

# DIVERSITY OF *BACILLUS CEREUS* IN EXTENDED SHELF LIFE

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## ABSTRACT

Characterisation of 49 *Bacillus cereus* strains obtained from extended shelf life (ESL) milk and filler nozzles was done using (GTG)<sub>5</sub> Rep PCR fingerprinting; determining the presence of virulence genes; *cytK*, *nheA*, *cer* and *hbla* and discrimination of psychrotrophic and mesophilic strains with 16S rDNA. Fourteen isolates were selected for 16S partial sequencing. Fingerprinting and sequencing showed evidence of filler nozzles contaminating ESL milk despite high heterogeneity existing between the isolates. While there is high prevalence of *cer*, *hbla* and *nheA*; *cytK* was not widely distributed. There was 100% and 8% prevalence of mesophilic and psychrotrophic signatures respectively. Despite the high diversity of the *B. cereus* in this study, there is evidence that filler nozzles and raw milk are a source of contamination of *B. cereus* in ESL milk.

**Keywords:** Extended shelf life milk, *Bacillus cereus*, cytotoxin K (*cytK*), nonhemolytic enterotoxin A (*nheA*), emetic toxin cereulide (*cer*) and enterotoxin hemolysin BL (*hbla*).

## 1. INTRODUCTION

*Bacillus cereus*, an ubiquitous spore-forming bacteria has been identified as the main spoilage organism in pasteurised milk, (Aouadhi, Maaroufi and Mejri, 2014). Its presence in milk products does not only cause spoilage concerns, but safety too as it is known to produce a number of toxins, (Hansen and Hendriksen, 2001; Bartoszewicz, Hansen and Swiecicka, 2008). Extended shelf life (ESL) milk is a product bridging the gap between UHT and pasteurised milk that has gained popularity over the past few years. Although previous studies have reported that *B. cereus* was not the dominating organism in ESL milk (Schmidt *et al.*, 2012; Mugadza and Buys, 2014), its ability to grow at 7 °C (Mugadza and Buys, 2014), makes it a significant organism in ESL milk.

While raw milk has been implicated as an important source of endospores in milk products, (Miller *et al.*, 2015) other studies have shown that a different population of endospore forming microorganisms exists between raw milk and milk products made from the same raw milk. This population change has been attributed to a number of reasons that include post process contamination among others (Scott *et al.*, 2007; Burgess, Lindsay and Flint, 2010; Hill and Smythe 2012). Post heat treatment contamination by processing equipment such as fillers has also proved to cause bacterial population variations between raw milk and milk products (Scott *et al.*, 2007; Burgess *et al.*, 2010; Hill and Smythe 2012). Other studies have shown that *B. cereus* isolates from the environments (soil, food, the dairy production chain) have a higher degree of heterogeneity as compared to clinical isolates (Helgason *et al.*, 2000; Ehling-Schulz *et al.*, 2005). While Banyko´ and Vyletelova, 2009 concluded that *B. cereus* do not originate exclusively in either the raw or pasteurised milk but from post-pasteurisation contamination and propagation during the manufacturing process.

A full understanding of the characteristics and relationship between *B. cereus* strains isolated in the ESL milk processing environment becomes important in determining the source of product contamination. The objective of this study was to characterise *B. cereus* isolates obtained from ESL milk processing and during shelf life under refrigeration with the aim of determining cross contamination routes within the ESL milk processing plant.

## **2. MATERIALS AND METHODS**

### ***2.1 Isolates and DNA extraction***

Milk samples and filler nozzles swabs were collected at the same time from an ESL milk processing plant (Gauteng, South Africa). Samples were selected from the same process line ensuring the raw milk is tracked to the final product. Samples of raw milk, pasteurised milk before packaging and packaged ESL milk were collected together with filler nozzles swabs during 3 visits. Furthermore, some packaged ESL milk samples were stored at 4 and 7 °C over a 21 day shelf life period. *B. cereus* isolates were obtained from these samples as described elsewhere (Mugadza and Buys 2014; Khoza 2015). Forty nine *B. cereus* isolates that were obtained from raw milk (2), pasteurised milk (4), filler nozzles (40) and ESL milk during storage at 7 °C (3), were grown in nutrient agar at 30°C together with a reference strain, *B. cereus* ATCC 10876 until the late exponential phase which required an incubation period of 16 – 24h. DNA was extracted using the ZR Fungal/Bacterial DNA MiniPrep™ (Zymo Research, California, USA) according to manufacturer's instructions for use in the PCR reactions.

## **2.2 (GTG)<sub>5</sub> Rep PCR Fingerprinting**

Fingerprinting PCR was carried out in 20µL reaction tubes in a Bio-Rad T100™ Thermal Cycler (Bio-Rad, Singapore, Singapore). The reaction mixture consisted of 10µL KAPA BIOSYSTEMS 2X KAPA Taq Ready Mix (Kapa Biosystems Cape Town, South Africa), 0.3µL GTGGTGGTGGTGGTG oligonucleotide primer (Versalovic *et al.*, 1994), 2µL DNA template, 7.3µL PCR-grade water and 0.4µL Dimethyl sulfoxide (DMSO). The PCR program was as follows: 95 °C for 5 min, 95 °C for 1 min, annealing at 52 °C for 1 min, extension at 72 °C for 3 min. The program was repeated for 34 cycles and a final extension at 72 °C for 10 minutes. PCR products were separated by Gel electrophoresis using a 1.5 % Agarose gel (55V for 4h) and the image viewed using a Bio-Rad Gel Doc™ EZ (Bio-Rad, California, USA). The GTG5 fingerprints were analysed using Gel-Compar II version 6.5 (Applied Maths NV, Sint-Martens-Latem, Belgium). The similarity of digitised bands patterns was calculated using Pearson's correlation coefficient, and unweighted pair group method with arithmetic means. Complete linkage algorithms were used to construct an average linkage dendrogram to show relationship of isolates. Isolates were considered to be within a clonal cluster if relatedness was 70% and above (Stackebrandt *et al.*, 2002). However, due to the close similarities that existed between the isolates sub clusters were further considered at 95%.

## **2.3 PCR to determine virulence genes and discriminate psychrotrophic from mesophilic *B. cereus***

Pathogenicity of the *B. cereus* was determined by observing the presence of the following genes; cytotoxin K (*cytK*), nonhemolytic enterotoxin A (*nheA*), emetic toxin cereulide (*cer*) and enterotoxin hemolysin BL (*hbla*). Discrimination of psychrotrophic and mesophilic *B. cereus* was done by targeting the 16S rDNA signatures. The primers used are described in

Table 1. All PCRs were carried out in 10µL reaction tubes in a Bio-Rad T100™ Thermal Cycler (Bio-Rad Singapore, Singapore). The reaction mix consisted of 1 µL bacterial DNA template, 5 µL KAPA BIOSYSTEMS 2X KAPA Taq Ready Mix (Cape Town, South Africa), 3.4 µL PCR-grade water and 0.3 µL of each primer. The PCR conditions for *cytK*, *cer*, *hblA* and *nheA* are described in a previous study (Swiecicka and Mahillon, 2006), while conditions for mesophilic and psychrotrophic *B. cereus* PCR are also previously described (von Stetten *et al.*, 1998). Correlation between isolates was also determined by assigning a scores 1 and 2 for the presence and absence of a gene respectively and similarity calculated using principle component analysis (PCA) and agglomerative hierarchical clustering (AHC) in XLSTAT an add-in programme of Microsoft excel.

**Table 1: Primers used to detect virulence, 16S mesophilic and psychrotrophic genes in *B. cereus* strains Isolated from Extended Shelf Life milk processing and during shelf life**

Primer Name	Sequence	Amplicon size (bp)	Gene amplified	Reference
Emetic toxin cereulide	F. ACGTCACCAGTNGATATWTC	1146	<i>cer</i>	Swiecicka <i>et al.</i> 2006
	R. CTCCACCATTCCCAWGCAAG			
Non hemolytic enterotoxin (A)	F. TACGCTAAGGAGGGGCA	499	<i>nheA</i>	Swiecicka <i>et al.</i> 2006
	R. TTTTATTGCTTCATCGGCT			
Hemolysin BL (B)	F. GTGCAGATGTTGATGCCGAT	1154	<i>hblA</i>	Swiecicka <i>et al.</i> 2006
	R. ATGCCACTGCGTGGACATAT			
Cytotoxin K	F. ACAGATATCGGTCAAATG	1011	<i>cytK</i>	Fagerlund <i>et al.</i> 2004
	R. CAACCCAGTTACCAAGTT			
Mesophilic MF/UR	MF – ATAACATTTTGAACCGCATG	249	16S rDNA	Stenfors and Granum, 2001
	UR – CTTTCATCACTCACGCGGC			
Psychrotrophic PR/UF	PR – GAGAAGCTCTATCTCTAGA	132	16S rDNA	Stenfors and Granum, 2001
	UF – CAAGGCTGAAACTCAAAGGA			

## 2.4 *B. cereus* 16S Sequencing

Sixteen *B. cereus* isolates that represented each cluster of (GTG)<sub>5</sub> were selected for 16S sequencing. Using the ZR Fungal/Bacterial DNA Kit™ (Zymo Research, California, USA), DNA was obtained from the cultures. The 16S target region was amplified using DreamTaq™ DNA polymerase (Thermo Scientific™, Johannesburg, South Africa) and the primers, (Amplicon size 1465bp) shown in Table 2. PCR products were gel extracted (Zymo Research, Zymoclean™ Gel DNA Recovery Kit, California, USA), and sequenced in the forward and reverse directions on the ABI PRISM™ 3500xl Genetic Analyser. Purified sequencing products (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, California, USA) were analysed using CLC Main Workbench 7 followed by a BLAST search (NCBI).

**Table 2: 16S Primers sequences used for *B. cereus* 16S sequencing**

Name of primer	Target	Sequence (5' to 3')
<b>16S-27F</b>	16S rDNA sequence	AGAGTTTGATCMTGGCTCAG
<b>16S-1492R</b>	16S rDNA sequence	CGGTTACCTTGTTACGACTT

## 3. RESULTS

### *3.1 (GTG)<sub>5</sub> Rep PCR Fingerprinting of B. cereus strains isolated from ESL milk processing and during storage*

All the *B. cereus* isolates under study showed close similarities above 70%. However they clustered into 6 groups at 95% similarity level (Figure 1). Groups one, three and five comprised of isolates from filler nozzles only. Groups 2 and 4 showed evidence of a very close relationship between raw milk and filler nozzles originating while group 6 showed a very close similarities between isolates from filler nozzles and pasteurised milk. All the

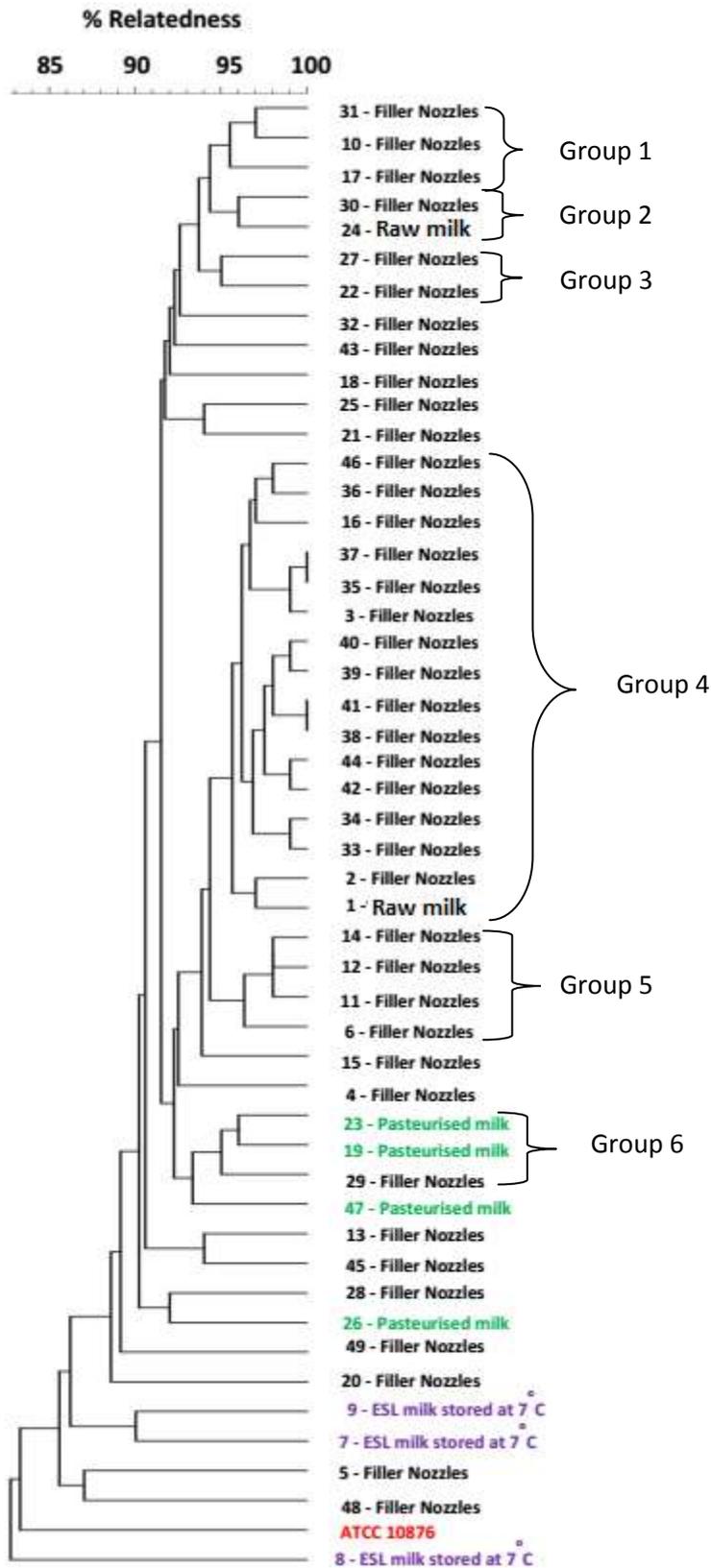


Figure 1: Dendrogram of Rep-PCR (GTG)<sub>5</sub> fingerprint patterns in *B. cereus* isolates from raw milk, ESL milk processing and during shelf life

isolates from ESL milk stored at 7 °C and the reference strain *B. cereus* ATCC 10876 did not cluster with any isolates at 95% although they showed some relationship with the other isolates at similarity level above 70%.

### ***3.2 Detection of virulence, mesophilic and psychrotrophic genes in B. cereus strains isolated from ESL milk processing and during storage***

While 65% of the isolates showed the presence of cereulide gene (*cer*) none were from ESL stored at 7 °C (Table 3). The cytotoxin K gene (*cytK*) was present in 33% of the isolates from all the sources and 88% of the isolates showed the presence of enterotoxin haemolysin BL (*hbla*). Ninety-eight percent of the isolates were positive for non-haemolytic enterotoxin A (*nheA*) while all the isolates showed the presence of the mesophilic 16S signature (MU/MF). Only 8% of the isolates showed the presence of psychrotrophic 16S signature of which the isolates originated from ESL milk stored at 7 °C, raw milk and pasteurised milk. Six groups were observed when isolates were clustered basing on these genes (Figure 2). Group 1 comprised isolates originating from raw milk, pasteurised milk and ESL milk filler nozzles that were positive to all genes under investigation except *cytK* and the psychrotrophic 16S signature. Groups 2, 3 and 4 comprised entirely of isolates from filler nozzles. Group 2 isolates were positive only for *nheA*, *hbla* and the mesophilic 16S signature while group 3 was only negative for the psychrotrophic 16S signature. Furthermore group 4 was negative for the psychrotrophic 16S signature and *hbla*. Group 5 was made up of isolates from ESL milk stored at 7 °C only while group 6 is a mixture of ESL milk stored at 7 °C and ESL milk filler nozzles isolates. While these two groups are the only ones that were positive for the psychrotrophic 16S signature the former was only negative for *cer* and *hbla* and in addition to the psychrotrophic 16S signature the latter was only positive to *nheA*. The principal component analysis (Figure 3) showed that *cytK* present in psychrotrophic strains was absent

in over 70% of mesophilic strains hence these two groups did not cluster together. Mesophilic strains showed 4 distinct clusters with the biggest cluster showing the presence of *hblA* and *cer* genes. *Cer* and *nheA* dominated the second cluster while *hblA* dominated the other cluster. The smallest cluster was more aligned to the presence of *cytK* and *nheA*.

**Table 3: Detection of virulence, mesophilic and psychrotrophic genes in *B. cereus* strains isolated from raw milk, ESL milk processing and during shelf life**

Isolate Source	Isolate (%)	Gene					
		<i>Cer</i>	<i>nheA</i>	<i>hblA</i>	<i>cytK</i>	MF/UR	PR/UF
Raw Milk	2	+	+	+	-	+	-
Pasteurised Milk	4	+	+	+	-	+	-
Filler Nozzles	33	+	+	+	-	+	-
Pasteurised Milk	2	+	+	+	+	+	-
Filler Nozzles	20	+	+	+	+	+	-
Filler Nozzles	22	-	+	+	-	+	-
Filler Nozzles	4	+	+	-	+	+	-
Filler Nozzles	2	-	+	-	-	+	+
ESL Milk	2	-	+	-	-	+	+
ESL Milk	4	-	+	-	+	+	+
Raw Milk	2	-	+	+	+	+	-
Pasteurised Milk	2	-	-	+	-	+	-
ATCC 10876	-	+	+	+	+	+	+

+ means positive, - means negative.

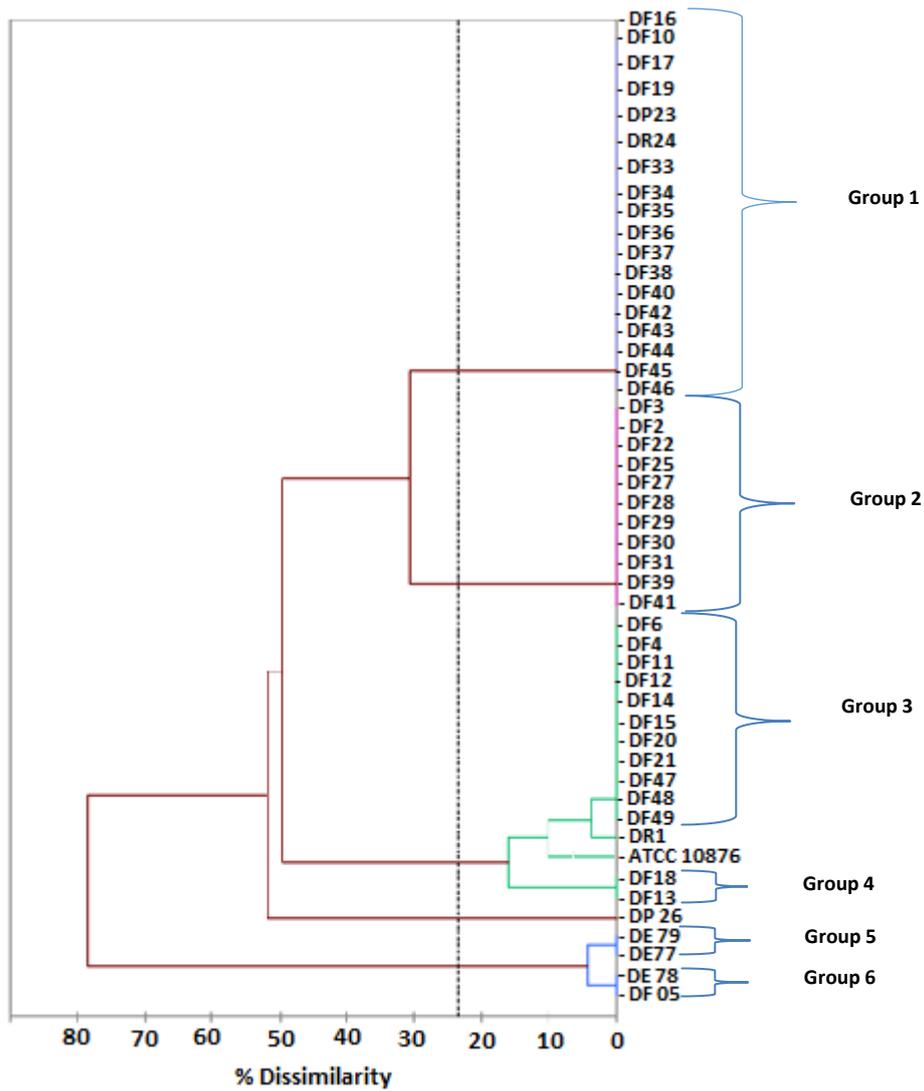
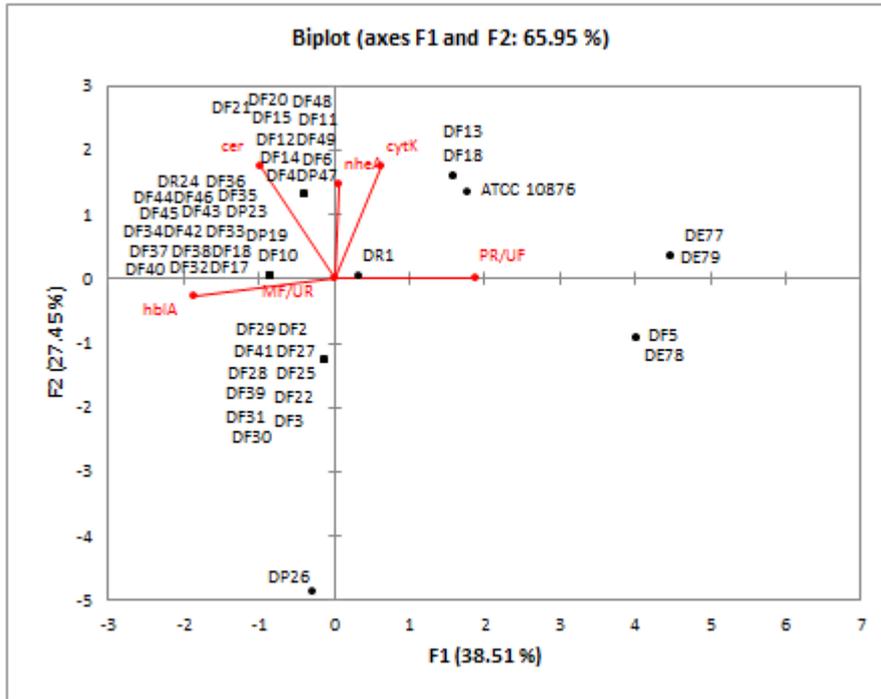


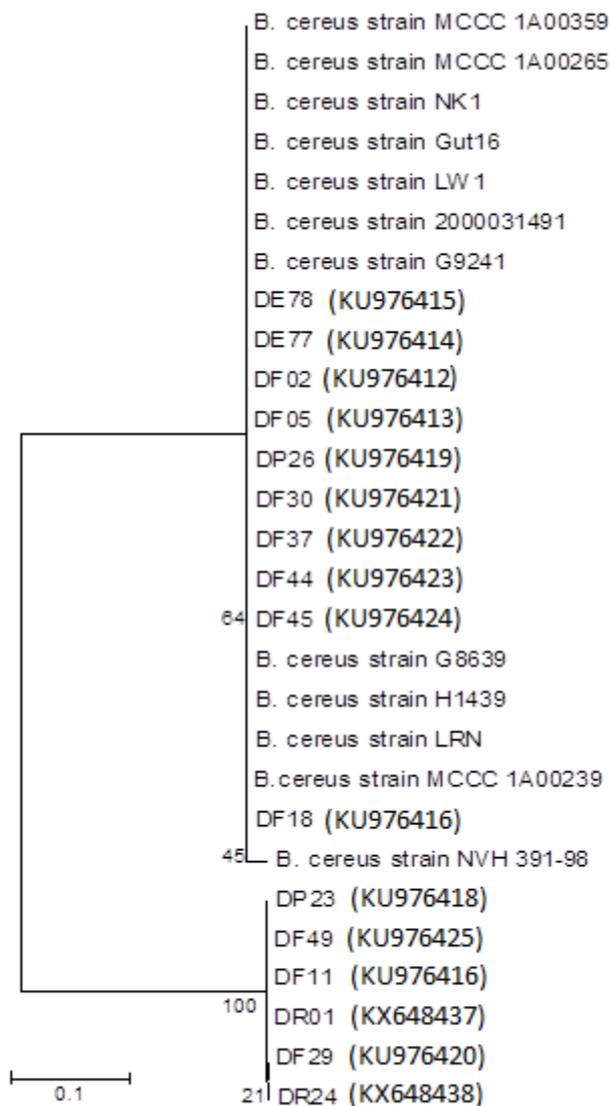
Figure 2: Agglomerative Hierarchical clustering of *B. cereus* isolates from ESL milk processing and during shelf life based on presence of virulent genes, 16S mesophilic and psychrotrophic signatures.



**Figure 3: Principal component analysis of virulent genes, 16S mesophilic and psychrotrophic signature data set consisting of 49 *B. cereus* isolates obtained from ESL milk processing and during storage.**

### 3.3 *B. cereus* 16S Sequencing

While the isolates had 2 distinct clusters (Figure 4), the biggest cluster showed that 7 isolates from filler nozzles, clustered together with 1 from pasteurised milk and 2 from ESL milk stored at 7 °C. Nine isolates out of the total 16 (56.25%) possessed a 16S rDNA sequence with 64% similarity to that of a previously characterised clinical and environmental bacterial species. The second cluster comprised of 3 isolates from filler nozzles, 1 from pasteurised milk and 2 from raw milk. The relatedness in this second cluster is high enough to prove that these isolates are the same strain. Although all the isolates did not show close relationship with previously characterized strains, 4 of them possessed a 16S rDNA sequence with  $\geq 99\%$  similarity.



**Figure 4: 16S rRNA sequence based maximum likelihood phylogenetic tree of *B. cereus* from ESL milk processing and during shelf life and other *B. cereus* strains**

\*Gene Bank accession numbers are given in parentheses.

#### 4. DISCUSSION

In this present study we determined the genetic diversity of *B. cereus* isolates from an ESL milk processing factory. Our results showed that there was relatedness amongst all isolates at 70% similarity level. Although this clustering does not mean the isolates belong to one strain, the similarity can be attributed to isolates belonging to the same species. Furthermore, as

previously described, (Aminov 2011; Böhm *et al.*, 2015) evolution and transfer of genetic material through horizontal gene transfer may also have contributed to these similarities. Although the isolates under study belonged to the same cluster at 70%, they exhibited high levels of genetic polymorphism resulting in several sub clusters. These results were similar to previous studies on *B. cereus*, (Helgason *et al.*, 2000; Ehling-Schulz *et al.*, 2005; Savic *et al.*, 2015), that revealed high heterogeneity among the *B. cereus* isolates from milk environment. This suggests that ESL milk contamination is not exclusively from one source but rather a number of them including raw milk and processing equipment among others, (Faille, Fontaine and Bénézech, 2001; Jan-Tová, Draňková and Vorlová, 2004; Miller *et al.*, 2015). These results were consistent with previous studies on *B. cereus* in pasteurised milk processing and farm environment that provided evidence for additional contamination of pasteurized milk in production lines, (Christiansson, Bertilsson and Svensson, 1999; Svensson *et al.*, 2000). Despite the high heterogeneity observed among sub-clusters, high similarities were also observed within sub-clusters on isolates from filler nozzles and from the milk. This cements the hypothesis of raw milk being a source of contamination among many sources (Svensson *et al.*, 2000; 2004). In an earlier study, specific subgroups of *B. cereus* were found in pasteurized milk, while most isolates obtained from the pasteurized milk and final products belonged to the cluster as the strains germinated from spores in raw milk (Lin *et al.*, 1998). The presence of the similar isolates of *B. cereus* in raw milk, filler nozzles and commercially pasteurized milk samples, further confirms the role of raw milk and filling machines as sources of *B. cereus* contamination (Eneroth *et al.*, 2001; Huck *et al.*, 2007; Banyko' and Vyletelova 2009).

Previous studies have reported that most food contaminants are mesophilic strains (Larsen and Jørgensen 1997; Francis *et al.*, 1998; Stenfors and Granum, 2001; Guinebretire *et al.*, 2008). This shows that the risk posed by psychrotrophic *B. cereus* in food is obviously low.

However, our results show an increased risk posed by psychrotrophic strains in ESL milk as they contained some virulent genes. This difference could be revealing that virulent genes are closely associated with mesophilic strains hence the coexistence of mesophilic and psychrotrophic signatures may have resulted in the presence of virulent genes in psychrotrophic strains. However, scientific evidence is needed to support this hypothesis.

Our study also revealed that all *B. cereus* isolates linked to ESL milk contamination showed mesophilic characteristics. This was inline with a previous study that demonstrated a clustering of *B. cereus* strains from a dairy plant and suggested a long-lasting contamination caused by mesophilic *Bacillus* strains early in the production chain (Svensson *et al.*, 1999).

We revealed that only 8% (4) isolates showed the presence of the 16S psychrotrophic signature. Similarly a previous study reported that most *B. cereus* strains obtained from the food chain environment were mesophilic while a few exhibited psychrotrophic properties (Stenfors and Granum, 2001). Although previous studies have indicated that most food contaminants and virulent *B. cereus* are mesophilic strains (Larsen and Jørgensen 1997; Francis *et al.*, 1998; Stenfors and Granum, 2001; Guinebretire *et al.*, 2008) our study revealed a contrary trend. This may be a result of the emergence of psychrotrophic *B. cereus* strains resulting from increased cold exposure to cold environments leading to increased adaptation and evolution (Guinebretire *et al.*, 2008). This indicates that the most problematic strains in ESL milk are not psychrotrophs only but mesophiles that have psychrotrophic signatures or vice versa.

Contrary to a previous study which concluded that emetic toxin, cereulide producing *B. cereus* are rare in the environment (Altayar and Sutherland, 2005) our results showed 65% prevalence of the emetic toxin producing *B. cereus*. However, in agreement with previous studies (Carlin *et al.*, 2006; Hoton *et al.*, 2009), all cereulide positive isolates were

mesophilic. The increased presence of the *cer* is probably due to adaptation as most *cer* containing strains usually propagate at low incidences in milk (Bartoszewicz *et al.* 2008). Furthermore the fact that toxigenic determinants spread horizontally among *Bacillus* species (Bartoszewicz, Hansen and Swiecicka, 2008) may have contributed to increased *cer* prevalence. Although cereulide synthesis is known to occur at a temperature range 12 to 37 °C, (Finlay, Logan and Sutherland, 2000; Häggblom *et al.*, 2002), depending on strain and media too (Apetroaie-Constantin *et al.*, 2008) cold chain abuse of ESL milk product therefore puts consumers at risk of this emetic toxin.

The distribution of enterotoxin gene *hblA* has not followed a definitive trend however, it has been hypothesised that many food-borne strains of *B. cereus* are less prone to cause diarrhoea (in't Veld *et al.*, 2001; Guinebretière and Broussolle, 2002). The high prevalence of the enterotoxin genes *hblA* and *nheA* in the total isolates and low prevalence of the genes in the final ESL milk product could be result of failure of the *hblA* and *nheA* possessing strains to withstand processing conditions and proliferate during sold storage (Hansen and Hendriksen, 2001; Ehling-Schulz *et al.*, 2006b; Bartoszewicz *et al.* 2008). The presence of the *hblA* does not directly translate to production of the toxin since other components are required to be present (Granum and Lund, 1997; in't Veld *et al.*, 2001; Arnesen, Fagerlund and Granum, 2008). However, our results show that consumers of cold-stored ESL milk product maybe at risk of the diarrheal causing toxins.

Although cytotoxin K is regarded as highly toxic to humans (Hardy, Lund and Granum, 2001; Fagerlund *et al.*, 2004), it is not widely distributed in *B. cereus* (Lund, De Buyser and Granum, 2000; Stenfors and Granum 2001; Fagerlund *et al.*, 2004; Swiecicka and Mahillon 2006). While the reason for low frequency of *cytK* in food related *B. cereus* isolates is not clear a possible explanation could be the failure of *cytK* containing strains to propagate in

the food matrix. However studies need to be done to prove the hypothesis. Despite the fact that enterotoxins are heat labile and prone to degradation by pH changes (Hansen and Hendriksen, 2001) the presence of the *cytK* in psychrotolerant isolates from the ESL milk poses a potential safety threat to consumers.

## 5. CONCLUSION

The simultaneous presence of psychrotrophic, mesophilic and at least 2 virulence genes in some of the isolates shows high diversity of *B. cereus* strains in ESL milk process that does not follow any distinguished pattern. This shows that contamination of ESL milk with *B. cereus* follows several routes. This study provided evidence that filler nozzles and raw milk are a source of contamination of ESL milk.

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