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Comparative genomic survey of *Bacillus cereus sensu stricto* isolates from the dairy production chain in Brazil

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Abstract

The genomes of 262 *B. cereus* isolates were analyzed including 69 isolates sampled from equipment, raw milk and dairy products from Brazil. The population structure of isolates showed strains belonging to known phylogenetic groups II, III, IV, V and VI. Almost all the isolates obtained from dairy products belonged to group III. Investigation of specific alleles revealed high numbers of isolates carrying toxin associated genes including *cytK* (53.62%), *hblA* (59.42%), *hblC* (44.93%), *hblD* (53.62%), *nheA* (84.06%), *nheB* (89.86%), *nheC* (84.06%) with isolates belonging to groups IV and V having significant higher prevalence of *hblACD* and group IV of *CytK* genes. Strains from dairy products had significantly lower prevalence of *CytK* and *hblACD* genes compared to isolates from equipment and raw milk/bulk tanks. Genes related to sucrose metabolism were detected at higher frequency in isolates obtained from raw milk compared to strains from equipment and utensils. The population genomic analysis demonstrated the diversity of strains and variability of putative function among *B. cereus* group isolates in Brazilian dairy production, with large numbers of strains potentially able to cause foodborne illness. This detailed information will contribute to targeted interventions to reduce milk contamination and spoilage associated with *B. cereus* in Brazil.

Keywords: population genomics; foodborne diseases; milk; next-generation sequencing

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1. Introduction

Bacteria belonging to the *Bacillus cereus* group are widely distributed in the environment and are commonly isolated from soil, air, water, plants and faeces. The ubiquity of these bacteria means that they frequently enter food production chains where human consumption of viable cells and preformed toxins can cause serious diarrhoeal disease and emetic syndrome respectively. Food poisoning outbreaks caused by this opportunistic pathogen have been widely reported (Zhou et al., 2014; Schimd et al., 2016) and *B. cereus* was responsible for an estimated 19% of reported foodborne outbreaks in the USA from 1998 to 2008 (Bennet et al., 2013). The actual number of outbreaks attributable to *B. cereus* may be higher, as detailed diagnostics to identify pathogenic strains are not widely used in clinical microbiology laboratories (Newell et al., 2010).

The genetic diversity within the *B. cereus* group (Raymond et al., 2010) and the mobility of virulence determinants among phenotypically defined species - often encoded on plasmids (Gonzalez et al., 1982; Okinaka et al., 1999; Raymond & Bonsall, 2013), have contributed to the difficulty in defining specific virulence and toxin production mechanisms in disease-causing lineages (Jeßberger et al., 2015). For example, the production of cereulide and diarrheal enterotoxin are not exclusive to *B. cereus sensu stricto* (Thorsen et al., 2006; Kovac et al., 2016) and some strains can harbor *B. anthracis* genes and are considered able to cause anthrax disease (Marston et al., 2016) and bacteremia (Sasahara et al., 2011; Schaefer et al., 2016). Detailed characterization of natural populations using comparative genomics techniques has the potential to identify strains that proliferate in food production systems and the genes associated with outbreak strains.

B. cereus group isolates have been identified in numerous foods but food poisoning is principally associated with heat-treated foods where the ability to produce metabolically inert resistant spores facilitates survival in adverse processing conditions (Daelman et al., 2013). The abundance of *B. cereus* in animals, including cows, makes it highly prevalent in the dairy farm environment, contaminating raw milk by simple transfer during milking (McAuley et al., 2014, Magnusson et al., 2007; Vissers et al., 2007; Coorevits et al., 2008; Masiello et al., 2014). Pasteurization may induce sporulation and the spores can subsequently survive the pasteurization process and contaminate dairy products (Bartoszewicz et al., 2008; Miller et al., 2015). Adoption of cleaning systems and good hygiene practices in dairy processing can reduce spore contamination, the proliferation of vegetative cells and toxin formation (Shaheen et al., 2010; Kumari & Sarkar, 2014) and *B. cereus* foodborne outbreaks are rarely linked with milk or dairy products consumption (Bennett et al. 2013). However, the risk for public health is dependent upon careful management.

Increasing demand for cheap milk and dairy products, and poor standards, such as the presence of feces in the dairy environment, improper bedding (Magnusson et al., 2007) and use of silage (Vissers et al., 2007) can contribute to milk contamination with *B. cereus* spores. This is exacerbated by the capacity to form biofilms which can lead to persistent recontamination during the dairy production process (Salustiano et al., 2009; Kumari & Sarkar, 2016). As a result, *B. cereus* remains a threat to public health as well as reducing production through spoilage of products. Furthermore, in countries such as Brazil, where there is higher consumption of dairy products sold through unregulated markets (Vidal-Martins et al., 2013), there is higher incidence of human infection from toxigenic *B. cereus* in raw milk and dairy products (Vidal-Martins et al., 2006; Salustiano et al., 2009; Reis et al., 2013, Aragon-Alegro et al., 2008; Chaves et al., 2011).

In this study we aim to clarify the population structure of *B. cereus* group isolates in Brazilian dairy production chains. Using structured sampling of *B. cereus* in dairy (farms, industries and products) and comparative genomics techniques we investigate if specific lineages and genes are significantly overrepresented in particular production stages and products. This study provides evidence of the genomic factors that contribute to survival of *B. cereus* from farm to fork in Brazil.

2. Material and Methods

2.1 Bacterial sampling and genomes

A total of 466 samples were obtained from the dairy production chain (dairy farms (331 samples), industries (58) and products on supermarkets (77)) in the municipality of Pirassununga, located in the central-eastern region of the state of São Paulo, Brazil, through July to November of 2016. A subset of 69 isolates were obtained from these samples and confirmed phenotypically to be *Bacillus cereus s.s.* through isolation on Mannitol Egg Yolk Polymyxin agar plates. The remaining 397 samples were not contaminated with *B. cereus* group or had other microorganisms concomitantly and were not sequenced. A set of 38 isolates were obtained from 26 distinct dairy farm

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environments in Pirassununga, isolated from the surface of bulk tanks (7), a manual milking can (1), milk cans (5), milk pipelines (4), raw milk (6), teats cups (5) and other utensils/equipment (10) that had direct contact with milk. Furthermore, 9 isolates were obtained in two dairy processing plants located in the same region from the dairy trucks dumping raw milk (2), equipment (3), milk pipeline (1) and utensils (3) used in dairy production. The samples were collected using swabs or sterile tubes only for raw milk. The other 22 isolates were obtained from dairy products available for consumption, such as Minas cheese (1), UHT dairy beverage (1), cream prepared with cheese (1), instant cappuccino (1) and Brazilian curd cheese "requeijão" (18). These 69 isolates were also categorized into three groups according to isolation origin as following: (i) Equipments and Utensils (34); (ii) Raw milk and bulk tanks (13); (iii) Dairy products (22). Furthermore, isolates were clustered into previously defined phylogenetic groups (Guinebretière et al. 2008).

Sequence data of 69 isolates obtained in Brazilian dairy production are archived in the NCBI GenBank repositor and the Short Read Archive (SRA) associated with BioProject: PRJNA390851 (Table S1). Assembled genomes are also available on FigShare (10.6084/m9.figshare.5120020). Full details and individual accession numbers of previously published genomes used as a reference (n=193 strains) can be found in Table S2.

2.2 Bacterial culture and biochemical typing

Pre-enrichment of the samples was performed using Tryptic-Soy Browth (TSB) (Oxoid, Hampshire, UK) with Polymyxin B addition (20µg/mL) (Stadhouders, 1992) and incubated during 24 hours on 30ºC. Afterwards, the samples were transferred to Mannitol Egg Yolk Polymyxin Agar (Oxoid, Hampshire, UK) plates (Mossel et al., 1967) using streaking technique and incubated during 24 to 48 hours on 30ºC. One presumptive colony was transferred to tubes containing inclined Trypticase Soy Agar (TSA) (Oxoid, Hampshire, UK) and incubated during 24 hours on 30ºC. These colonies were characterized using Gram staining and biochemical tests. Strains classified as *B. cereus s.s.* were those with the following biochemical characteristics: catalase (+), glucose fermentation (+), Voges-Proskauer (+), rhizoid growth (-), sheep's blood hemolysis (+), crystals production (-), motility (+/-) and nitrate reduction to nitrite (+/-) (MacFadin, 1976; Sharif & Alaeddinoglu, 1988; APHA, 2001).

2.3 DNA extraction and genome sequencing

Bacteria were transferred from tubes containing TSA (Oxoid, Hampshire, UK) to tubes containing TSB (Oxoid, Hampshire, UK) and were incubated during 24 hours at 30ºC. Aliquots of 1.5mL of suspended sample were transferred to eppendorf tubes to perform DNA extraction using GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, St. Louis, MO) according to manufacturer's instructions for Gram positive bacteria. DNA quality and concentration were evaluated by fluorimetric quantitation using a DeNovix DS-11 system. Sequencing libraries were prepared using the Illumina Nextera® XT Library Prep Kit (v3) according to the manufacturer's protocol. Briefly, this process involves fragmentation of 1 µg genomic DNA by enzymatic fragmentation, labeling with adapters' ligation and PCR using the Illumina 3 primer set. AMPure® XP paramagnetic beads (Beckman Coulter, Inc., USA) were used to perform DNA cleanup after each step to remove small fragments of DNA. Libraries were normalized and pooled for sequencing in an Illumina MiSeq.

2.4 Genome assembly and analysis

Contiguous sequences (contigs) were assembled using SPAdes v.3.8.0 (Bankevich et al., 2012). Individual genes were aligned to *B. cereus* reference strain (ATCC 14579) using default BLAST parameters in BIGSdb (Jolley & Maiden, 2010). The BLAST algorithm was used to scan all genomes for gene orthologs at each locus in the reference genome. An ortholog was defined as a reciprocal best hit of the sequence with >70% nucleotide identity over 50% of the difference in alignment length. MAFFT software was used to align gene orthologs on a gene-by-gene basis, and these data concatenated into contiguous sequence for each isolate genome (Sheppard, et al. 2013). Gene discovery, sequence export and gene-by-gene alignments were performed using MUSCLE (Edgar, 2004).

Neighbour joining (NJ) trees were constructed using rapidnj (Simonsen et al., 2008) from 69 whole genome sequences compared with 193 publicly available *B. cereus* group genomes. For comparison, a maximum-likelihood (ML) phylogeny was also constructed using FastTree version 2.1 (Price et al., 2010) and an approximation of the maximum-likelihood algorithm and visualized using Phylo.io (Robinson et al., 2016) (Figure S3). A set of 69 isolates also was classified according into seven distinct phylogenetic groups according to *panC* sequence (Guinebretière et al., 2008) in the online tool https://www.tools.symprevius.org/bcereus/english.php. The multilocus sequence type of each isolate was defined based upon allelic variation of 7 housekeeping genes (*glp, gmK, ilvD, pta, pur, pyc* and *tpi)* (Priest et al., 2004). Available nucleotide sequences from NCBI were used to identify toxigenic genes from *B. cereus* group, as follow: *cesA* (NC_010924), *cesB* (NC_010924.1), *cytK* (NC_005957.1), *hblA* (NC_005957.1), *hblC* (NC_005957.1), *hblD* (NC_005957.1), *nheA* (NC_005957.1), *nheB* (NC_003909.8) and *nheC* (NC_005957.1).

2.5 Statistical Analysis

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Fisher Exact Test using 5% of significance ($p<0.05$) was used in order to compare the prevalence of genes among phylogenetic groups and according to isolates origin.

3. Results

3.1 Population structure and epidemiology of milk-associated *B. cereus* group isolates

The clustering of isolates is consistent between NJ and ML genealogies between the phylogenetic groups (Figure S3). The population structure of 262 strains belonging to *B. cereus* group generally agreed with traditional genotyping designations based on the sequence of *panC* gene (Guinebretière et al., 2008) (Figure 1), except by one strain that was classified as group IV using *panC* sequence but it was clustered with the strains belonging to group V using whole genome sequencing. No strain belonging to phylogenetic groups I or VII was detected in this study in which *B. pseudomycoides* and *B. cytotoxicus* are included (Guinebretière et al., 2008; Guinebretière et al., 2010). Only one strain belonged to group VI, composed of *B. weihenstephanensis*, *B. mycoides and B. thuringiensis* strains. The other isolates obtained from samples of raw milk and bulk tanks, and those obtained from samples of utensils and equipment from dairy farms and industries were distributed in different clusters belonging to phylogenetic groups II, III, IV or V (Guinebretière et al., 2008). However, almost all the isolates obtained from dairy products (mainly curd cheeses “requeijão” cheeses) belonged to group III, which is composed of *B. cereus s.s.* (including emetic strains), *B. thuringiensis* and *B. anthracis* species. Using MLST, we detected sequence types: ST18; ST46; ST144; ST182; ST223; ST247; ST554 on milking cans, teat-cups and other equipment and utensils from dairy farms and industries. In bulk tanks, ST18, ST73 and ST144 were detected. In dairy products, ST5, ST26 and ST92 were detected.

3.2 Assessment of toxigenic potential by genotyping

The potential to produce four toxins (cytotoxin Cytk, hemolysin BL, non-hemolytic enterotoxin and cereulide) was evaluated in isolates. The presence of putative toxin producing genes was investigated in the genome including *cesA, cesB, cytK, hblA, hblC, hblD, nheA, nheB* and *nheC* (Ehling-Schulz et al., 2005; Ehling-Schulz et al., 2006; Ngamwongsatit et al., 2008). A total of 66 (95.65%) isolates carried at least one of these genes. All three (4.35%) isolates that did not carry any of these genes were obtained from curd cheese requeijão. High numbers of isolates carried *cytK* (53.62%), *hblA* (59.42%), *hblC* (44.93%), *hblD* (53.62%), *nheA* (84.06%), *nheB* (89.86%) and *nheC* (84.06%) genes. The prevalence of putative toxin genes was evaluated based upon isolate origin (Table 1) and phylogenetic group (Table 2).

The strains included in group IV and V had significantly higher prevalence of *hblA* ($p = 9.457 \times 10^{-11}$), *hblC* ($p = 8.846 \times 10^{-9}$) and *hblD* ($p = 2.432 \times 10^{-12}$) compared with those included in group III, using Fisher’s exact Test (5% of significance). Also, a significantly ($p = 1.082 \times 10^{-7}$) higher prevalence of *CytK* was observed in isolates included in group IV than in groups III and V. Fisher’s exact test was also performed to evaluate statistical differences of gene prevalence among groups according to isolation origin. *CytK* was significantly ($p = 0.01$) more prevalent in isolates obtained from milk samples than those from processed dairy products. Also, *hblA* ($p = 8.312 * 10^{-5}$), *hblC* ($p = 0.001$) and *hblD* ($p = 0.0002$) were significantly more prevalent in isolates obtained from equipment and utensils than in those obtained from dairy products. The presence of *cesAB* genes, related to emetic-toxin production, was observed in two isolates (2.89%); one from curd cheese requeijão and other from milk pipeline in a dairy farm.

3.3 Accessory genes associated with the later stage of dairy production chain

Some clues about the genes that may confer an advantage in survival through dairy processing can be obtained by quantifying genes that are enriched in strains isolated at the latter stages of the production chain. The difference in gene prevalence was determined by comparing *B. cereus* group isolates from three distinct categories: equipment and utensils; raw milk and silo tanks; dairy products. The greatest difference in gene prevalence between isolates obtained from raw milk and silo tanks and those from equipment and utensils was among genes putatively involved in sucrose metabolism. Specifically the gene cassette composed of genes putatively encoding fructokinase (*FruC*); sucrose-6-phosphate hydrolase (*SacA);* sucrose-specific PTS (*SacP*) and sucrose open repressor (*sacR*) (Fouet et al., 1987) – that had the highest difference in prevalence among isolates obtained from milk compared to those from equipment (57%). The prevalence was 92% (11/12) in isolates from raw milk while the value was 35% (11/34) from those obtained from equipment. Furthermore, the main difference (79%) comparing the prevalence of genes among the isolates from milk/tanks (92% - 11/12) with those from dairy products (13% - 3/22) was observed in the *amyS* gene, putatively responsible by alpha-amylase production and supports growth on starch/glycogen (Mols et al., 2007).

4. Discussion

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B. cereus group isolates sampled from the Brazilian dairy production chain were distributed in several known phylogenetic groups (Guinebretière et al., 2008; Caamaño-Antelo et al., 2015). The population structure of strains belonging to *B. cereus* group generally agreed with traditional genotyping designations based on the sequence of *panC* gene (Guinebretière et al., 2008), except by one isolate. This difference could be attributed to the methods used in this study (Neighbour joining tree and traditional genotyping) as previously reported (Carroll et al., 2017) or detection of new putative B. cereus group species (Liu et al., 2017). The presence of only one strain belonging to group VI can be explained by the psychrotrophic characteristic of bacteria included in this group, which are rare in tropical countries (Von Stetten et al., 1999). Furthermore, the exclusion of colonies with rhizoid morphology contributes to explain the non-detection of phylogenetic group I. Within these branching phylogenetic groups, specific strains of STs have been shown to be involved in food-borne outbreaks (Cardazzo et al., 2008; Yang et al., 2017). These include ST26, ST92 and ST144, isolated from curd cheese in this study, that have previously been involved in outbreaks (Hoffmaster et al., 2008). The genes *cytK*, *entFM*, *nhe* and *hbl* are reported in food-borne *Bacillus cereus* STs (Cardazzo et al., 2008). ST26 and ST144 have also been associated with emesis (Priest et al., 2004; Hoffmaster et al., 2008; Didelot et al., 2009) pneumonia (Hoffmaster et al., 2008) and contaminated infant formula in China (Yang et al., 2017). Other STs detected in this study (ST173 and ST18) have been linked to food poisoning outbreaks (Hoffmaster et al., 2008). With such a diversity of STs potentially causing disease, further research is required to understand the infection potential of the isolates described in this study.

Different phylogenetic groups have been shown to have different potential to cause disease. For example, group IV strains obtained from dairy origin pose a higher potential to produce hemolysin BL (Kovac et al. 2016). In our study, groups IV and V strains had higher prevalence of *hblA hblC* and *hblD* compared to those from group III. However, toxin production is dependent upon regulatory mechanisms (Kovac et al., 2016) and intestinal conditions (JeBberger et al., 2017). In our study, a high proportion of isolates belonged to phylogenetic group III (mainly curd cheeses “requeijão” cheeses). This group is considered to be of high risk for foodborne disease (Guinebretière et al., 2008), containing *B. cereus s.l.* lineages, including those considered as emetic, cytotoxic, and ‘highly cytotoxic’ (Guinebretière et al., 2008; Guinebretière et al., 2010). The presence of strains belonging to group III in heat-treated dairy products, such as observed in this study, could be related with a higher capacity of spores in resisting heat-treatment processes (Luu-Thi et al., 2014).

Alignment of sequences of whole genomes allows the identification of genes putatively involved in foodborne illness. For example, isolates carrying genes related to toxin production such as the *nhe* and *hbl* genes that are associated with diarrheal syndrome (Guinebretière 2010), and have been reported in isolates from milk (Granum et al., 1993). In our study, there was a high prevalence of the *cytK, hblA, hblC* and *hblD* genes in isolates from milk and equipment and utensils compared with those from dairy products. This potentially indicates different potential for toxin production in Brazilian dairy production, with consumption of raw milk being a potential risk for public health. The *ces* gene, associated with emetic-toxin production, was observed in two isolates in our study, one from curd cheese and the other from a milk pipeline in a dairy farm. The presence of emetic strains in dairy production chain is considered rare (Svensson et al., 2006, Cui et al., 2016) and the detection of a potential emetic strain in refrigerated “requeijão” is a concern for consumers as production of cytotoxic isocereulide A is higher at low temperatures (Kranzler et al., 2014).

In addition to detecting putative toxin genes, the comparative genomic analysis allowed the identification of genes that may confer an advantage for survival or growth in dairy products. We detected a high prevalence of genes related to the sucrose pathway in isolates obtained from raw milk and bulk tank samples compared to those from equipment and utensils and dairy products. Sucrose utilization has been demonstrated among *B. cereus* strains isolated from milk (Wong et al. 1998) but it is difficult to explain functionally as sucrose it is not a natural component of milk (Jenkins & McGuire, 2006). Sucrose is, however, a common plant carbohydrate and the main *B. cereus* contamination sources for raw milk are spores in grazing systems from cow udders contaminated with soil, feces and bedding material (Christiansson et al., 1999, Magnusson et al., 1999). This potentially explains the presence of strains able to metabolize sucrose in raw milk and bulk tanks.

The metabolism of carbohydrate is also related to toxin production (Ouhib et al., 2006). The induction of macrophage death at infection sites, and access to carbon sources and nutrients for bacterial growth, can occur through mechanisms requiring glucose and iron availability (Sineva et al.2009, Tran et al., 2011; Guillemet et al. 2013, Tran et al., 2013). This potentially provides alternative explanations for the prevalence of putative sucrose pathway genes among *B. cereus* group isolates in raw milk. First, because macrophage killing potentially provides a competitive advantage within the microbial community (Ceuppens et al., 2013) of raw milk where there are high numbers of viable macrophages (Langoni et al., 2011, Li et al., 2015). Second, because milk cows infected with subclinical mastitis, and hence elevated macrophage numbers, are a major source of milk contamination (Bhatt et al., 2012). However, these explanations are not tested in this study.

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In conclusion, we have demonstrated the utility of whole genome sequencing for characterizing the population structure of potentially toxigenic *B. cereus* group isolates in the dairy production in Brazil. Detailed information about the differential abundance of strains and specific genes across the production chain provides a basis for attributing the source of contamination and the detection of genetic elements that may underlie important phenotypes associated with persistence and pathogenicity. Clearly, the presence of potentially pathogenic strains in dairy production in Brazil requires the adoption of hygienic practices and avoidance of raw milk to improve food safety and reduce spoilage of dairy products.

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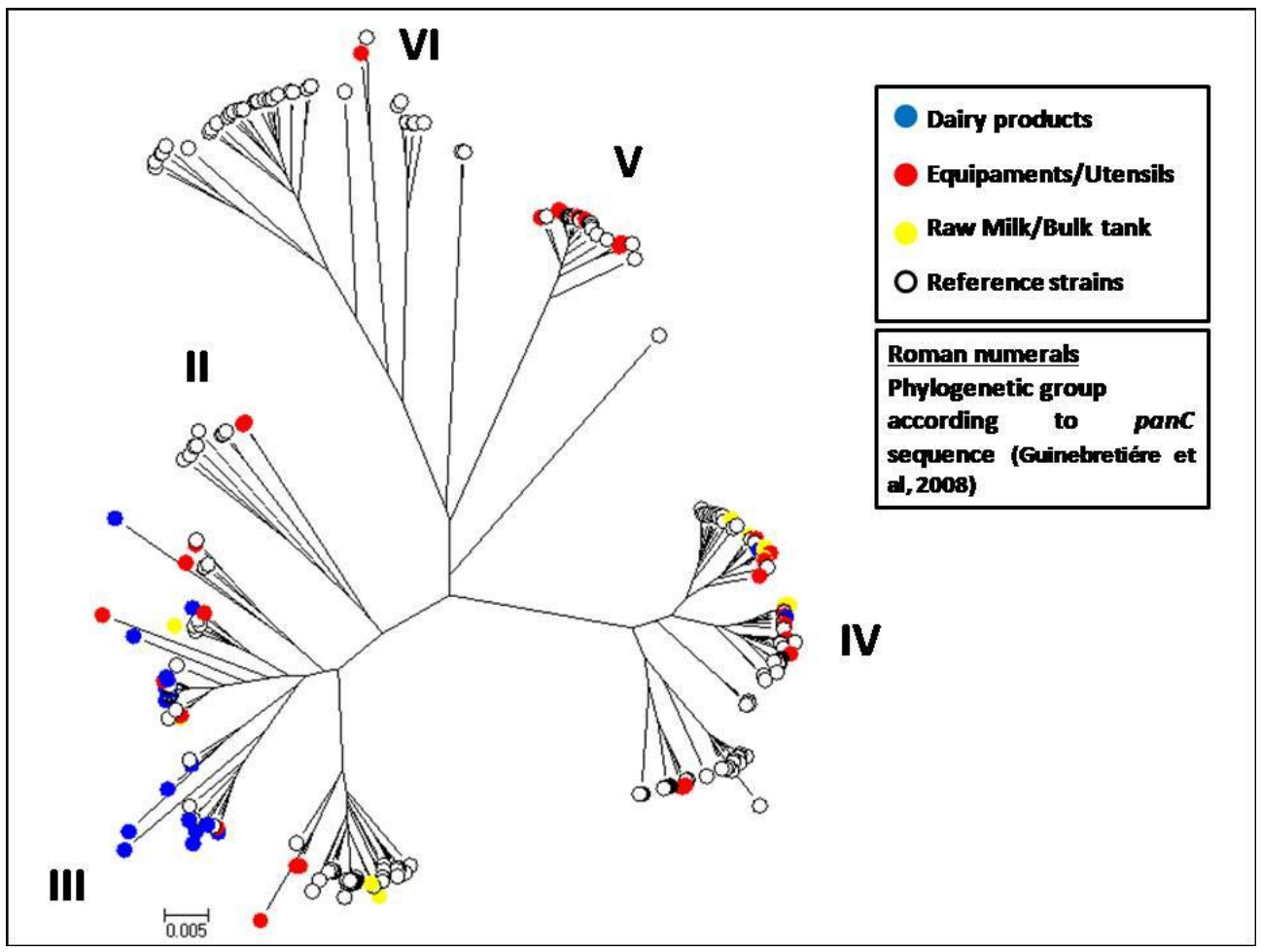

Figure 1. Population structure of 262 isolate genomes (69 from Brazilian dairy production chain) belonging to *B. cereus* group. The Neighbour joining trees was constructed using rapidnj (Simonsen et al., 2008) from whole genome sequencing (5,210 genes). Isolate origin is presented in the leaves on the tree, classified in dairy products (blue), equipment and utensil (red), raw milk and bulk tank (yellow) and the background population comprised 193 strains from published collections (white). The phylogenetic groups are defined according to genotyping information of the *panC* gene (II to VI) (Guinebretière et al., 2008).

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Table 1. Prevalence of genes related to toxins production (*cesAB, cytK, hblACD* and *nheABC)* in *B. cereus* group obtained from equipment and utensils, raw milk or bulk tank and dairy products in the state of São Paulo, Brazil, during 2016.

	Equipments/Utensils				Raw milk/Bulk tank				Dairy products				Total		
	+	N	P (%)	C. I 95%	+	N	P (%)	C. I 95%	+	N	P (%)	C. I 95%	+	N	P (%)
cesA	1	34	**2,94**	0,52 to 14,92	0	13	**0**	0,00 to 22,81	1	22	**4,55**	0,81 to 21,80	2	69	2,9
cesB	1	34	**2,94**	0,52 to 14,92	0	13	**0**	0,00 to 22,81	1	22	**4,55**	0,81 to 21,80	2	69	2,9
cytK	19	34	**55,88**	39,19 to 72,57	11	13	**84,62**	57,76 to 95,67	7	22	**31,82**	16,36 to 52,68	37	69	53,62
hblA	27	34	**79,41**	65,82 to 93,00	9	13	**69,23**	42,37 to 87,32	5	22	**22,73**	10,12 to 43,44	41	69	59,42
hblC	21	34	**61,76**	45,43 to 78,10	7	13	**53,85**	29,14 to 76,79	3	22	**13,64**	4,75 to 33,34	31	69	44,93
hblD	24	34	**70,59**	55,27 to 85,90	9	13	**69,23**	42,37 to 87,32	4	22	**18,18**	7,31 to 38,52	37	69	53,62
nheA	30	34	**88,24**	73,38 to 95,33	12	13	**92,31**	66,69 to 98,63	16	22	**72,73**	51,85 to 86,85	58	69	84,06
nheB	32	34	**94,12**	80,91 to 98,37	13	13	**100**	77,19 to 100,00	17	22	**77,27**	56,56 to 89,88	62	69	89,86
nheC	29	34	**85,29**	69,87 to 93,55	13	13	**100**	77,19 to 100,00	16	22	**72,73**	51,85 to 86,85	58	69	84,06

Legend: + = Number of positive isolates; N = Number of isolates; P= prevalence and C. I 95% = Confidence interval 95%

Table 2. Prevalence of genes related to toxins production (*cesAB, cytK, hblACD* and *nheABC)* in *B. cereus* phylogenetic groups (Guinebretière et al., 2008) in the state of São Paulo, Brazil, during 2016.

	II				III				IV				V				VI			
	+	N	P (%)	C. I 95%	+	N	P (%)	C. I 95%	+	N	P (%)	C. I 95%	+	N	P (%)	C. I 95%	+	N	P (%)	C. I 95%
cesA	0	2	0	0,0 – 65,76	1	34	2,94	0,52 – 14,92	1	22	4,55	0,81 – 21,80	0	8	0	0,0 – 32,44	0	1	0	0,0 – 79,35
cesB	0	2	0	0,0 – 65,76	1	34	2,94	0,52 – 14,92	1	22	4,55	0,81 – 21,80	0	8	0	0,0 – 32,44	0	1	0	0,0 – 79,35
cytK	0	2	0	0,0 – 65,76	12	34	35,29	21,49 – 52,09	22	22	100	85,13 – 100,0	3	8	37,5	13,68 – 69,43	0	1	0	0,0 – 79,35
hblA	2	2	100	34,24 – 100,0	8	34	23,53	12,44 – 40,0	22	22	100	85,13 – 100,0	8	8	100	67,56 – 100,0	1	1	100	20,65 – 100,0
hblC	2	2	100	34,24 – 100,0	4	34	11,76	4,67 – 26,62	16	22	72,73	51,85 – 86,85	8	8	100	67,56 – 100,0	1	1	100	20,65 – 100,0
hblD	2	2	100	34,24 – 100,0	5	34	14,71	6,45 – 30,13	22	22	100	85,13 – 100,0	7	8	87,5	52,91 – 97,76	1	1	100	20,65 – 100,0
nheA	2	2	100	34,24 – 100,0	27	34	79,41	63,20 – 89,65	20	22	90,91	72,18 – 97,47	8	8	100	67,56 – 100,0	1	1	100	20,65 – 100,0
nheB	2	2	100	34,24 – 100,0	30	34	88,24	73,38 – 95,33	22	22	100	85,13 – 100,0	7	8	87,5	52,91 – 97,76	1	1	100	20,65 – 100,0
nheC	2	2	100	34,24 – 100,0	29	34	85,29	69,87 – 93,55	19	22	86,36	66,67 – 95,25	7	8	87,5	52,91 – 97,76	1	1	100	20,65 – 100,0

Legend: + = Number of positive isolates; N = Number of isolates; P= prevalence and C. I 95% = Confidence interval 95%

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