SHORT COMMUNICATION

Source tracking *Bacillus cereus* in an extended shelf life milk processing plant using partial sequencing of *rpo*B and multilocus sequence typing

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SUMMARY

In this study we characterized seven *Bacilllus cereus* strains obtained from an ESL milk processing plant. The objective was to source track *B. cereus* contamination in ESL milk. Multiloccus sequencing and *rpoB* partial sequencing was used to source track. Due to similarities observed among isolates we suggest 3 routes of *B. cereus* contamination in ESL milk. The study showed that *B. cereus* contamination of ESL milk might be through raw milk and biofilms from filler nozzles. In addition, *rpoB* partial sequencing and MLST can be used as tools for source tracking in ESL milk processing.

ABSTRACT

We used *rpoB* partial sequencing and multilocus sequence typing (MLST) to characterize 7 *Bacillus cereus* strains obtained at the following points: ESL milk during shelf life, pasteurized milk, raw milk, and filler nozzles after cleaning in place. The objective of the study was to determine relatedness among *B. cereus* isolates from several sampling points along an ESL processing plant with the aim of source tracking. The study revealed that isolates from filler nozzles shared 100% similarity with isolates from ESL milk and raw milk using *rpoB* sequencing. It also revealed that isolates from pasteurized milk shared 100% similarities with isolates from filler nozzles and ESL milk using MLST. We suggest 3 routes of *B. cereus* contamination in ESL milk. We showed that *B. cereus* contamination of ESL milk might be through raw milk and biofilms from filler nozzles. In addition, *rpoB* partial sequencing and MLST can be used as tools for source tracking in ESL milk processing.

Keywords: Extended shelf life milk, *Bacillus cereus*, source tracking, housekeeping genes

INTRODUCTION

Despite advancement in preservation and processing technologies, *B. cereus* remains a shelf life and consumer safety challenge for the dairy industry (Ranieri et al. 2012; Aouadhi et al. 2014; Mugadza and Buys, 2017a). *B. cereus* is a ubiquitous spore-forming bacterium (Ranieri et al., 2012; Aouadhi et al., 2014) that is reported to produce various toxins responsible for diarrhoeal and emetic food poison (Hansen and Hendriksen, 2001; Arnesen et al., 2008; Bartoszewicz et al., 2008). Studies conducted at an ESL milk processing plant revealed the presence of

psychrotrophic *B. cereus* in milk during processing as well as during storage and in filler nozzles after CIP (Khoza, 2016; Mugadza and Buys, 2017a). A follow up study on these *B. cereus* strains then showed close relatedness between these strains using repPCR and 16S partial sequencing (Mugadza and Buys, 2017b).

B. cereus endospores can germinate at refrigeration temperatures. In addition, some strains can also grow, as well as produce toxins under these refrigeration conditions (Larsen and Jørgensen, 1997; Stenfors and Granum, 2001; Thorsen et al., 2006). *B. cereus* has also been reported to be able to attach to stainless steel (Eneroth et al., 1998, 2001; Khoza, 2016) the main material used in manufacturing processing equipment. In addition, other studies have isolated *B. cereus* on the processing equipment, suggesting the potential to form biofilms which can later be responsible for post process contamination of processed milk (Eneroth et al., 1998, 2001).

Although raw milk has been implicated as a source of *B. cereus* contamination in pasteurized milk (Huck et al., 2007a,b), post processing contamination by processing plant equipment has also been described (te Giffel et al., 1997; Svensson et al., 2000; Huck et al., 2007a,b). Identification of points of entry for these bacteria may allow the development of effective strategies for reducing or eliminating their presence in milk production systems (Huck et al., 2008).

Despite several methods being suggested for source tracking (Fu and Li, 2014), multilocus sequence typing (MLST) has been described as a source tracking tool that determines exact nucleotide differences for conserved loci (Cardazzo et al., 2008). MLST studies have previously been used to examine the phylogeny of the *B. cereus* complex (Helgason et al., 2000, 2004;

Barker et al., 2005), identifying three distinct lineages that largely correspond to the species distribution (Cardazzo et al., 2008). In addition to MLST, partial sequencing of protein coding genes such as *rpoB* have also been successfully used to discriminate closely related species that are difficult to distinguish with other methods that are based on the 16S rRNA (Adékambi et al., 2009; Jiménez et al., 2013).

The objective of this research was to source track *B. cereus* in an ESL milk processing plant using partial sequencing of *rpoB* and MLST with the aim of determining the route of *B. cereus* contamination in ESL milk.

MATERIALS AND METHODS

Bacteria strains and DNA preparation

Seven *B. cereus* isolates obtained from various sampling points in an ESL milk processing plant; ESL milk during shelf life (BC7, BC8), pasteurized milk (BC23, BC26), raw milk (BC24) as well as filler nozzles after CIP (BC5, BC29) and described in previous studies (Khoza, 2016; Mugadza and Buys, 2017a), were selected for sequencing. The isolates were selected based on previously described $(GTG)_5$ fingerprints (Mugadza and Buys, 2017b), ensuring representation of each sampling point. Bacterial cultures were grown on nutrient agar at 30 ^{o}C for period of 16-24h. DNA was extracted from the *B. cereus* isolates using the ZR Fungal/Bacterial DNA MiniPrep (California, USA) according to manufacturer's instructions for use in the PCR reactions.

DNA amplification and sequencing

Protein coding gene *rpoB* together with 5 housekeeping genes; glycerol uptake facilitator protein (glpF), guanylate kinase, putative (gmk), dihydroxy-acid dehydratase (ilvD), phosphate acetyltransferase (pta) and phosphoribosylaminoimidazolecarboxamide (pur) distributed around the chromosome of *B. cereus*, were chosen for partial sequencing and MLST respectively and amplified using a Bio-Rad T100 Thermal Cycler (Singapore, Singapore). Details of primers have been previously described (Miyoshi-Akiyama et al., 2013; www.mlst.net). The reaction mixture consisted of 8 µL distilled PCR grade water, 1 µL each of the forward and reverse primer, 10 µL PCR mix (containing dNTPs, buffer, $MgCl_2$ and Taq polymerase) 1 µL gDNA. PCR protocol was as follows; 95 ^oC for 5 min, 95 ^oC for 30 s, 50 ^oC for 30 s, 45 Cycles, 72 ^oC for 30 s, 72 ^oC for 10 min, 4 ^oC hold. The PCR product was cleaned using USB ExoSAP-IT PCR Product Cleanup (Singapore, Singapore) according to manufacturer's instructions. Gel electrophoresis was conducted using a 1% agarose gel (with 5 µl of EZ-Vision In-Gel solution (Solon, Ohio USA) for every 50 ml of melted agarose). PCR product was mixed with GelRed® loading dye. The gel was run for 30 min at 100 V. DNA sequencing was done using Big Dye V3.1 as per manufacturer's instructions on the ABI 3500 XL with POP-7 and a 50 cm array.

RpoB gene phylogenetic analysis

Sequence analysis was performed at Inqaba Biotechnologies, Pretoria, South Africa and the chromatograms from the ABI 3100 sequences were exported, visually examined and gene sequences were analyzed using FinchTV version 1.4.0 (Geospiza). The *rpoB* gene sequences were aligned using BIOEDIT version 7.2.1. Multiple sequence alignment was performed using

Clustal Omega (EMBL-EBI, Hinxton). The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model (Jukes and Cantor, 1969). The tree with the highest log likelihood (-955.9639) was selected. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The proportion of sites where at least 1 unambiguous base is present in at least 1 sequence for each descendent clade is shown next to each internal node in the tree. The analysis involved 8 nucleotide sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

MLST data analysis

The sequences of the five housekeeping genes (*gmk*, *ilv*, *pur*, *pyc*, *tpi*) were edited to allele lengths (between 348 and 504 bp). The genes sequences were then assigned allele numbers based on the already described alleles of *B. cereus* MLST database (http://www.pubmlst.org/cereus). Isolates were assigned sequence type (ST) based on the combination of 5 alleles. The five gene fragments of each of the 7 isolates were concatenated and downloaded from the MLST website. An eighth isolate, *B. cereus* 2053 from the database was used to provide an outgroup sequence. The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model (Jukes and Cantor, 1969). The tree with the highest log likelihood (-940.3564) was selected. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using

the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 8 nucleotide sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

RESULTS AND DISCUSSION

In this present study *B. cereus* contamination routes in an ESL milk processing plant were determined using partial sequencing of the proten coding gene *rpoB* and MSLT. MLST showed close similarities between isolates from ESL milk (2), pasteurized milk (1) and filler nozzles (1). Two of these isolates (ESL milk and Pasteurized milk) had 100% similarity showing they belonged to the same strain. Two other isolates from pasteurized milk and filler nozzles also showed 100% similarity (Figure 1).

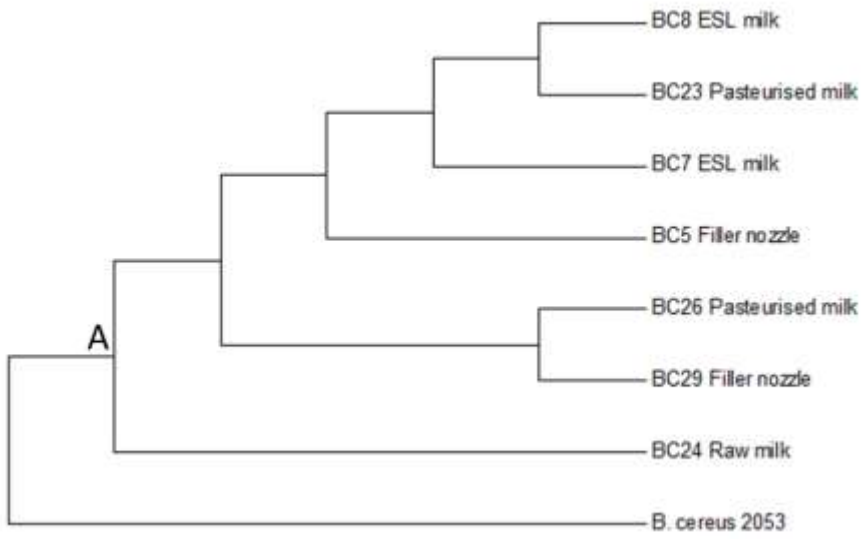

Figure 1: MLST profiles using five housekeeping alleles showing relationship among *B. cereus* strains isolated from raw milk, pasteurized milk, ESL milk processing and during shelf life.

These results show that *B. cereus* in ESL milk originated from raw milk among other sources. This is similar to previous studies that reported raw milk as the major source of *B. cereus* contamination in milk products (Huck et al., 2007a,b). The *B. cereus* strain from pasteurized milk was closely related to strains from ESL milk. This shows that these strains might be surviving throughout the ESL milk process until they get into the final product. *B. cereus* strains have successfully used endospores as a strategy to survive processing hurdles as compared to their vegetative forms (Lin et al., 2017).

MLST also revealed relatedness between *B. cereus* isolates from filler nozzles and pasteurized milk suggesting possible post processing contamination by processing equipment. This is consistent with previous studies that reported the contribution of processing equipment to milk contamination (Svensson et al., 2000; Huck et al., 2007a,b). These results were also consistent with previous studies on *B. cereus* in pasteurized milk processing and farm environment that provided evidence for additional contamination of pasteurized milk in production lines, (Christiansson et al., 1999; Svensson et al., 2000). In addition to endospore formation some *B. cereus* strains also use biofilm formation together with resistance to acids and alkali as strategies of survival during CIP (Lin et al., 2017). The presence of *B. cereus* isolates from raw milk that did not cluster closely with other isolates (Figure 1) may possibly show that contamination of ESL milk is from a diverse range of sources including raw milk and ESL milk filler nozzles. This is in line with previous studies which concluded 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 et al., 2001; Jan·Tová et al., 2004; Miller et al., 2015). MLST results indicate that ancestral lineage of all the isolates was point A (Figure 1), excluding BC24. This

shows that the various contaminations along the production line originated from some isolates from the raw milk. Slight genetic differences may have occured in *B. cereus* isolates from the raw milk and those in other parts of the production line because of microevolution taking place in biofilms. This is in line with findings from previous studies that confirmed adaptation as well as horizontal gene transfer and deletion of some determinants among *B. cereus* strains (Bartoszewicz et al., 2008; Guinebretire et al., 2008; Aminov, 2011; Böhm et al., 2015).

The *rpoB* partial sequencing showed that 3 pairs of isolates with 100% similarity existed (Figure 2). In Figure 2, first pair consisted of isolates from pasteurized milk, second pair consisted of isolates from raw milk and filler nozzles while the third pair had isolates from filler nozzles and ESL milk. Partial sequencing of *rpoB* further confirmed that *B. cereus* raw milk and pasteurized milk contributed to ESL milk contamination. This route of transmission from raw milk to pasteurized products is consistent with previous studies tracking spore-forming microbial contaminants from raw milk to finished fluid milk products (Huck et al., 2007a, 2008). Although *rpoB* has high discriminatory power in species and strain identification it is important to highlight that supplementary methods might be necessary for those species that cannot be delineated by sequence comparison of a single gene (Spanu et al., 2011). Using both *rpoB* partial sequencing and MLST the following 3 possible routes of *B. cereus* contamination in the ESL milk processing plant are suggested as follows; (1) *B. cereus* from raw milk survives the process and contaminate the final product without attaching filler nozzles, (2) *B. cereus* from raw milk survives the process, attaches to the filler nozzles and later contaminate the product, (3) *B. cereus* from biofilms on filler nozzles contaminate the product during packaging.

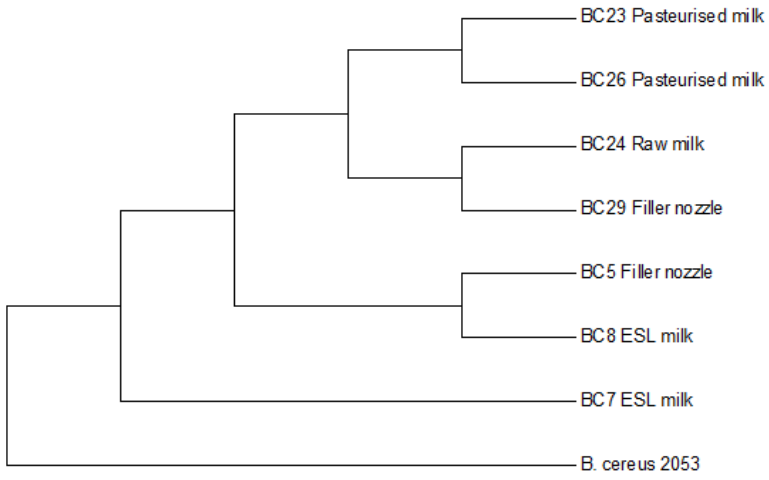

Figure 2: Neighbor-joining *rpoB* dendrogram representing the phylogenetic relationships of *B. cereus* strains isolated from raw milk, pasteurized milk, ESL milk processing and during shelf life.

CONCLUSIONS

The study established three possible routes of *B. cereus* contamination in ESL milk processing. *B. cereus* contamination of ESL milk is through raw milk and biofilms associated with filler nozzles after CIP. However, further work will be needed using more isolates to get a more robust picture about these possible routes of *B. cereus* contamination in ESL milk. In addition, the study has also shown that *rpoB* partial sequencing and MLST can be used as a tool for source tracking in ESL milk processing.

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