance of *Enterobacter* spp. in comparison with other members of the Enterobacteriaceae. Findings from this present study also agree with the work of O'Brien *et al.* (2009a) where these researchers did not detect *E. sakazakii* in 468 samples representative of 31 different milk and soya-based infant formula products commercially available in European countries. However, these researchers did recover *E. sakazakii* from two cereal-based infant drinks. While Torres-Chavolla *et al.* (2007) reported on the frequent isolation of *E. sakazakii* from powdered infant milk formulae that were sold in Mexico. O'Brien *et al.* (2009b) also recently demonstrated the merits of using a new one-step enrichment protocol consisting of a combined pre-enrichment and enrichment broth (Cronobacter enrichment broth [CEB]) in conjuction with selective-differential agar ChromID Sakazakii to facilitate a shortened 2-day culture method for detection of this pathogen in PIF. Moreover, all artificially spiked PIF samples were recovered using CEB, and a significantly higher bacterial concentration was obtained with CEB than with other enrichment broths. In order to explore a possible relationship between environmental samples and link with illness in infants from contaminated PIF, Molloy *et al.* (2009) showed that while no *E. sakazakii* (*Cronobact*er spp.) was recovered from food production animals, this enteric pathogen was present in a range of diverse sample types (positive for 33 of 518 samples), with particular association with the environment. While Wang *et al.* (2009) recently described a rapid DNA microarray-based detection technique to identify 10 different pathogenic bacteria including *B. cereus*, *E. sakazakii*, *Salmonella enterica* and *L. monocytogenes* in PIF.

The bacteriological quality of PIF retailed in Ireland is of satisfactory quality (i.e., less than $10^3$ cfu/g) and is consistent with the type of organisms isolated by previous researchers, consisting predominatly of aerobic spore formers and thermoduric cocci (Kwee *et al.* 1986; Becker *et al.* 1994; Rowan *et al.* 1997; Usama *et al.* 2007). Maximum allowed levels for *B. cereus* in dried infant feeds vary depending on the recommendations and policies set by differerent countries. For example, acceptable thresholds of $10^3$ and $10^4$ cfu/g have been recommended by Finland and Sweden, respectively, for *B. cereus* (Shaheen *et al.* 2006). Veda *et al.* (1980) also showed that the most frequently isolated organisms from dried baby formulae in Japan were *B. licheniformis* and *B. subtilis*, while other *Bacillus* recovered included *B. cereus*, *B. pumilus*, *Bacillus megaterium, Bacillus circulans* and *Bacillus coagulans.* By far, the greatest factors influencing the bacteriological quality of each infant feed were the number of organisms initially present, and the temperature and duration of incubation, which corroborates previous studies carried out by Rowan *et al.* (1997) who investigated the bacteriological quality of PIF retailed in Scotland. Reconstituted PIF containing approximately $10^2$ *B. cereus* spores/g became unfit for consumption when subjected to storage at or above 25C for 14 h, reaching levels of $1.3 \times 10^3$ cfu/g. These authors noted that the levels of *B. subtilis* in some reconstituted PIF decreased when *B. cereus* was also present. However, findings from investigating the bacteriological quality of reconstituted PIF retailed in Ireland in the current study demonstrated the frequent emergence of *B. cereus* as the predominat organism in these high-risk feeds under a range of storage abuse conditions. In addition, the current study also clearly demonstrated that *B. cereus* inhibited the growth of a wider range of *Bacillus* species (and the growth of other unrelated potentially pathogenic gram-positive bacteria such as *L. monocytogenes*) when either naturally present as contaminants or artificially co-cultured in these reconstituted PIF. While Becker *et al.* (1994) revealed that reconstituted infant formulae containing the same initial concentration of viable cells may reach levels as high as $10^5$ *B. cereus* cells/g in 7–9 h when incubated at 27C. Usama *et al.* (2007) also showed that 100 samples of commercial infant formulae bought in the Poznan region (Poland) and Cairo region (Egypt) were of satisfactory bacteriological quality, exhibiting an aerobic plate count lower than $10^4$ cfu/g (mean $4.9 \times 10^2$) and a *B. cereus* count lower than $10^3$ cfu/g (mean $1.1 \times 10^2$).

Stadhouders *et al.* (1980) showed that heating of milk at temperatures from 65 to 95C for various holding times heat activated slow germinating endospores of *B. cereus*, which, for the main part, did not germinate within 24 h in high-temperature short-time milk stored under similar conditions. However, it was observed that greater numbers of *B. cereus* and other members of the genus *Bacillus* emerged in reconstituted PIF when held under storage for 8 h or more at 20C compared with numbers presented in PIF when examined immediately after reconstitution. Incubation of reconstituted PIF, which initially contained members of the *B. subtilis* subgroup at $\geq$25C, often resulted in the emergence of *B. cereus* as the dominant organism, which frequently grew to the exclusion of the former *Bacillus* spp. Irish retailed PIF reconstituted and stored at $\leq$10C for 24 h did not increase in microbial number. Crielly *et al.* (1994) examined *Bacillus* populations in milk-based powders reconstituted and also found that storage at 15C for 24 h showed no further growth of *B. cereus*. However, the numbers of *B. cereus* increased in the range $10^{\circ}$–$10^6$ cfu/mL between 8 and 24 h at 20 h, which is in agreement with findings from this present study. Wong *et al.* (1988)

reported that when *B. cereus* organisms started to multiply in milk products, the growth of other bacteria was inhibited. They attributed this inhibitory effect to the bacteriostatic activity of the organic acids produced by *B. cereus*. Nutricia, a large Netherlands-based infant nutrition and healthcare company, has published outcomes of predictive modeling work it has undertaken for abused reconstituted infant formulae. It concluded from predictive simulations that 2-h storage at 30C followed by 24 h at 10–12C would result in a five generation increase in *B. cereus* numbers in rehydrated infant formulae. This infers that, even if the formula powder had a level of 100 *B. cereus* per gram, the final level in rehydrated formulae after the latter storage abuse would be less than $10^4$ cfu/mL (a level that is not considered hazardous and unlikely to cause illness even in a vulnerable population group such as infants). Because of the marked variation (including absence of growth inhibition for many co-cultured indicator strains) in antagonistic activity exhibited by *B. cereus* in this present study, it is unlikely that organic acid production plays a significant role in this antagonism. Rowan and Anderson (1998) reported that chemical disinfection procedures failed to eliminate enterotoxinic *B. cereus* on surfaces of infant feeding bottles. Another potential source of *B. cereus* is contaminated water used to prepare the feed (Griffiths and Schraft 2002).

However, the findings from this study corroborate the work of other researchers who demonstrated that *B. cereus* produced antagonistic activities toward gram-negative and other gram-positive foodborne pathogenic bacteria (Yilmaz *et al.* 2006; Kevaney *et al.* 2009) and toward plant pathogenic microorganisms (Stabb *et al.* 1994; Kevaney *et al.* 2009). In the present study, test strains of *B. cereus* isolated from PIF also exhibited antagonism toward unrelated gram-positive bacteria, namely, *L. monocytogenes*. However, the authors of the present study have not conducted a sufficient range of appropriate tests to statistically prove that the inhibitory substance(s) present in PIF or in filtered overnight LB-extracts from test *B. cereus* strains are bacteriocin in nature. Altayar and Sutherland (2006) observed that that fungicidal or bactericidal effect was not attributed to cereulide, where the researchers tested autoclaved extracts from *B. cereus* emetic toxin standard strain F4810/72. Indeed, *B. cereus* has been previously used as a probiotic for humans (Sánchez *et al.* 2009), for marine aquaculture (Ravi *et al.* 2007) and for livestock (Gil de los Santos *et al.* 2005; Lodemann *et al.* 2008; Schierack *et al.* 2009). Futhermore, *B. cereus* and *Bacillus thuringiensis* can produce bacteriocins that can suppress the growth of other foodborne microbial pathogens (Kevaney *et al.* 2009). However, this present work constitutes the first report of antagonistic interactions among *Bacillus* spp. that contaminate PIF.

In conclusion, PIF commercially available to Ireland are of satisfactory bacteriological quality and should not present any health problems to consumers if properly reconstituted at the recommended water temperature of 70C under hygienic conditions. However, it is important that reconstituted PIF are consumed within 4 h or preparation as bacterial numbers present may proliferate to unacceptable level. Storage abuse of PIF may also lead to the proliferation and predominace of *B. cereus* of toxigenic potential.

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