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Influence of pH and storage period on Haemolysin BL (HBL) production by *B.cereus* from pasteurized milk during refrigerated storage

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----------**ABSTRACT**----------

A study was conducted to determine the effects of pH and storage period on the ability of B.cereus to produce haemolysin BL diarrhoeal enterotoxin (HBL) in Brain heart infusion broth (BHI). Wild B.cereus strains standardized to 1 Mcfarlands standard (about 8.5 log $_{10}$ CFU/ml) isolated from raw cow milk was inoculated into pasteurized milk samples at pH levels of 6.3, 6.4, 6.7 and 6.8. Milk samples were stored at refrigeration temperature (4-10^{o}C) and isolates tested for their ability to produce the HBL enterotoxin in Brain heart infusion (BHI) broth at intervals of 24 hours for a period of 72 hrs. Enterotoxin assay was carried out with RPLA-BCET toxin detection kits (Oxoid) which is specific for the L_2 component of the HBL diarrhoeal enterotoxin. Toxin titres of for isolates ranged from < 2 ng/ml for B.cereus cells from milk samples at pH 6.8 following 48 hours of storage to ≥64ng/ml for isolates from milk at pH 6.3 following 24 hours of storage and pH 6.7 after 48 hours of storage. Pearson correlation established a positive correlation between toxin titre production and B.cereus counts at the end of storage periods. DNA amplication by PCR revealed the presence of at least one hbl gene in isolates from milk samples at varying pH levels and storage periods, including non-enterotoxin producing isolates. Results imply the HBL diarrhoeal enterotoxin expression could be suppressed in some B.cereus milk isolates following refrigerated storage at some pH levels. Results also infer the possibility of sustained heritable traits of suppressed HBL toxin expression in some B.cereus milk isolates. Results also highlight the impact of storage conditions on hbl genes detection by PCR in the same B.cereus strain from milk samples.

KEYWORDS; *B.cereus, pasteurized milk, refrigeration, HBL diarrhoeal enterotoxin.*

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INTRODUCTION

Food-borne diseases (FBD) or illnesses are defined as diseases of infectious or toxic nature caused by agents that enter the body through the consumption of food[1] . FBDs are caused by many different disease - causing pathogens that can contaminate foods, or by toxins produced by these pathogens that are present in food[2]. *Bacillus cereus*, the model species of the "*Bacillus cereus* group", also known as *Bacillus cereus sensu lato*[3], is a gram positive, rod shaped bacterium capable of facultative aerobic metabolism. The organism is widely distributed in the environment, mainly in the soil, from which it iseasily spread to many types of foods especially those of vegetable origin, as well as meat, milks and dairy products [4,5,6,2,7]. *B. cereus* is known to cause two different types of food poisoning which are characterized by either diarrhoea or emesis.

The ability of *B. cereus* to form spores ensures its survival through all stages of food processing, and subsequent time/temperature abuse enables low levels of *B. cereus* to multiply to dangerous levels [8]. It has been referred to as the major problem in convenience foods and mass catering[9]. A three component complex system, designated haemolysin BL (HBL) is believed to be the major diarrhoeal toxin of *B. cereus*[10]. Reports of some studies have shown the influence of variations in environmental conditions on the growth, survival and production of diarrhoeal enterotoxins by *Bacillus cereus* [11,5,12,7]. It has been documented that changes in gene expression or cellular phenotype in organisms occur in some instances without any changes to underlying DNA sequences of the organism[13] but can be associated with chemical modifications of DNA, or of the structural and regulatory proteins bound to it[14]. These changes may remain through cell divisions for the remainder of the cell's life and may also last for multiple generations[15]

Material and methods

Determination of HBL diarrhoeal toxin production in Brain Heart infusion (BHI) broth: Wild *B.cereus* strains standardized to 1 Mcfarlands standard (about 8.5 log $_{10}$ CFU/ml) previously isolated from raw cow milk and identified using a microgen™ *Bacillus* ID, was inoculated into pasteurized milk samples at pH levels of 6.3, 6.4, 6.7 and 6.8 . Milk samples were stored at refrigeration temperature (4-10^{o}C) and isolates tested for their ability to produce the HBL enterotoxin in Brain heart infusion (BHI) broth at intervals of 24 hours, for a period of 72 hrs. Enterotoxin assay was carried out using an RPLA-BCET toxin detection kit (Oxoid) which is specific for the L_2 component of the HBL diarrhoeal enterotoxin following the manufacturer's instructions.

This involved titrating BHI culture filtrates of test isolates against purified antiserum from rabbits immunized with purified *B.cereus* diarrhoeal enterotoxin in V- well microtitre plates(Titertech®). Plates were then examined for agglutination, and toxin titers (concentrations) recorded after 20 to 24 hours at room temperature. The sensitivity of this test in detecting enterotoxin is 2ng/ml in test extracts.

Statistical analysis of data: Pearson Product Moment Correlation was used to determine the correlations between toxin titres and counts at the end of storage periods. Analysis of variance was also employed to determine level of significance of differences in toxin titres of isolates (SPSS 16).

PCR detection of *hbl* genes in *B.cereus* milk isolates: Enterotoxin and non enterotoxin producing isolates above were subjected to assay for the presence of HBL enterotoxin DNA by PCR as described by Rowan *et al*., (2001). DNA sequences of diarrhoeagenic genes *hblD* , *hblC and hblA* were used to design primers that were used amplify segments of the genes where present in DNA extracts from the test isolates. DNA extraction from *B.cereus* isolates was carried out using ZR fungal/bacterial DNA miniprep™ DNA extraction kits (Zymo research) as described by the manufacturers. Primers for the test genes were designed by Inqaba biotechnical industries limited, South Africa and amplification was carried out in a PCR thermal cycler (Techne®) as described in the PCR programmes in table 1.

Table 1: Primers and PCR programmes for amplification of genes encoding for the HBL diarrhoeal enterotoxins in *B. cereus* pasteurized milk isolates

Genes	Primer sequence	Expected amplicons	PCR programme	Reference
hblA	5´GCTAATGTAGTTTCACCTAGCAAC3´ 3´AATCATGCCACTGCGTGGACATATAA5´	873bp	1	Rowan *et al*., 2001
hblC	5´AATAGGTACAGATGGACAGG3´ 3´GGCTTTCATCAGGTCATACTC5´	339bp	2	
hblD	5´ AATCAAGACCTGTCACGAAT3´ 3´ CACCAATTGACCATGCTAAT5´	439bp	3	

Programme	Cycling procedure
1	36 times (94^{o}C, 30S; 63^{o}C, 60S; 72^{o}C, 60S)
2	36 times (94^{o}C, 30S; 62^{o}C, 60S; 72^{o}C, 60S)
3	36 times (94^{o}C, 30S; 54^{o}C, 60S; 72^{o}C, 60S)

Results and discussion

Toxin titre values for *B.cereus* from pasteurized milk (table 2) following 24 hours refrigerated storage ranged from 4ng/ml at pH 6.4, to ≥64 ng/ml at pH 6.3. In addition, it was observed toxin titres were lower for isolates from samples where bactericidal effects (pH 6.4, 6.7, 6.8) occurred following the 24 hour storage period than for samples where bacteriostatic effects were observed (pH 6.3). Toxin titres of < 2 ng/ml were recorded for *B.cereus* cells from milk samples at pH 6.8 following 48 hours of storage. There appeared to be a general pattern of positive correlation between toxin titres and counts recorded for milk samples at the end of the storage periods. Toxin titres for the isolates were however not significantly different (P>0.05).

The *hblD* gene was detected in isolates following 24 hours of storage (table 3) except for the isolate at pH 6.8 where none of the genes was detected by PCR. Isolates at 48 hours refrigeration all had the *hblD* gene detected. The *hblC* gene was however not detected by PCR in all isolates during the storage periods. *T*he *hblA* gene was detected only in isolates at pH 6.7 following 48 hours of storage..

Table 2: Combined effects of pH, temperature and storage time on enterotoxin production by *B.cereus* isolates from pasteurized cow milk

Temperature (^{0}C)	pH	Storage Time (H)	Counts (log_{10}CFU/ml)	Toxin Titre (ng/ml)*	Correlation coefficient (r)	P**
4-10	6.3	24	8.5±0.3	≥64	0.40	0.11
	6.4		8.2±0.5	4		
	6.7		8.0±0.5	32		
	6.8		7.7±0.2	32		
	6.3	48	6.6±0.3	4	0.70	
	6.4		8.4±0.5	8		
	6.7		8.7±0.1	≥64		
	6.8		7.2±0.2	<2		
	6.3	72	7.7±0.4	16	0.71	
	6.4		7.9±0.4	2		
	6.7		8.4±0.5	2		
	6.8		8.0±0.3	8		

CFU/ml ; colony forming units per milliliter, * ; Sensitivity at 2ng/ml, ** ; level of significance at $P<0.05$ for toxin titres at all storage periods; Initial inoculum levels of 8.5 log_{10}CFU/ml; counts are means of triplicate samples ± standard deviations.

Table 3: *hbl* genes detected by PCR in *B.cereus* isolate R3M1 from pasteurized milk at varying pH during refrigeration*

pH	Storage time (H)	Toxin titre (ng/ml)**	Diarrhoeagenic genes detected by PCR *hblD*	*hblC*	*hblA*
6.3	24	≥64	+	-	-
6.4	24	4	+	-	-
6.4	48	8	+	-	-
6.4	72	2	+	-	-
6.7	48	≥64	+	-	+
6.8	24	32	-	-	-
6.8	48	<2	+	-	-

*Refrigeration temperature of 4-10^{o}C; **toxin titre sensitivity at 2ng/ml ; PCR = Polymerase Chain Reaction ; *hblD* =haemolysin blDgene; *hblC* = haemolysin blC gene; *hblA* = haemolysin blA gene;

Cross reactivity between immune rabbit globulins and cell free BHI culture filtrates obtained from *B.cereus* isolates grown in refrigerated pasteurized milk, showed toxin titer appeared to correlate positively with counts. There appeared to be a general pattern of toxin production increasing or decreasing for isolates, under conditions of increases or decreases in cell density respectively, during storage periods. Positive correlation coefficients (r) as shown between toxin titres and counts at the end of storage period intervals of 24 hours corroborate these observations.These observations therefore appear to infer a positive impact of cell count on HBL production by *B.cereus* from milk during storage at refrigeration. Expression of HBL genes have been shown to be regulated by a number of proteins, such as the PlcR (Phospholipase C Regulator)[16] which controls most known virulence factors in *B.cereus*[17]. Its transcription is auto induced by PapR, an auto inducer peptide that accumulates inside the bacteria when high density cells are reached[18]. It has also been proven that plcR- strains are not able to produce the enterotoxin HBL, while their plcR+ equivalent did show HBL production[19]. Increases in toxin titres were recorded for isolates from milk samples at pH 6.3 and 6.8, as cell population increased following 72 hours of storage. This may have resulted from *B.cereus* cells adapting by way of general stress response mechanisms, which have been described to be involved in cross protection of *B.cereus* cells exposed to stress conditions[20].

Molecular analysis by PCR showed presence of at least one of the *hbl* genes in both enterotoxin and non enterotoxin producing *B.cereus* isolates in this study. None of the isolates in this study appeared to simultaneously encode for all three *hbl* genes after storage periods as shown by PCR studies. Whilst studies have documented variations in the distribution of diarrhoeagenic genes in *B.cereus* isolates from environmental and food samples[21,22,23], no reports were found in the course of this study that suggested varying growth environment affected the number of *hbl* genes detected by PCR in the same *B.cereus* strain from a food sample, as was observed in this study. The non detection of other *hbl* genes may be attributed to polymorphism among genes [24], a phenomenom where multiple forms of a single gene exist in an organism. High polymorphism has been reported in *hbl* genes in food associated *B. cereus* strains than diarrhoeal strains[25], where in *B.cereus* isolates which tested negative for *hbl* genes with PCR, had the genes confirmed by southern blotting. The southern analysis showed that genetic heterogeneity among *B.cereus* strains, particularly environmental strains, were more truly associated with sequence polymorphism, than with the lack of the genes composing the *hbl* operon. Genes contained in an operon are either expressed together or not at all. Several genes must be both co-transcribed and co-regulated to define an operon. Polymorphism may therefore explain why isolates in this study with had positive results for the L_2 component of the HBL enterotoxin with the BCET-RPLA assay, did not appear to encode for some of the *hbl* genes as shown by PCR.

It was also observed in this study that non enterotoxin producing isolates also encoded for at least one of the *hbl* genes; *B.cereus* isolates from milk samples at pH 6.8, following refrigeration for 48 hours, tested negative for the L_2 component of the HBL complex with the BCET-RPLA kit when cultured in BHI broth, despite encoding for the *hblD* gene, inferring that expression of the gene was suppressed when isolates were exposed to these storage conditions.

CONCLUSION

The above findings significantly highlight the possibility of sustained heritable traits of toxin suppression in *B.cereus* milk isolates as a result of some environmental conditions during refrigerated storage. The patterns of responses exhibited by these isolates as regards HBL production in response to environmental conditions during milk storage suggests the possibility of induced suppression or expression lasting through some generations of these *B.cereus* milk isolates. As such, the significance of extensively studying the effects of environmental stresses on production of toxins by *B. cereus* in different food matrices could not be over emphasized. Results of such studies could provide platforms that could be exploited for control of food borne infections and intoxications.

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