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Phenotypic properties and genotyping analysis of *Bacillus cereus* group isolates from dairy and potato products

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ABSTRACT

Bacillus cereus group (*B. cereus sensu lato*) are ubiquitously distributed in diverse environments. In this study, eight isolates including *B. cereus*, *B. paranthracis* and *B. toyonensis* species, from dairy and potato products, were assessed for biofilm formation, sporulation and genetic information including biofilm-related genes and toxin genes. The isolates varied in their ability to form biofilm (either at the stainless steel-liquid-air interface or floating pellicles). The amounts of biofilms of *B. cereus s.l.*, were increased when incubated in agitation condition varied between isolates. Sporulation within the planktonic and biofilm modes of growth was compared, suggesting that biofilm is a favourable environment for *B. cereus s.l.* to form spores. Whole genome sequencing (WGS) was used to compare these *B. cereus s.l.* isolates. New sequence types (STs) of *B. cereus* were found in this study. Isolates that shared similar genomes had different biofilm-forming and sporulation abilities. Most of isolates tested, possessed biofilm-related genes. Different combinations of toxin-producing genes were identified in different isolates, with all isolates containing *nhe* while only some contained *hbl* and *cytK*. None of the food isolates contained the emetic *ces* gene. This study highlights the diversity of *B. cereus s.l.* in biofilm formation, sporulation and their genetic variables.

1. Introduction

The *Bacillus cereus* group, also called *B. cereus sensu lato* (*B. cereus s.l.*) comprise diverse species, including well-known *B. cereus sensu stricto* (*B. cereus*), *B. thuringiensis*, *B. mycoides*, *B. anthracis*, *etc.* and recently identified *B. toyonensis*, *B. paranthracis*, *etc.* (Guinebretière et al., 2010; Jensen, Hansen, Eilenberg, & Mahillon, 2003; Jiménez et al., 2013; Lechner et al., 2009; Liu et al., 2017). *B. cereus* is frequently identified in various food products, such as vegetables, milk, meat products, rice and cereals, therefore, it is considered as a significant challenge for food safety (Chang, Lee, Han, Kwak, & Kim, 2011; Eglezos, Huang, Dykes, & Fegan, 2010; Guinebretiere, Girardin, Dargaignaratz, Carlin, & Nguyen-The, 2003; Park et al., 2009; Samapundo, Heyndrickx, Xhaferi, & Devlieghere, 2011; Wijnands, Dufrenne, Rombouts, In't Veld, & Van Leusden, 2006). Biofilm and sporulation are two survival strategies used by *B. cereus* to adapt and survive with environmental stresses (Flemming et al., 2016). *B. cereus* is well-known as a potential foodborne pathogen, mainly due to the production of heat-liable enterotoxins: hemolysin BL (HBL) (Beecher, Schoeni, & Lee Wong, 1995), non-hemolytic enterotoxin (NHE) (Granum, O'Sullivan, & Lund, 1999), cytotoxin K (CytK) (Lund, De Buyser, & Granum, 2000) and emetic toxin cereulide (Rajkovic et al., 2008).

B. cereus is common in the dairy industry as it can grow at low-temperatures used to store raw milk and dairy products, and it produces spores that survive after milk pasteurisation (Bartoszewicz, Hansen, & Swiecicka, 2008; Gopal et al., 2015; Watterson, Martin, Boor, Kent, & Wiedmann, 2014; Wijnands, Dufrenne, Rombouts, In'tVeld, & Van Leusden, 2006). Potato is a popular food and common staple as a source of starch and abundant nutrients. *B. cereus* has been found in potato-based products including raw unpeeled potatoes, dehydrated potato flakes, ready-to-eat potato products, refrigerated processed potato puree, cooked mashed potato (Heini, Stephan, Ehling-Schulz, & Johler, 2018; Rajkovic, Uyttendaele, Courtens, Heyndrickx, & Debevere, 2006; Thomas et al., 2002; Turner, Whyte, Hudson, & Kaltovei, 2006).

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B. cereus, as a heat resistant spore-forming bacterium, can survive during the processing (heating and dehydration) of potato products and is regarded as a significant foodborne pathogen related to processed potato-based foods (Doan & Davidson, 2000; Heini et al., 2018). Unlike *B. cereus*, *B. toyonensis* and *B. paranthracis* are less well studied.

The purpose of this present study was to study some phenotypic properties (biofilm and sporulation) and genetic information (biofilm-related and toxin genes) within the *B. cereus* group isolates from dairy and potato products. This research highlights the microbiological issues in the food industry caused by *B. cereus s.l.*, and the diversity of the *B. cereus* strains in the food industry.

2. Materials and methods

2.1. Bacterial isolates and culture conditions

Eight food isolates, including potato isolates (P2, P4 and P5) and dairy isolates (M1, M2, M3, M4 and M5) were used in this study. These isolates were firstly selected from *B. cereus* selective MYP (mannitol yolk polymyxin) agar plate and were further identified as *B. cereus s.l.* based on 16S ribosomal RNA sequencing (Massey Genome Service, Massey University, New Zealand). Two reference strains, ATCC 14579 and F4810/72 (DSMZ, Germany), were also included in this study. Stock cultures were streaked on tryptic soy agar (TSA, Difco™, Becton, Dickinson and Company, the USA) plates and incubated for 24 h at 30 °C to obtain single colonies. The overnight culture was obtained by inoculating a single colony into TSB and incubated overnight (17–18 h) at 30 °C with 120 rpm shaking. The overnight culture was used for subsequent experiments. TSB (Tryptic soy broth) media was used throughout this study.

2.2. WGS of eight food isolates

WGS analysis was performed to study the genetic information of these food isolates. DNA from eight *B. cereus s.l.* food isolates (P2, P4, P5, M1, M2, M3, M4 and M5) was extracted using the QIAamp® BiOstic® Bacteremia DNA kit (QIAGEN, Germany) according to the manufacturer's instructions. The purity and concentration of the DNA were determined by NanoDrop spectrophotometers (Thermo Fisher Scientific, USA) and Qubit Fluorometer 3.0 (Thermo Fisher Scientific) with Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific), respectively. The qualified genomic DNA was used for the library construction according to the instructions of the QIAseq FX DNA Library Kit (QIAGEN). The library fragment size was then measured using an Agilent 2100 Bioanalyzer and DNA High Sensitivity Assay Kit (Agilent, USA). After standardization, the samples were pooled and library concentrations were determined using the Qubit™ dsDNA HS Assay Kit. After library quantification, paired-end sequencing was performed using the NextSeq 500/550 High Output Kit v2 (300 cycles) and Illumina NextSeq 550 platform (Illumina, USA). The raw data of sequencing were first quality trimmed using Trimmomatic v 0.39 software (Bolger, Lohse, & Usadel, 2014), then assembled by SPAdes software v 3.12.0 (Bankevich et al., 2012) and annotated by Prokka v1.11 (Seemann, 2014).

Pairwise genome calculations of the average nucleotide identity (ANI) were performed using the Python module Pyani v 0.2.8 with default parameters (https://github.com/widdowquinn/pyani), shifting the genomic gold standard for the prokaryotic species definition (Richter & Rosselló-Móra, 2009). A standard MLST scheme with seven house-keeping genes according to the MLST database (http://pubmlst.org/bcereus) were extracted from WGS data to determine the sequence type (ST). Furthermore, more genetic information including the presence of some reported biofilm-related and toxin genes within those food isolates were analysed using the genome sequences of different isolates.

2.3. Biofilm formation on stainless steel coupons and pellicle formation

Biofilm formation by all eight *B. cereus s.l.* isolates and two reference strains was assessed in 48-well plates (Costar®, Corning, USA) which were half-filled with 0.5 mL TSB broth in each well and inoculated with 1% (vol/vol) overnight-grown cultures. Biofilms were developed on stainless-steel coupons (10 mm × 10 mm x 1 mm; 304-2B stainless steel; Advanced Sheetmetals Ltd., Palmerston North) placed vertically into the wells, as stainless steel is the ideal material to use for equipment in various food manufacturing plants. Around half (100 mm^2 surface in total; 50 mm^2 on each side) of the coupons was submerged into the liquid medium. Plates were wrapped with parafilm (Bemis®, the USA) to prevent evaporation and then incubated for one, two and 3 day at 30 °C to allow the growth of biofilm cells.

The pellicle formation of ten *B. cereus s.l.* isolates was also investigated. Pellicles of these isolates were formed in a 48-well plate filled with 0.5 mL TSB broth and inoculated with 1% (vol/vol) overnight cultures. Wells containing only media were included as the negative controls. The pellicle formation was compared between isolates visually and photographing the wells after one-, two-, and three-days incubation. The plates were incubated at 30 °C either in static or 120 rpm shaking incubation for both biofilm formation on stainless steel coupons and pellicle formation. This biofilm test was performed with three independent biological repetitions.

2.4. Enumeration of total cells and spores within planktonic and biofilm populations

The number of biofilm cells grown on stainless steel coupon was quantified using spread plating. Each coupon was washed by dipping three times in 1% BPW and placed in a sterile glass bottle containing 5 mL BPW with 5 g sterile glass beads (D = 3 mm, Paul Marienfeld GmbH & Co., Czech Republic). Bottles were mixed by vortex at maximum speed (Scilogex, Germany) for 1 min to detach the biofilm cells from the coupon. Hayrapetyan, Muller, Tempelaars, Abee, and Nierop Groot (2015) indicated that this methodology can effectively separate cells from the substratum without affecting cell viability. Planktonic cultures were grown in a 250 mL shaking flask filled with 50 mL TSB broth and inoculated with 1% of overnight-grown culture. The enumeration of biofilm cells and the corresponding spores were investigated in either static or 120 rpm shaking incubation, while the planktonic culture was only tested in 120 rpm shaking incubation to avoid sedimental of cells.

For spore counts, both the planktonic cultures and biofilm suspensions were heated at 80 °C for 10 min in a heating block (Ori-Block® 08-3, Techne, Germany) to inactivate all vegetative cells. The number of total cells and their spores were determined by standard spread plating counting grown on TSA plates after 24 h incubation at 30 °C. Total CFU and spore counts within biofilm and planktonic populations were determined in independent biological triplicates for each isolate tested. The sporulation percentages within either planktonic and biofilm population were calculated based on the equation below:

$$\text{Sporulation percentage (\%)} = (\text{spores' counts/total cells counts}) \times 100\%$$

2.5. Statistical analysis

All graphs were created and analysed by GraphPad Prism 7. The data expressed in the figures were generated from the average value of three independent biological repetitions. Standard deviations represent the variation among replicates. One-way ANOVA or Two-way ANOVA (Tukey's multiple comparison test) by GraphPad Prism 7 were used to determine the statistically significant differences between treatments.

3. Results

3.1. Bacterial species identification

The genomes of eight food isolates were compared with all trusted type genome assemblies of *B. cereus* group species in the GenBank database. The results confirmed that P2, P4, P5, M1, M3 and M4 were *B. cereus* species, while M5 was identified as *B. toyonensis*. M2 showed 97.5% identity with 81% coverage to type strain of *B. paranthracis* and only 91.8% identity with 76% coverage to type strains of *B. cereus*, therefore, M2 was assigned as *B. paranthracis*. The results are summarised in Table 1.

3.2. Genetic diversity between isolates based on WGS

3.2.1. ANI analysis and MLST scheme

The genetic distances of all ten tested isolates in this study (eight food isolates and two reference strains) were analysed. The genomes of ATCC 14579 (GCF_006094295.1) and F4810/72 (GCF_000021225.1) were obtained from GenBank. The alignment coverage (%) between isolates were calculated (Supplementary data). Based on the coverage, the pairwise genome comparisons of percent identities (ANI metrics) between isolates were analysed, shown in Fig. 1. An ANI above 95% between the two genomes is an indication that the two strains with highly similar genetic background. The genome of M2 was very similar to the emetic reference strain *B. cereus* F4810/72 (ANI > 99%), although it was assigned as *B. paranthracis*. P4, M1 and M3 can be clustered with ATCC 14579 (ANI > 98%). Another two isolates, P2 and P5, were genetically different from others and present a relative similarity (ANI > 96%) between each other. The genome of M4 presented 95% similarity (ANI) with M2 and F4810/72, while was different from other isolates (ANI < 91%). M5 was different from all other isolates (ANI < 91%) and was assigned as *B. toyonensis*.

To further identify the genetic diversity and ST of these *B. cereus s.l.* isolates, the corresponding sequences of seven house-keeping genes based on the standard MLST database (http://pubmlst.org/bcereus) were extracted from the WGS data, shown in Table 2. Not all the loci were identical for all isolates from the MLST database, expressed with "b" in Table 2. For example, there was no exact allelic profile match for the *glp* loci for P2. Two of the food isolates, P4 and M1, matched precisely to the database and they were assigned to the same ST-427. *B. paranthracis* M2 was assigned to ST-26, which was the same as *B. cereus* F4810/72. Although, P4, M1, M3 and ATCC 14579 can be clustered (ANI > 98%), their allelic profiles of house-keeping genes could be different resulting in different ST. P4 and M1 seem to share the same ST (ST-427) which is different from ATCC 14579 (ST-4), while M3 could be a new ST of *B. cereus*. P2, P5 and M4 could be new STs of *B. cereus* as well. *B. toyonensis* M5 showed a unique ST when compared with the database.

3.2.2. Biofilm-related and toxin gene profiling

The existence of biofilm- and toxin-related genes in tested isolates were identified by extracting the data from WGS. Summary information is shown in Table 3. The genes existed in ATCC 14579 and F4810/72 were identified by extracted the genome from GenBank. Most of our food isolates contain variety of biofilm-regulated genes, with exceptions. Interestingly, M4, F4810/72, *B. paranthracis* M2 and *B. toyonensis* M5 all lack *plcR* gene, which is a well-known pleiotropic transcriptional regulator (Hsueh, Somers, Lereclus, & Wong, 2006). Eight food isolates showed various combinations of toxin genes (Table 3). None of these food isolates had the emetic *ces* gene. Isolates P4, P5, M1 and M3 harboured a combination of all three enterotoxin genes (*hbl, nhe* and *cytK*), while isolates P2 and M4 contained only the *nhe* and *cytK* genes. *B. toyonensis* M5 contained both *hbl* and *nhe* genes, while *B. paranthracis* M2 contained only the *nhe* gene.

3.3. Biofilm formation on stainless steel coupons in static or shaking incubation

Eight food isolates and two reference strains (ATCC 14579 and F4810/72) were tested for biofilm formation in static and 120 rpm shaking conditions, shown in Fig. 2 A and Fig. 2 B, respectively. The numbers of biofilm cells grown on stainless steel coupon were expressed in log CFU/cm^2 (Fig. 2).

All of ten isolates showed different numbers of culturable biofilm cells in both conditions and the time to reach the highest amount of biofilm varied between isolates. In static incubation (Fig. 2 A), M1 showed relatively higher biofilm-forming capacity after one-day incubation (around 7 log CFU/cm^2) however it showed lower numbers of biofilm cells (less than 5 log CFU/cm^2) after two days compared with other isolates. While, M3 showed significantly lower ($p < 0.05$) biofilm formation at day one (around 3 log CFU/cm^2) but increased at day two and three (about 6 log CFU/cm^2). P2 was a strong biofilm-former (Fig. 2 A), especially after two days incubation, reaching around 7 log CFU/cm^2 showing higher biofilm formation than other isolates. *B. toyonensis* M5 showed strong biofilm formation in static incubation as well, reached 7 log CFU/cm^2 after two days (Fig. 2 A). Most of the isolates tested formed stable or increased amounts of biofilm within three days in static incubation, except for M1 and M2, which showed decreases in biofilm cells after two and three days, respectively (Fig. 2 A). The two reference isolates showed either comparable or slightly lower biofilm formation than the food isolates in a static condition (Fig. 2 A).

For biofilm in a shaking condition (Fig. 2 B), P5 was the strongest biofilm-former ($p < 0.05$) at days one, two and three, producing around 7.5 log CFU/cm^2 biofilm on the stainless steel coupon after one day. P2 and *B. toyonensis* M5 were still strong biofilm formers (7 log CFU/cm^2) compared with other isolates tested in the shaking condition. The statistical comparison of biofilm formation in static and agitation conditions was expressed as "H" in Fig. 2 B indicating "higher" ($p < 0.05$) cell numbers with shaking compared with the static incubation. It showed that either comparable or significantly higher ($p < 0.05$) amounts of biofilm cells formed with agitation incubation, especially at the early time points (one- or two-day incubation). However, there was a difference between the agitated and static biofilms in that the agitated coupons tended to show some release of biofilm over time compared with the static coupons where the biofilm was a little more stable over time. Isolates P2 and M4 differed from this trend (Fig. 2 B).

3.4. Pellicle formation in static and shaking condition

Pellicles formed by *B. cereus s.l.* isolates were formed in 48-well plates filled with TSB medium and incubated at 30 °C for one, two and three days in either static or 120 rpm shaking conditions. After incubation, pictures of the two representative wells were taken (Fig. 3). The pellicle formation varied between isolates. In static incubation (Fig. 3 left), most of the isolates started to produce visible pellicle after two days incubation, except for the *B. paranthracis* M2 which showed

Table 1
The identification and summary of food isolates in this study.

Isolated source	Potato-based products			Dairy products				
Name of isolate	P2	P4	P5	M1	M2	M3	M4	M5
Species identification	*B. cereus*			*B. cereus*	*B. paranthracis*	*B. cereus*	*B. cereus*	*B. toyonensis*

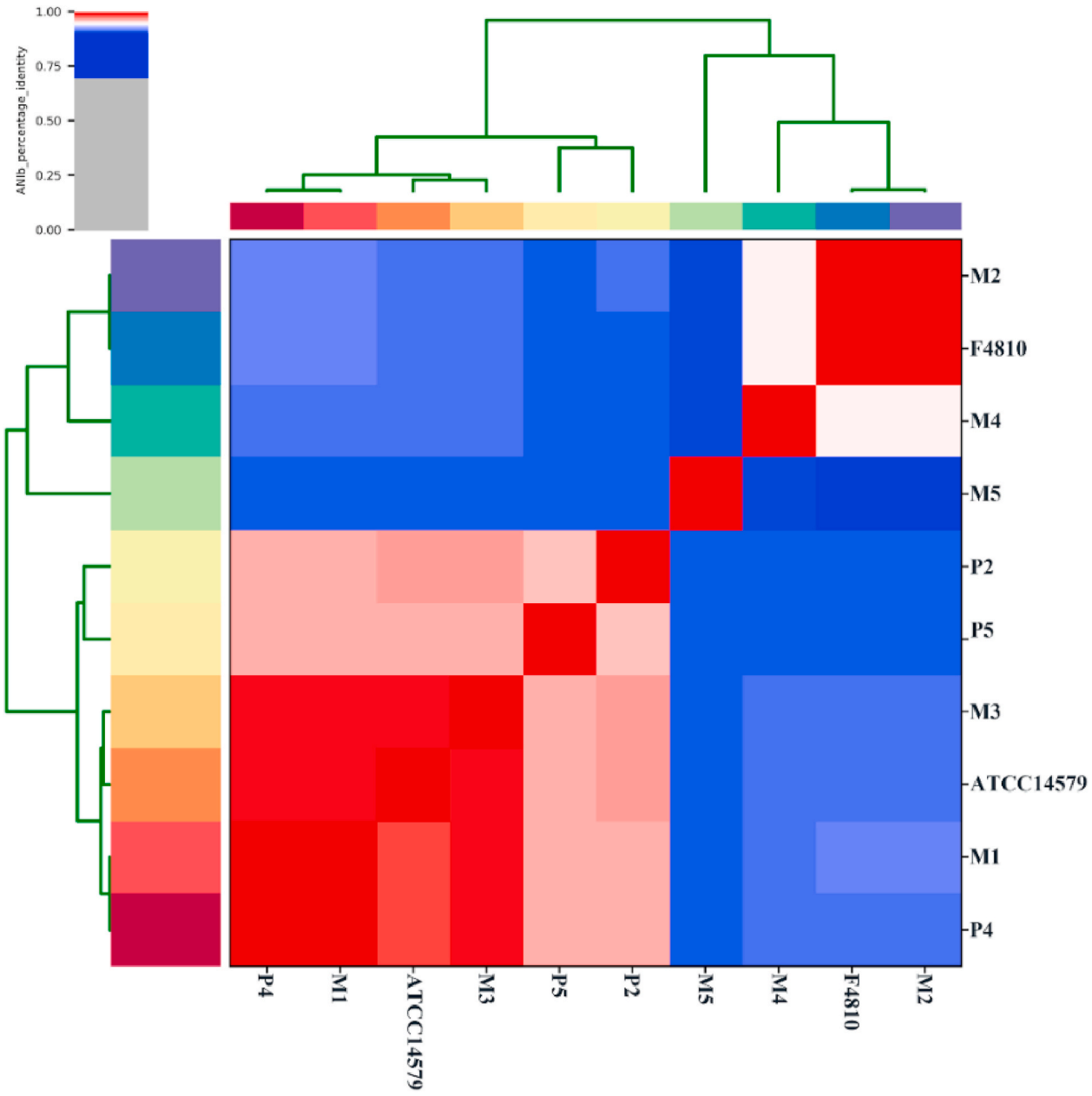

Fig. 1. Average nucleotide identity (ANI) among eight B. cereus s.l. food isolates; the dendrogram directly reflects the degree of identity between genomes.

Table 2
The allelic profiles of seven house-keeping genes in MLST scheme of these isolates based on WGS.

Locus	P2	P4	P5	M1	M2	M3	M4	M5	ATCC1579	F4810/72
glp	37[b]	122	93	122	3	12	65	43	13	3
gmk	9	8	6	8	2	8	1	26	8	2
ilv	76	8	170[b]	8	31	9	93	35	8	31
pta	18	11	9	11	5	14	1	42	11	5
pur	12	9	4	9	16	11	51	39	11	16
pycA	14	12	7	12	3	12[b]	52	41	12	3
tpi	7	10	21	10	4	10	24	63[b]	7	4
ST[a]	ND	427	ND	427	26	ND	ND	ND	4	26

ND Not determined.
[a] ST Sequence Types.
[b] Allele no[b] found in the B. cereus MLST database, shown as the closest matched alleles.

large amounts of pellicle at day one. Interestingly, P2 did not show strong pellicle formation in the static conditions even after three days incubation (Fig. 3 left), which was contradictory to the results obtained from biofilm formed on stainless steel coupons in that P2 was one of the strongest biofilm formers in the same condition (Fig. 2 A). This shows that the ability to form pellicle is not necessarily related to biofilm

Table 3
The existence of biofilm- and toxin-related genes selected based on current database.

	Gene	ATCC 14579	F4810/72	P2	P4	P5	M1	M2	M3	M4	M5	Function/Protein product	References
Biofilm-related gene	*Spo0A*	+	+	+	+	+	+	+	+	+	+	Biofilm regulator; Sporulation transcription factor	Fagerlund et al. (2014)
	ComER	+	+	+	+	+	+	+	+	+	+	Biofilm and sporulation regulator	Yan et al. (2016)
	CodY	+	+	+	+	+	+	+	+	+	+	GTP-sensing pleiotropic transcriptional regulator	Lindbäck et al. (2012)
	PlcR	+	–	+	+	+	+	–	+	–	–	Pleiotropic transcriptional regulator	Hsueh et al. (2006)
	AbrB	+	+	+	+	+	+	+	+	+	+	DNA-binding domain-containing protein	Fagerlund et al. (2014)
	CalY	+	+	+	+	+	+	+	+	+	+	Biofilm extracellular matrix fibre protein and cell surface adhesion	Caro-Astorga, Pérez-García, de Vicente, and Romero (2014)
	SipW	+	+	+	+	+	+	+	+	+	+	Matrix protein encoding gene; Signal peptidase I	
Toxin-related gene	*Hbl*	+	–	–	+	+	+	–	+	–	+	Haemolytic toxin	Ehling-Schulz et al. (2006)
	Nhe	+	+	+	+	+	+	+	+	+	+	Non-haemolytic toxin	
	CytK	+	–	+	+	+	+	–	+	+	–	Cytotoxin	
	Ces	–	+	–	–	–	–	–	–	–	–	Emetic toxin	

+, gene is present; -, gene is absent.

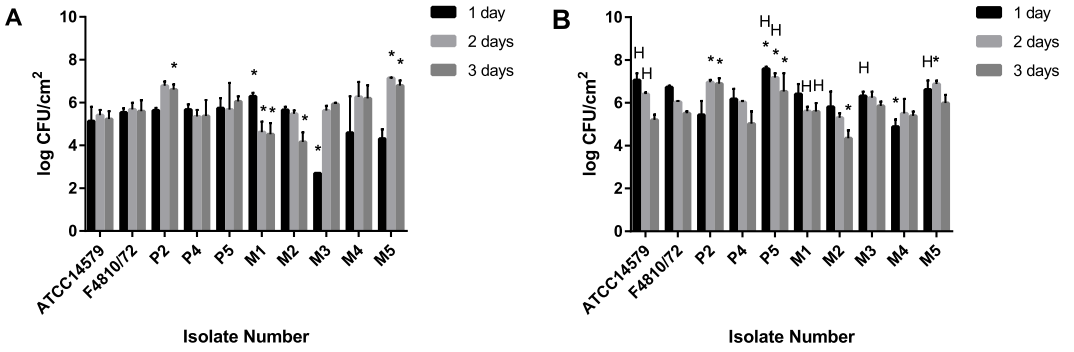

Fig. 2. Biofilm cells of ten B. cereus s.l. isolates incubated in static (A) or 120 rpm shaking condition (B). The numbers of culturable cells based on plating method. The growth of biofilms was checked after one-, two- and three-days. Biofilms were grown on stainless steel in TSB medium and incubated at 30 °C. Each column indicates the average number within biological replicates and error bars represent standard deviation. "*" means the significant difference ($P < 0.05$) between isolates in each condition, while the significant difference between static and shaking of corresponding isolates and time was expressed with "H" in Fig. B, meaning higher cell counts in shaking condition compared with static.

formation on a solid surface. When incubating plates under 120 rpm shaking, all isolates showed relatively higher and/or earlier pellicle formation compared with static incubation, which was comparable with biofilms formed on stainless steel coupons described previously. Most of the isolates started to produce pellicle from day one in shaking conditions, especially P5 and *B. paranthracis* M2. P5 was a strong biofilm former on stainless steel coupons in the same conditions (Fig. 2 B). The location and status of pellicle formation varied between isolates as well. For example, P5 formed a complete piece of pellicle in the air-liquid interface under shaking incubation (Fig. 3 right), while other strains such as ATCC 14579 and F4810/72 formed fragmented pellicles floating within the medium. Similar to the stainless steel coupon where biofilm was partially released, the pellicles also disintegrated over time.

3.5. The sporulation within planktonic or biofilm populations

The sporulation percentage in planktonic and biofilm communities was calculated based on the total cell number and spore counts. This is because the numbers of total viable cells of planktonic and biofilm are different therefore it is hard to compare the spore-forming ability of these two types of cells with only spores counts. Furthermore, the units used for the two types of cells were different as planktonic cells were expressed as log CFU/mL while biofilms were expressed as log CFU/cm^2. In our study, the biofilm were grown on stainless steel coupons inserted in the 48-well plate filled with TSB and incubated in either static or 120 rpm shaking condition, while the planktonic culture was grown in shaking flask filled with 50 mL TSB and incubated only in 120 rpm shaking condition to avoid cells' sedimentation. Both planktonic and biofilm cells were grown at 30 °C, and the total cells and spores were tested after one, two and three days (Fig. 4).

The sporulation percentages of planktonic culture were below 1% for all tested isolates at day 3 (Fig. 4). TSB used in this study is a nutrient-rich medium, and this may explain why sporulation levels were low in the planktonic state after three days incubation. However, a considerably higher sporulation percentage was observed within biofilms grown in both static and 120 rpm shaking incubation, although the differences were not all statistically significant. The sporulation of the *B. toyonensis* M5 was low compared with other isolates within three days incubation, for both planktonic and biofilm cultures. While, *B. paranthracis* M2 showed high sporulation level within biofilm populations, representing over 50% of spores within the total biofilm cells at day two and three. Most of isolates started to produce spores within their biofilm populations from day two, especially for F4810/72, P2, M1, M3 and M4 showing statistically significant higher sporulation percentages in biofilms compared with in planktonic culture. The effect of shaking incubation in sporulation varies between isolates. Most of tested isolates reached higher or comparable amounts of spores when placed in shaking compared with the static incubation, including F4810/72 (both day 2 and 3), M2 and M3 at day two (Fig. 4), P4, M1 and M4 at day three (Fig. 4).

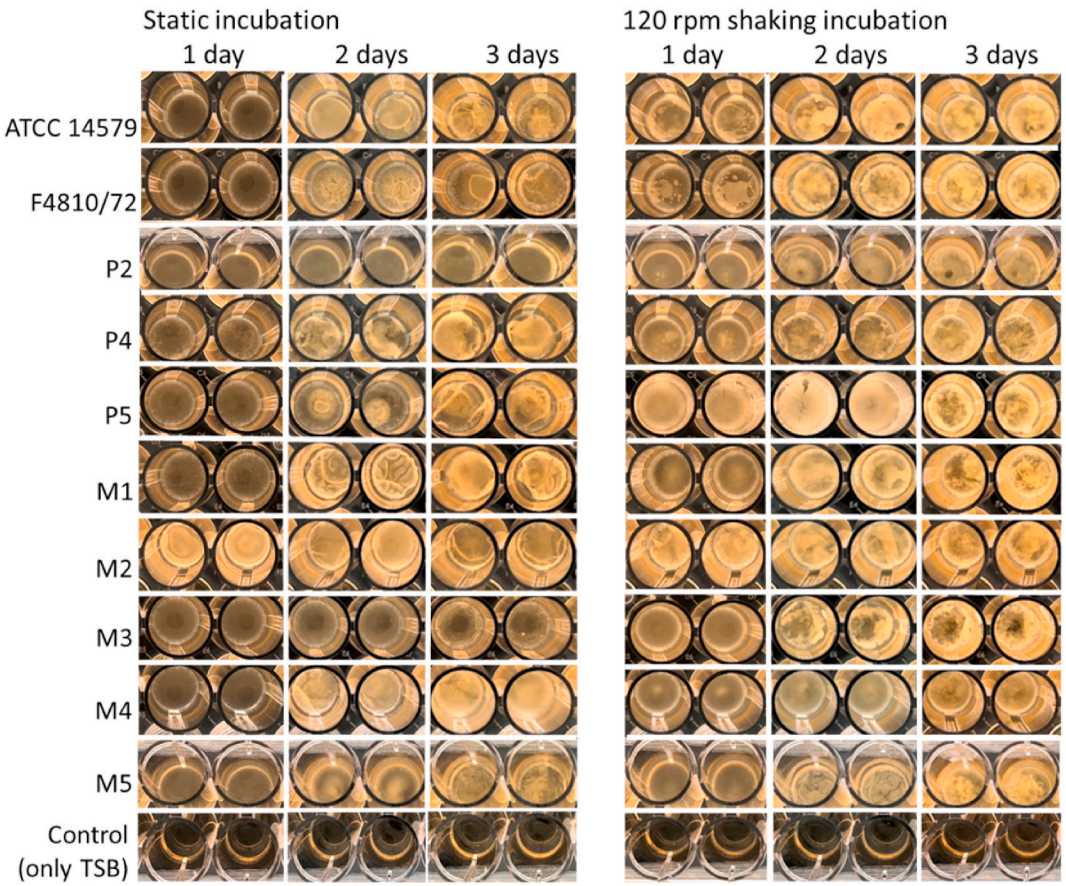

Fig. 3. The floating pellicle of ten B. cereus s.l. isolates in TSB medium. Pellicle was grown in 48-well plates filled with TSB medium and incubated at 30 °C for one, two and three days in static (left) and 120 rpm shaking incubation (right). Pellicle formation of each isolate in each condition was performed with three biological repetitions, although only two representative wells are shown here. Negative controlled wells with only TSB medium are also shown in the last row.

4. Discussion

There is an extensive nucleotide diversity among *B. cereus* group strains therefore there is potential to find previously unidentified sequence types (Carter et al., 2018). Our data showed that the isolates tested showed considerable diversity with many of them not previously observed and characterized by the *B. cereus* MLST database. Two recently identified *B. cereus s.l.* species, *B. paranthracis* and *B. toyonensis*, were found for the first time in dairy isolates. This is the first time that the two species have been reported in New Zealand, although it is possible that strains belonging to these species have already been existed in New Zealand but under the name of *B. cereus* species, as these two species have been described recently. The dairy-associated *B. cereus* group isolates have been shown to possess high genetic diversity with novel STs and new allelic types of housekeeping genes (Kovac et al., 2016). WGS is still the preferred and most comprehensive method to determine the genetic information of bacteria. However, isolates that share highly similar genomes, could have different biofilm-forming and sporulation ability.

Most of tested *B. cereus s.l.* isolates showed the presence of biofilm-related genes, however, with exceptions. *PlcR* is a pleiotropic transcriptional regulator involved in biofilm formation and also a virulence factor (Hsueh et al., 2006; Ramarao & Lereclus, 2006). However, F4810/72, M4, *B. paranthracis* M2 and *B. toyonensis* M5 all lack the *plcR* gene. Although the biofilm-related genes existed in the genome of isolates, the actual expression level of these genes is unknown. Our *B. cereus s.l.* isolates showed a variety of combinations of toxin genes, which fits our expectation that toxin gene prevalence and distribution among the *B. cereus* group strains varies greatly (Carter et al., 2018). All our food isolates contained *nhe* genes while only some isolates contained *hbl* and *cytK* genes, which is in line with previous studies (Park et al., 2009; Wijnands et al., 2006). Unsurprisingly, none of our food isolates (potato- or dairy-associated isolates) contained the emetic toxin-related *ces* gene, as the presence of emetic *B. cereus* strains is rare (Agata, Ohta, & Yokoyama, 2002; Altayar & Sutherland, 2006). This result could be due to the limited numbers of isolates tested in our study. We observed that dairy isolates contained various combinations of enterotoxin genes, which agrees with other studies (Saleh-Lakha et al., 2017; Wijnands et al., 2006). Potato-associated *B. cereus* isolates could carry enterotoxin-related genes including *hbl*, *nhe* and *cytK*. *B. paranthracis* and *B. toyonensis* isolates found in our study showed the presence of *hbl* and *nhe* toxin gene as well. *B. toyonensis* has been used as a probiotic (Jiménez et al., 2013), however, the presence of the enterotoxin genes within the strain should be considered as a potential food safety risk, although studies showed that the enterotoxin levels in *B. toyonensis* were much lower than *B. cereus* strains and in some cases, even absent (Abdulmawjood et al., 2019). Of course, the presence of toxin genes can only mean the potential for toxin production. Kovac et al. (2016) indicated that the presence of *hblACD* and *nheABC* is not enough to produce detectable Hbl and Nhe toxin. Therefore, what types of toxins and how much of these toxins can be produced in these *B. cereus s.l.* isolates should be examined in future.

The biofilm formation of *B. cereus s.l.* isolates on stainless steel-liquid-air interface and floating pellicles varies between isolates, which is in line with Hussain and Oh (2017). In our study, P2, P5 and *B. toyonensis* M5 isolates were considered as strong biofilm formers on stainless steel coupons, while P5 and *B. paranthracis* M2 isolates were strong pellicle formers in TSB medium. This is the first study indicating

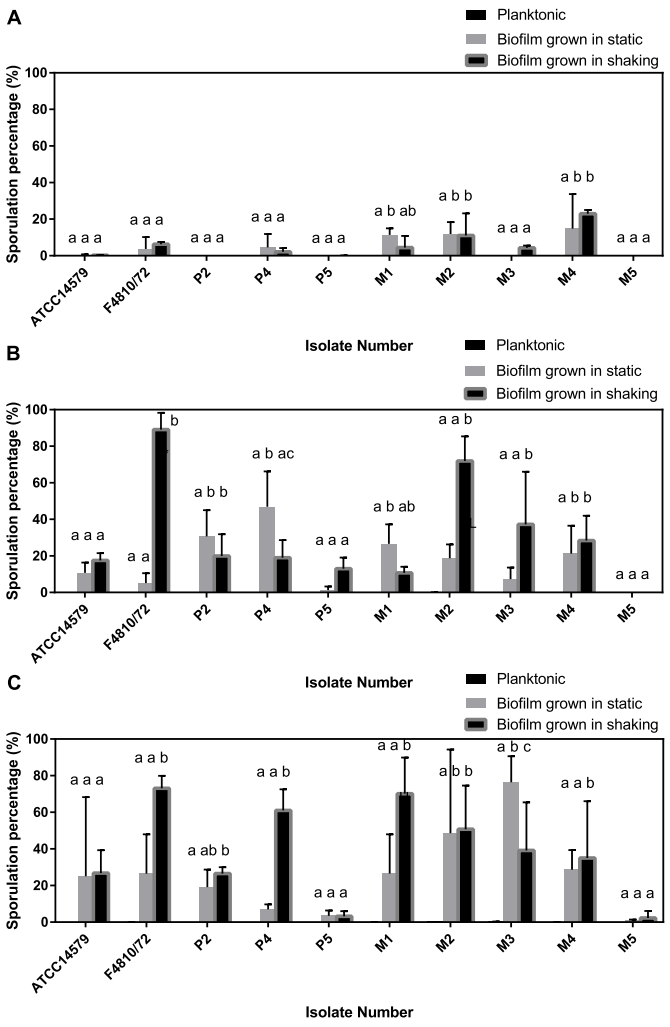

Fig. 4. The sporulation percentages of ten B. cereus s.l. isolates in planktonic culture, biofilm grown in static and in 120 rpm conditions after one day (A), two days (B) and three days (C) incubation. Biofilms were grown on stainless steel coupons vertically inserted into 48-well plate filled with TSB broth, while planktonic culture was grown in shaking flask filled with 50 mL TSB broth. The sporulation percentages were calculated by (spores' numbers/total cell numbers) %. Each column indicates the average number within replicates and error bars represent standard deviation. To compare the sporulation percentages of planktonic population, biofilm grown in static and shaking condition for each isolate, Turkey's multiple comparison test was performed. Different alphabets within each condition indicate significant difference ($p < 0.05$).

the biofilm formation of *B. toyonensis* and *B. paranthracis*, indicating the potential biofilm-forming ability of these two species. Interestingly, P2 that can produce large amounts biofilm on stainless steel coupons, was not necessarily a strong pellicle former, which suggests that the mechanism behind pellicle formation of *B. cereus* strains could be different from other types of biofilms. Okshevsky et al. (2018) reveals that the genes required for submerged biofilm and pellicle formation are different. Pellicles were also regarded as one type of "air-liquid biofilm", although it is a floating structure and lacks a solid surface for attachment (Branda, Vik, Friedman, & Kolter, 2005; Chabane et al., 2014). The amount, location and the status of pellicle formation varies between isolates. However, a standard quantification method is needed to study pellicle formation, as most of pellicle studies focus on visualization (Bridier et al., 2011; Chabane et al., 2014; Gao, Foulston, Chai, Wang, & Losick, 2015). Dairy-associated *B. cereus* strains could be strong biofilm formers (Ostrov, Sela, Belausov, Steinberg, & Shemesh, 2019), which was observed in our result where some dairy-related *B. cereus s.l.* isolates could not only form strong biofilm on stainless steel but also formed strong pellicle. Therefore, food safety issues caused by *B. cereus* in the dairy industry may not only focus on the contamination on the stainless steel processing line but pellicle formation in tanks and other similar environments may also be important. Unlike well-studied dairy-related *B. cereus*, the biofilm formation of potato isolates has not been reported. We observed that *B. cereus* isolates such as P2 and P5 from potato products could produce large amounts of biofilm. This was especially true for the P5 isolate, that not only produced a strong biofilm on stainless steel coupons but also produced considerable amounts of floating pellicles. This encouraged us to focus on the biofilm formation of *B. cereus* in the potato-based food industry.

Spores within biofilms of *B. cereus* can be released (Wijman, De Leeuw, Moezelaar, Zwietering, & Abee, 2007) and form new biofilms, causing recontamination in the food processing line. The sporulation of *B. cereus s.l.* isolates in our study shows a variety, which is agrees with Wijman et al. (2007) and Faille et al. (2014) indicating sporulation is a strain-dependent characteristic for *B. cereus*. In our study, we found higher sporulation within biofilms than planktonic cells, which fits our hypothesis that biofilm could be a better reservoir for spores compared with planktonic cells and agrees with observations with other *Bacillus* species (Burgess, Flint, & Lindsay, 2014; Hayrapetyan et al., 2015). The high cell density reached in biofilm may cause nutrient limitation influencing sporulation (bib_Van_Gestel_et_al_2012Van Gestel, Nowak, & Tarnita, 2012). Furthermore, we found that isolates considered as strong spore formers in a biofilm population may not necessarily be good spore formers in the planktonic state. We therefore hypothesized that different mechanisms may exist for the regulation of sporulation within the biofilm and planktonic states. Some genes or proteins have been identified relating to both sporulation and biofilm formation in *Bacillus* species (Aguilar, Vlamakis, Guzman, Losick, & Kolter, 2010; García et al., 2018; Huang, Flint, & Palmer, 2020). Intertwined regulatory pathways of sporulation and biofilm formation have been proposed for *B. subtilis* (Vlamakis, Chai, Beauregard, Losick, & Kolter, 2013), however, this remains to be determined for *B. cereus*.

Agitation seems to be a preferred condition for *B. cereus* to form biofilms, both at the stainless steel-liquid-air interface or as pellicle. It is generally regarded that *B. cereus* strains prefer to develop biofilms at the air-liquid interface with typical ring biofilms (Hayrapetyan et al., 2015; Wijman et al., 2007), which could be due to the aerotaxis (migration towards oxygen) property of *B. cereus* strains (Daniel J. Laszlo, 1984). We hypothesise that shaking could increase the availability of oxygen therefore help *B. cereus* to form more biofilm in the air-liquid interface. More intensive/efficient nutrient exchange during shaking (Moreira et al., 2013; Oosthuizen et al., 2002) may also accelerate or support biofilm formation of *B. cereus*. Motility has been identified as an important factor to form strong biofilms especially in the air-liquid interface biofilms (Houry, Briandet, Aymerlch, & Gohar, 2010), while agitation could provide additional movement enhancing *B. cereus* interaction with a surface. In addition, there will be a slightly larger surface for attachment on a stainless steel coupon by bacterial cells when shaken. This extra surface area may also be considered as a nutrient-limited surface (as this part is only partially submerged in the medium during shaking) and this may stimulate biofilm formation (Elhariry, 2011). The inoculum (overnight culture) was also grown in a shaking condition where the cells have adapted to the agitated environment, which could also be a reason for higher growth and biofilm formation in the shaking condition. Some *B. cereus* strains are known to form submerged biofilms (Oosthuizen et al., 2002). It is unknown whether agitation can affect the submerged biofilms of *B. cereus*. The biofilm formed in shaking conditions has been investigated with *Escherichia coli*, showing that higher shear forces produced during shaking promote the formation of interface biofilms (Gomes, Moreira, Simões, Melo, & Mergulhão, 2014). However, to our knowledge, this is the first paper comparing the biofilm formation in static and agitation conditions for *B. cereus*.

5. Conclusion

Dairy and potato products, as two important daily foods, are the sources of *B. cereus s.l.* isolates. *B. toyonensis* and *B. paranthracis* were reported from dairy products in the New Zealand market for the first time, showing the biofilm and spores forming ability and the presence of enterotoxin genes. To the best of our knowledge, this is the first paper indicating the potato-related isolates as strong biofilm formers, suggesting that controlling biofilm of *B. cereus* should be included in the potato-based food industry. *B. cereus* isolates from dairy and potato products could also form strong floating pellicles, which could be a safety risk related to liquid food products filled in containers. This is also the first study to compare the biofilm formation of *B. cereus* including pellicle formation in static and agitation conditions suggesting that food processing conditions with agitation such as stirred storage tanks, pumping of product and transportation could influence biofilm formation of *B. cereus*. WGS is the most comprehensive method to determine the genetic information of bacteria. *B. cereus s.l.* is highly diverse at the genetic level. Genetic closely related *B. cereus s.l.* isolates may display significant differences in biofilm and sporulation ability.

Accession number

The accession numbers of ATCC 14579 and F4810/72 at GenBank are GCF_006094295.1 and GCF_000021225.1, respectively. The draft genome sequences (Whole Genome Shotgun project) of eight isolates have been deposited at DDBJ/ENA/GenBank under the accession numbers of JAAVIP000000000 (P2), JAAVIQ000000000 (P4), JAAVIR000000000 (P5), JAASAB000000000 (M1), JAAVIL000000000 (M2), JAAVIM000000000 (M3), JAAVIN000000000 (M4), and JAAVIO000000000 (M5).

CRediT authorship contribution statement

Yiying Huang: Conceptualization, Methodology, Software, Writing - original draft. **Steve H. Flint:** Supervision, Conceptualization. **Shubo Yu:** Supervision, Conceptualization, Funding acquisition. **Yu Ding:** Resources, Formal analysis. **Jon S. Palmer:** Resources, Formal analysis.

Declaration of competing interest

This manuscript is original, and no part of the manuscript has been published before. There are no conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2021.110853.

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