1 **SHORT COMMUNICATION**

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3 **GROWTH AND INHIBITION BY SPICES OF GROWTH FROM**
4 **SPORES OF ENTEROTOXIGENIC *BACILLUS CEREUS* IN COOKED**
5 **RICE**

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Abstract

Bacillus cereus is a pathogenic spore-forming bacterium implicated in cases of diarrheal and emetic type of foodborne illness. We previously found that enterotoxigenic *B. cereus* is widely present in retail spices. Here we analyzed the spore heat resistance of nine diarrheal strains isolated from spices. Seven had $D_{95^{\circ}\text{C}}$ values ranging from 0.64-3.53 min while two emetic strains had $D_{95^{\circ}\text{C}}$ values of 7.04 min and 6.64 min. The ability of selected strains to grow in cooked rice at temperatures 20°C, 17°C and 12°C was determined as well as their toxin expression capability. After 48 h, *B. cereus* levels of 1.26×10^7 and 3.8×10^7 CFU/g were obtained in cooked rice maintained at 17°C and 20 °C respectively. At 12 °C, counts did not reach 10^4 CFU/g even after 48 h of incubation. The increase in the aerobic, mesophilic bacterial population (APC) and *B. cereus* population naturally present in 0.1% crushed pepper added to cooked rice was determined over a period of 48 h at 20°C and 17°C. Levels of *B. cereus* in pepper/rice samples reached a maximum of 1600 MPN/g at 20 °C while the APC was 2.4×10^8 /g at this temperature. When thyme, containing the same initial natural level of *B. cereus*, was added to rice in place of pepper, more than a five-fold greater level of *B. cereus* was detected at 20°C. Since thyme contained initial APC of 2.5 $\log_{10}$ less than pepper we conclude that the high APC functions in a competitive-exclusion manner, minimizing the growth of *B. cereus* and potentially other agents of foodborne illness. This is particularly relevant in instances of temperature abuse of foods and may explain the low incidence of foodborne illness due to *B. cereus* despite its widespread presence shown in previous surveys of market spices.

Key words: *Bacillus cereus*, spices, spores, enterotoxin, rice

1. Introduction

B. cereus, a pathogenic, endospore forming organism, has been implicated as the cause of two types of foodborne illness, characterized by diarrhea and emesis. The emetic type is caused by a 45-kDa cyclic heat- and pH-stable peptide that is pre-formed in food. The diarrheal type is caused by three component heat-labile enterotoxins, non-hemolytic enterotoxin (NHE), and hemolysin BL (HBL) (Granum and Lindback, 2013; Lindback and Granum, 2013). Foods associated with the emetic type are commonly farinaceous while the diarrheal type is associated with meat, sauces, pudding, vegetables and milk products (Kramer and Gilbert, 1989).

Spices have recently received attention due to cases of foodborne illness caused by *Salmonella*. The U.S. is the largest importer of spices which are obtained from developing countries that have minimal processing steps post-harvest and where workers receive marginal training in safe handling and sanitation, thus presenting a risk of contamination of spices which naturally contain high levels of microorganisms. Between 1973 and 2010 fourteen spice-associated foodborne outbreaks have been documented worldwide. *Salmonella* has been implicated in 10 of the outbreaks and *Bacillus* spp., the cause of the other four (FDA, 2013). In Hungary, from years 1960 to 1966, 88 outbreaks of foodborne illness were caused by *B. cereus*. Seasoned meat dishes were the implicated cause of three out of every five outbreaks caused by this organism. It was proposed that Hungarian meat dishes are highly seasoned with dried spices that usually contain large numbers of aerobic, bacterial spore formers including *B. cereus*. The spores survive cooking temperatures and, due to improper holding conditions, germinate and multiply to cause foodborne illness (Geopfert et. al., 1971).

We previously found widespread prevalence of enterotoxigenic *B. cereus* spores in retail spices at levels ranging from <3 MPN/g to 1600 MPN/g (Hariram and Labbe, 2015). Since rice is the most commonly consumed cereal and is associated with *B. cereus* (Ankolekar et. al, 2009a,b; Dommel et. al., 2009; Gilbert et. al., 1974; Martinez-Blanch et. al., 2009; Parry and Gilbert et. al., 1980), we determined here the growth and enterotoxin expression in cooked rice, and the heat resistance of spores of selected strains isolated from spices. Considering spices are added to dishes during and after cooking, we also

determined the kinetics of growth of *B. cereus* and aerobic, mesophilic bacterial population (APC) from pepper using cooked rice as the model food.

2. Materials and Methods

2.1. *B. cereus* strains

Control strains ATCC 14579 (HBL complex) and F4810/72 (*ces*-positive, originally obtained from an outbreak associated with cooked rice) (Turnbull et. al., 1979) were kindly provided by A. Wong, Univ. of Wisconsin. Isolate no. 10 from seafood, isolated by our laboratory, was used as another emetic control strain (Rahmati and Labbe, 2008). *B. cereus* diarrheal strains no. 182, 221, 66, 158, 226, 23, 210, 109 and 31 used in this study were isolated from retail spices (Hariram and Labbe, 2015).

2.2. Rice

Nature's Promise white organic rice purchased from a local supermarket was used as the food matrix in this study. An electric rice cooker (model no. ARC-733G, Aroma Housewares Co., San Diego, CA) was used to prepare cooked rice. Levels of *B. cereus* were enumerated immediately after preparation of rice (pH 6.2) and also after incubation for 48 h at 20°C to determine the presence of any naturally-occurring *B. cereus*. For this, a 3-tube MPN method using tryptic soy broth (TSB) with 0.1% polymyxin B was used (FDA, 2012). These preliminary experiments showed the absence of growth after 48 h, indicating that any growth in cooked rice in these studies was due to the external addition of *B. cereus* or the microbial flora in spice.

2.3. Preparation of spores

Overnight cultures of selected spice isolates were spread-plated on sporulation medium (nutrient agar and CaCl₂ and MnSO₄, 0.001% each), incubated at 37° C for 4-5 days, and examined by phase microscopy for the presence of mature spores. Sterile spreaders were used to collect the lawn of bacterial spores into chilled, sterile sodium-potassium phosphate buffer (0.05M, pH 7.0). Samples were centrifuged at 5000 g for 10 min at 4° C to remove vegetative cells and other debris. The precipitate was re-suspended

in chilled buffer and the process was repeated (at least four times) until the suspension contained $\geq 95\%$ free spores. Spore samples were refrigerated in phosphate buffer until used.

2.4. Heat resistance of spores

Spore suspensions were thoroughly shaken by hand to disrupt clumps (verified by microscopic observation) and diluted with chilled, sodium phosphate buffer to an A_{600} of 0.1 to 0.2 at 600 nm ($\sim 1 \times 10^7$ CFU/mL). Two hundred μ l of the sample were transferred to 2 mL glass ampoules and heat sealed. Ampoules containing spore suspensions were heated at 95°C. Duplicate ampoules were removed at specific times and immediately cooled in an ice-water bath. Heat-treated spores from each ampoule were serially diluted and enumerated in triplicate using nutrient agar supplemented with 0.1% glucose. Plates were covered with a thin overlay with the same medium to prevent spreading. Preliminary experiments with random samples showed an increase in number of CFU/mL from 24 h to 48 h at 32°C but not from 48 to 72 h of incubation in random samples. Therefore plates were incubated for 48 h at 32°C in these studies.

2.5 Statistical methods

Log transformations of numbers of spores that survived heat treatment were plotted against time in Microsoft Excel 2013. Duplicate heat-inactivation curves for each sample were plotted, and the negative reciprocal of the slopes (of the log portion) was used to compute the $D_{95^\circ\text{C}}$ values. To determine significance of observed differences in D-values between emetic and diarrheal strains, an independent sample t-test was run using IBM SPSS Statistics 22 Software.

2.5. Growth of B. cereus in rice

Preliminary experiments were conducted to compare the germination and growth rates of spores of selected strains. This involved the preparation of a cocktail of spores of two strains (isolate nos. 221 and 182) with similar germination and growth rates (not shown). Each was mixed at equal concentration

using 0.1% peptone as the diluent and heat activated for 10 min at 80° C. Two hundred µl of an inoculum (1.0 x 10⁴ total spores/mL) were added to 10 g samples of tempered, cooked rice in 30 mL beakers to obtain an initial concentration of 200 cells/g. Beakers were held at 20°C and at moderately abusive temperatures of 17° C and 12° C. Two samples were removed at time points every four h and enumerated as above. Duplicate growth curves were prepared for each temperature and mean generation times were obtained from the exponential growth phases.

2.6. Enterotoxin production

The production of NHE enterotoxin was detected in the cooked rice samples using a commercial toxin assay kit (Tecra Diagnostics, Roseville, Australia). Beakers of rice inoculated with a heat-activated spore mixture were removed at time points 24, 41, 45 and 48 h. Samples for the immunoassay were prepared as previously described (Ankolekar and Labbe, 2009b). In addition to the positive and negative controls provided in the kit, a sample containing only rice was prepared and used as a negative control.

2.7. Growth of *B. cereus* in rice in the presence of pepper

Ground spice sample #158, Whole Black Peppercorns (Hariram and Labbe, 2015) was inoculated at a level of 0.1% onto 10 g of cooked rice contained in 30 mL beakers (pH 6.0). Beakers were held at 20° C and 17° C. Duplicate samples were removed at intervals of four h, diluted in 0.1% peptone and enumerated for *B. cereus* using a chromogenic agar (R & F Laboratories) since the spice contained aerobic spore-formers in addition to *B. cereus*. Aerobic mesophilic bacterial counts were obtained by pour plating with nutrient agar in duplicate and incubating at 32°C for 48 h. Mean generation times were computed from the exponential phase of growth.

3. Results and Discussion

3.1. Heat resistance of spores

The D_{95°C} values obtained in this study are listed in Table 1. D_{95°C} values of six different diarrheal strains tested ranged from 0.64 min to 3.53 min while the two emetic strains had D_{95°C} values of 7.04 min

and 6.62 min (Table 1). A significant difference in the average heat resistance has also been reported between diarrheal and emetic strains of *B. cereus* (Ankolekar and Labbe, 2009b; Parry and Gilbert, 1977). $D_{95^{\circ}\text{C}}$ values of 1.2 to 36.2 min in phosphate buffer were previously reported for *B. cereus* spores (Kramer and Gilbert, 1989). The mean $D_{95^{\circ}\text{C}}$ values between the diarrheal and emetic strains, 1.8 min and 6.8 min, were statistically significant ($p < 0.05$). Fig. 1 shows the inactivation of spores of representative diarrheal and emetic isolates of *B. cereus*.

3.2. Growth of *B. cereus* in cooked rice

Data on growth of a mixture of two spore strains of *B. cereus* in cooked rice at 20 °C, 17 °C and 12 °C are shown in Fig. 2. Viable counts reached 5 log₁₀/g within 20 h of incubation at 20 °C and within 24 h at 17°C. At 12 °C, counts of the spore mixture did not reach 4 log₁₀/g even after incubation for 48 h. After 40 and 48 h viable counts reached 7.0 log₁₀/g within 40 h and 48 h at 20 °C and 17 °C respectively. Mean generation times were 85.6 ± 0.1 min at 20°C and 116.8 ± 1.1 min at 17°C. Lag times were nine h for samples at 20°C and 17°C, and 25 hr for samples at 12°C.

3.3. Pepper-rice study

Table 2 shows levels of *B. cereus* and APC in pre-cooked white rice inoculated with 0.1% of crushed peppercorns and held at 20°C and 17°C. The APC and *B. cereus* spore count of the pepper used as the inoculum were 6.5 x 10⁶ CFU/g and 460 MPN/respectively (Table 3). There was variation between duplicate experiments in the levels of *B. cereus* during the study (Table 2). The natural, i.e. uneven, distribution of *B. cereus* in pepper can be one reason for the fluctuating counts of *B. cereus* demonstrated in the pepper-rice study.

Within 48 and 44 h the APC of the pepper-rice samples reached 8 log₁₀/g and 6 log₁₀/g at 20°C and 17°C respectively (Table 2). The lower levels of *B. cereus* in the rice/pepper experiment compared to the levels in rice alone after 40 h at 20°C (i.e. $<10^4$ vs $>10^7$ (Tables 2 and Fig 2) could be due to the

competing microflora as shown by the high APC that were detected (Table 2). As shown in Fig. 2 pure cultures of *B. cereus* are readily able to grow in cooked rice. In a separate experiment 0.1% thyme, rather than pepper, was used as the inoculum (adjunct) in cooked rice. Like the pepper sample, thyme also contained an initial level of 460 *B. cereus* spores/g but had an initial APC of 1.39×10^4 /g, i.e., about 2.5 $\log_{10}$ fewer than the APC in pepper (Table 3). After 40 h the thyme-rice samples held at 20°C showed levels of *B. cereus* which were >five-fold greater than in the rice/pepper study but which had a lower APC at that time (Table 3). Thus the difference in the APC between thyme/rice and pepper/rice samples after 40 h of incubation (3.09×10^7 /g vs 5.49×10^5 /g) may explain the low *B. cereus* levels at that time and may reflect the effect of competing microflora on the levels of *B. cereus* in cooked rice

3.4. Enterotoxin expression

Enterotoxin was not detected in pre-cooked rice samples that had $\log_{10} 5.1$ CFU/g of *B. cereus* (after 24 h) (not shown). However low levels (toxin index of 3) of enterotoxin were detected when counts reached $\log_{10} 7.2$ CFU/g and $\log_{10} 6.5$ CFU/g (after 40 h) at 20 °C and 17 °C respectively. Lee et. al. (2006) and van Netten et. al. (1990) reported HBL enterotoxin production in rice cakes (22 °C) and rice meal (17 °C) when levels reached $6.6 \log_{10}$ CFU/g and $7.1 \log_{10}$ CFU/g respectively. Additionally, we previously demonstrated low levels of enterotoxin production in salmon only when levels reached $8 \log_{10}$ CFU/g and higher (Rahmati and Labbe, 2008). Many reports have demonstrated that growth of *B. cereus* is influenced by pH, carbohydrate sources and, in the case of enterotoxin, growth rate (Ouhib et. al., 2006; Rowan and Anderson, 1997; Sutherland and Limond, 1993), suggesting that enterotoxin production is not only dependent on cell numbers but also is affected by extrinsic factors. Although here enterotoxin was detected in temperature-abused rice, foodborne illness due to *B. cereus* is believed to result from the production of one or more enterotoxins in the intestine following ingestion of large numbers of *B. cereus* cells from temperature-abused foods (Granum and Lindback, 2013).

Our results suggest that, despite the widespread presence of enterotoxin-positive *B. cereus* in spices, the low incidence of confirmed outbreaks of foodborne illness due to *B. cereus*-contaminated spice added

to foods may be due to competing microflora including those enumerated by the aerobic plate count as well as by other non-toxigenic spore-formers. For example of the 248 retail spice samples we previously examined, 53% and 19% contained aerobic spore counts of $>10^3/\text{g}$ and $>10^5/\text{g}$ respectively (Hariram and Labbe, 2015). This is in addition to the non-spore population. Such high counts can effectively function in a competitive-exclusion manner, minimizing the growth of *B. cereus*. The role of competing microflora may also be of importance for other foodborne pathogens, such as *Clostridium perfringens*, where elevated levels of vegetative cells in outbreak foods are required for foodborne illness.

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Table 1. Heat resistance of *B. cereus* spores in this study

Strain	Toxin type	D _{95°C} (min)
ATCC 14579 (control)	Diarrheal	1.36 ± 0.22
182 (spice)	Diarrheal	0.64 ± 0.08
221 (spice)	Diarrheal	2.56 ± 0.19
66 (spice)	Diarrheal	3.53 ± 1.11
158 (spice)	Diarrheal	0.75 ± 0.03
226 (spice)	Diarrheal	2.46 ± 0.04
23 (spice)	Diarrheal	1.20 ± 0.41
F4810/72 (control)	Emetic	7.04 ± 0.51
10 (seafood)	Emetic	6.62 ± 0.73

Table 2. Growth of *B. cereus* and aerobic mesophilic population^a in pepper-rice samples

Time (hr)	20 °C			17 °C		
	<i>B. cereus</i> (MPN/g)		APC (log ₁₀ CFU/g)	<i>B. cereus</i> (MPN/g)		APC (log ₁₀ CFU/g)
	Exp ^b 1	Exp. 2		Exp 1	Exp. 2	
0	7.4	3.6	5.15 ± 0.00	7.4	3.6	5.15 ± 0.01
8	14	9.2	ND ^c	3.6	0	ND
12	15	23	ND	3.6	9.2	ND
16	7.4	3.6	5.28 ± 0.01	23	3.6	ND
20	9.2	9.2	ND	3.6	9.2	5.22 ± 0.16
24	3.6	93	6.04 ± 0.04	9.2	9.2	5.33 ± 0.10
24	15	15	6.33 ± 0.05	14	9.2	5.29 ± 0.00
32	1100	1100	ND	9.2	15	ND
36	1100	20	ND	20	20	5.87 ± 0.15
40	540	1600	7.49 ± 0.00	20	460	ND
44	220	1600	ND	1110	1110	6.40 ± 0.10
48	920	1600	8.39 ± 0.02	1110	460	6.65 ± 0.0

^aAPC

^bExp., experiment number

^cND, not determined

Table 3. Initial levels of *B. cereus* and APC in adjuncts and in rice following challenge study^a

	<u>Initial levels</u>		<u>in spice-rice mixture following challenge study</u>	
<u>Adjunct</u>	<u><i>B. cereus</i>/gm</u>	<u>APC^b/gm</u>	<u><i>B. cereus</i>/gm</u>	<u>APC^b/gm</u>
pepper	460	6.50 x 10 ⁶	1.10 x 10 ³	3.09 x 10 ⁷
thyme	460	1.39 x 10 ⁴	6.02 x 10 ³	5.49 x 10 ⁵

^aafter incubation at 20°C for 40 hr

^b aerobic plate count

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332 Figure Legend

333

334 Fig. 1. Inactivation of spores of representative diarrheal and emetic *B. cereus* strains of *B. cereus*

335 isolated from spices. Spores were heated at 95° C in 0.05 M sodium phosphate buffer, pH 7.0.

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337 Fig. 2 Growth of *B. cereus* in cooked rice. Inoculum was a mixture of spores of two diarrheal-type

338 isolates from spices. Duplicate results at the 24 h time point reflect the continuation of the growth

339 experiment on a subsequent day

Fig. 1

Fig. 2