The spore formation and toxin production in biofilms of *Bacillus cereus*

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Abstract

Bacillus cereus (*B. cereus*) is a foodborne pathogen causing diarrhoea and emesis which are the consequences of enterotoxin and emetic toxin production, respectively. Sporulation and biofilm formation are used as survival strategies by *B. cereus* protecting cells from harsh environments. However, these survival strategies also make *B. cereus* more difficult to control in the food industry. The aim of this study is to investigate the spore formation and toxin production in the biofilm of *B. cereus*.

In this study, higher sporulation and higher spore heat resistance were demonstrated in biofilms grown on stainless-steel (SS) compared to planktonic populations. The structure of coat in spores isolated from biofilms, the upregulated germination genes in planktonic cells and upregulated sigma factor B in biofilm cells are possible explanations for these observations. The levels of dipicolinic acid (DPA) did not affect the heat resistance of spores harvested from biofilms in this study.

Haemolytic toxin (Hbl) was mainly secreted by cells into surrounding media while emetic toxin (cereulide) was associated with cells. Higher Hbl toxin was observed in the presence of biofilms grown on SS compared to either planktonic culture or biofilm grown on glass wool (GW) using the *Bacillus cereus* Enterotoxin Reverses Passive Latex Agglutination test (BCET-RPLA). This was supported by the significant ($P < 0.05$) increase in *HblACD* expression in biofilm cells on SS, using both real-time quantitative PCR (RT-qPCR) and RNA sequencing. The transcriptomic analysis also revealed that biofilms grown on SS had an upregulated secretion pathway, suggesting biofilms of *B. cereus* grown on SS are more pathogenic than planktonic cells. Unlike the Hbl toxin, cereulide was associated with biofilm cells/structures and attached to the biofilm-forming substrates including SS and GW used in this study. The expression of *cerA* and *cerB* was similar between biofilms and planktonic cells using RT-qPCR. This project highlights the importance of biofilms by *B. cereus* in food safety through the enhanced heat resistance of spores, the higher Hbl toxin production and attached cereulide toxin.

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Declaration

The presented thesis is comprised of nine chapters including five chapters with research work in this study. Partial contents of chapter 2, 4, 5, 6, 7 and 8 are structured as manuscript that have either been published, submitted or intended to be submitted to a journal.

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List of publications and conference presentations

- **Huang, Y.**, Flint, S.H., Trevor S.L., Palmer, J.S., 2021. Emetic toxin production of *Bacillus cereus* in a biofilm. LWT-Food Science and Technology. Under review.
- **Huang, Y.**, Flint, S. H., Gagic, D, Palmer, J., 2021. The spores and toxins in the biofilms of *Bacillus cereus*. New Zealand Institute of Food Science and Technology Conference; Poster presentation and three-minute oral competition.
- **Huang, Y.**, Flint, S.H., Palmer, J.S., 2021. The heat resistance of spores from biofilms of *Bacillus cereus* grown in tryptic soy broth and milk. International Dairy Journal. Vol: 123. Page: 105169. Doi: https://doi.org/10.1016/j.idairyj.2021.105169
- **Huang, Y.**, Flint, S.H., Yu, S., Ding, Y., Palmer, J.S., 2021. Phenotypic properties and genotyping analysis of *Bacillus cereus* group isolates from dairy and potato products. LWT-Food Science and Technology. Vol: 140. Page: 110853. Doi: 10.1016/j.lwt.2021.110853.
- **Huang, Y.**, Flint, S. H., Gagic, D, Palmer, J., 2020. The effect of biofilm on spore and toxin production for *Bacillus cereus*. New Zealand Microbiological Society Conference; Poster presentation.
- **Huang, Y.**, Flint, S.H., Palmer, J.S., 2020. *Bacillus cereus* spores and toxins – The potential role of biofilms. Food Microbiology. Vol: 90. Page: 103493. Doi: 10.1016/j.fm.2020.103493.
- **Huang, Y.**, Flint, S., Palmer, J., 2019. Biofilms, Sporulation and Toxin Genes Profiling of *Bacillus cereus* isolates. New Zealand Microbiological Society Conference; Poster presentation.

Abbreviations

ANI	Average nucleotide identity
B. cereus s.l.	*Bacillus cereus sensu lato*
B. cereus	*Bacillus cereus sensu stricto*
B. paranthracis	*Bacillus paranthracis*
B. subtilis	*Bacillus subtilis*
B. toyonensis	*Bacillus toyonensis*
BcET	Enterotoxin T
BCET-RPLA	*Bacillus cereus* Enterotoxin Reverses Passive Latex Agglutination test
BHI	Brain Heart Infusion
CHO	Chinese hamster ovary
CIP	Clean-in-place
CV assay	Crystal Violet assay
CwpFM	Cell wall peptidases
CytK	Cytotoxin K
DAVID	Database for Annotation, Visualization and Integrated Discovery
DPA	Dipicolinic acid; pyridine-2,6-dicarboxylic acid; PDC
eDNA	Extracellular DNA
EFSA	European Food Safety Authority
EntFM	Enterotoxin FM
EPS	Extracellular polymeric substances
FDA	Food and Drug Administration
FDR	False discovery rate
GIT	Gastrointestinal tract
GSEA	Gene Set Enrichment Analysis
GW	Glass wool
Hbl	Heamolysin BL; haemolytic enterotoxin

HEp-2	Human carcinoma of the larynx
Int 407	Intestine 407, embryonic intestine
kDa	Molecular weight
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LC	Liquid chromatography
MLST	Multi-locus sequence typing
MS/MS	Q Exactive™ Focus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer
MTT	Metallization test; 3-(4,5,-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MYP	Mannitol yolk polymyxin agar plates
Nhe	Non-haemolytic enterotoxin
NRPS	Non-ribosomal peptide-synthetase
NZ	New Zealand
PCR	Polymerase chain reaction
QACs	Quaternary ammonium compounds
REPFED	Refrigerated Processed Foods of Extended Durability
RT-qPCR	Real-time quantitative PCR
Saline	0.85% NaCl solution
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
SS	Stainless-steel
SSW	Stainless-steel wool
STs	New sequence types
Tb^{3+}	Terbium ions
TECRA-BDE	Diarrheal Enterotoxin Visual Immuno Assay
TEM	Transmission electron microscopy
TSB/TSA	Tryptic soy broth/agar
WGS	Whole genome sequencing

Chapter 1 General introduction

1.1 Rationale and importance

1.1.1 Why *Bacillus cereus*

Bacillus cereus sensu stricto (referred to *B. cereus* for the whole thesis) is a Gram-positive bacterium distributed widely in the environment. The natural reservoirs of the species include fresh and marine water, decaying organic matter, soil, sediments, dust and rhizosphere of plants (Halverson et al., 1993; Jensen et al., 2003; Heath et al., 2009). Although a few strains of *B. cereus* are considered as beneficial strains and used as a bio-control method to prevent plant disease and animal probiotics (Emmert, 2002; Hong et al., 2005), most *B. cereus* strains are known as foodborne pathogens causing gastrointestinal diseases in humans. The pathogenicity of *B. cereus* is due to the production of several virulent substances, including haemolysins, phospholipase C, the emetic toxin, enterotoxins, metalloproteinases, collagenases and beta-lactamases (Agata et al., 1994; Beecher and Wong, 2000; Turnbull et al., 2008). In the gastrointestinal tract (GIT), specifically in the small intestine, vegetative cells/spores with over 10^5 CFU/g are ingested followed by the production and secretion of the protein enterotoxin(s), inducing diarrhoea (Ceuppens et al., 2013). The emetic toxin (called cereulide, a plasmid-encoded cyclic peptide) is produced by *B. cereus* cells present in foods and ingested as a preformed toxin (Granum and Lund, 1997).

B. cereus is a ubiquitous microorganism and can grow in the pH range of 4.9-9.3 and temperatures between 10°C to 50°C (Raevuori and Genigeorgis, 1975; Wijnands et al., 2006a). Psychrotolerant strains of *B. cereus* grow down to 6°C (Beno et al., 2019). *B. cereus* is found in various raw foods and is encountered in a wide range of processed foods, such as vegetables, dairy products, meat products, rice, potatoes, spices, cooked chilled food and Refrigerated Processed Foods of Extended Durability (REPFED) (Becker et al., 1994; Wijnands et al., 2006a; Park et al., 2009; Samapundo et al., 2011). *B. cereus* is a well-known spore former commonly found in the environment as spores. The contamination of the food can occur from "soil-to-table". One example of a "soil to table" contamination was reported by Guinebretiere et al., (2003), showing that courgettes contaminated with soil containing 4.0×10^4 *B. cereus* spores per gram of soil on the surface, followed by cutting of

the courgette resulting in the contamination of the finished food product. Storage of the cut courgettes at ambient temperatures for 1-2 days can allow *B. cereus* to grow to levels above 10^5 CFU/g (Guinebretiere et al., 2003). Processed and refrigerated foods are especially high-risk for *B. cereus* food poisoning, as other non-spore forming competitive bacteria are inactivated by processing such as drying or heating (pasteurization). It is assumed that the consumption of food containing over 10^5 *B. cereus* cells/spores will cause disease (Granum and Lund, 1997). However, food poisoning is not simply linked to the amounts of cells. The "infective dose" is largely influenced by the properties of strains (e.g., toxin-producing ability, mesophilic or psychrotolerant), host physiology (e.g., specific intestinal microbiota and health status) and the food matrix (e.g., storage conditions and nutritional compositions) (Ceuppens et al., 2013).

The two foodborne symptoms caused by *B. cereus* are diarrhoea and emesis, which are generally of limited duration (24 h or less) and normally self-limiting (Granum and Lund, 1997). Illness caused by *B. cereus* can be misdiagnosed because its symptoms are like those caused by *Staphylococcus aureus* (vomiting) and *Clostridium perfringens* (diarrhoea) (Bennett et al., 2013). A total of 98 outbreaks involving 1539 people were reported in the European Union (EU) in 2018 caused by *B. cereus*, representing 1.9% of all foodborne outbreaks in EU countries (EFSA, 2019). This number of outbreaks increased to 155 outbreaks involving 1636 cases with 44 hospitalisations and 7 deaths in 2019 (EFSA, 2021). *B. cereus* was reported as a major cause of foodborne disease in the Netherlands in 2006 and Norway in 2000, representing 5.4% and 32% of the foodborne outbreaks, respectively (Wijnands, 2008). Recent outbreaks have also been reported in other countries including China in 2018 with 200 students affected in a school (Chen et al., 2019). According to the Centres for Disease Control and Prevention in USA, 235 foodborne related outbreaks due to *B. cereus* resulted in 2050 cases with an estimated cost of $0.35 million occurred from 1998 to 2008 (Bennett et al., 2013). Food poisoning related to *B. cereus* is not a notifiable disease in most countries including Australia and New Zealand (NZ), and data is limited (https://www.foodstandards.govt.nz/). In Australia, it is estimated that 0.5% of foodborne illnesses are caused by *B. cereus* (Hall et al., 2005), however, an outbreak causing 45 cases was reported in Canberra in Australia in 2019 (Thirkell et al., 2019).

Reported outbreaks of *B. cereus* are rare with only three outbreaks in 2008 to 2014 in NZ, with the largest outbreak in 2017 causing 51 cases (https://www.mpi.govt.nz/) which was suspected relating to the Asian meals. *B. cereus* contamination resulted in, a dessert company recalling 2 batches of "Every Entertainer Chocolate Bavarian" as the products may have contained *B. cereus* in NZ. In 2020, Soyummy Ltd recalled specific batches of fermented bean paste as the product contained *B. cereus* (https://www.mpi.govt.nz/food-safety/food-recalls/). All the data above indicate the importance of studying *B. cereus*.

1.1.2 Spore formation and toxin production in the biofilms of *B. cereus*

Biofilms are surface-attached microbial communities formed by many bacteria including *B. cereus*, to adapt and survive in variable environmental conditions (Flemming et al., 2016). Biofilms formed by *B. cereus* comprise polysaccharides, proteins and extracellular DNA (eDNA), and these substances contribute to the structure of biofilms and act as a protective layer for *B. cereus* (Vilain et al., 2009; Karunakaran and Biggs, 2011).

Sporulation, toxin production and biofilm formation of *B. cereus* have been studied intensively, however, there is a lack of information on the role of biofilm in toxin production and sporulation of *B. cereus*. As a foodborne pathogen, toxin production is important, and sporulation and biofilm formation may exacerbate the risk of food poisoning. A greater understanding of the possible synergy between biofilm formation, toxin production and spore formation, may be important to understand the risk in food manufacture.

This PhD project used *B. cereus* isolates from dairy and potato sources, to investigate the sporulation and toxin production when grown in the presence of biofilms. This project aimed to understand the possible link between spore formation/toxin production and biofilm formation and how the biofilms affect the sporulation and toxin production of *B. cereus*.

1.2 Research questions and hypotheses

The research questions and overall idea of this PhD project are illustrated in Fig. 1. 1 below. The PhD project focussed on comparing biofilm and planktonic cultures of *B. cereus*, in terms of sporulation (Fig. 1. 1A) and toxin production (Fig. 1. 1B).

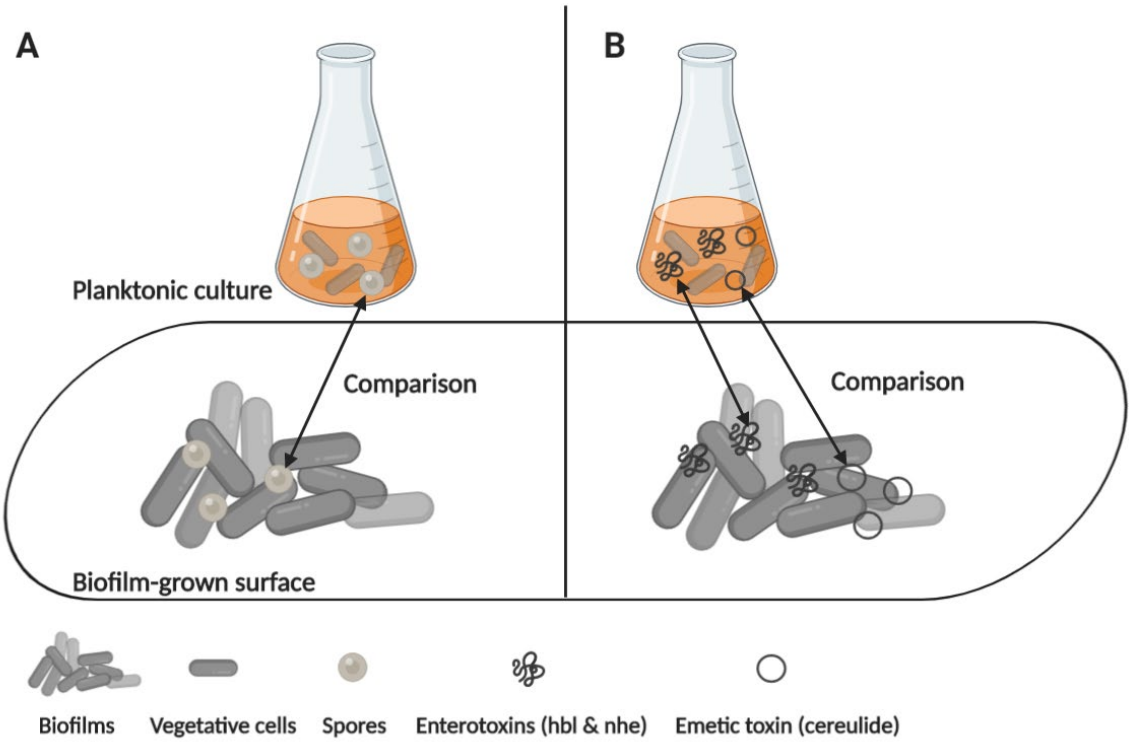

Figure 1. 1 The overall illustration of the PhD project. A and B indicate the spores' properties and toxin production, respectively, between the planktonic culture and the biofilm community. This figure was created using BioRender (https://biorender.com/).

1.2.1 Questions

- How diverse are *B. cereus* food isolates in biofilm formation?
- What are the differences between biofilm and planktonic cells at the transcriptomic level?
- Is biofilm a better reservoir for spores than planktonic cultures of *B. cereus*?
- Are spores isolated from biofilms more heat resistant than those from planktonic culture?
- Can biofilm cells of *B. cereus* produce enterotoxin and/or emetic toxin?

- How does the presence of biofilms affect toxin production (both enterotoxin and emetic toxin) of *B. cereus*?

1.2.2 Hypotheses

- *B. cereus* isolated from different food sources vary in their ability to form biofilm.
- Biofilms of *B. cereus* contain significantly different gene expression patterns to planktonic cells.
- Biofilm of *B. cereus* is a better reservoir for spores than planktonic culture with higher sporulation percentages and more heat resistant spores.
- Biofilm cells of *B. cereus* can produce enterotoxin and emetic toxin.
- The presence of biofilm affects the toxin production by *B. cereus* and the toxins may attach to the biofilm structure.

Chapter 2 Literature review

2.1 *Bacillus cereus*

The *Bacillus cereus sensu lato* group (refer to *B. cereus s.l.* below) comprise several closely related species including *Bacillus cereus* (*B. cereus sensu stricto*, refer to *B. cereus* below), *Bacillus thuringiensis* and *Bacillus anthracis* (Jensen et al., 2003; Lechner, 2009; Guinebretière et al., 2010), and recently identified *Bacillus toyonensis* and *Bacillus paranthracis* (Jiménez et al., 2013; Liu et al., 2017). The phylogenetic tree indicating the relationships between *B. cereus s.l.* species is shown in Fig. 2. 1 (Baek et al., 2019).

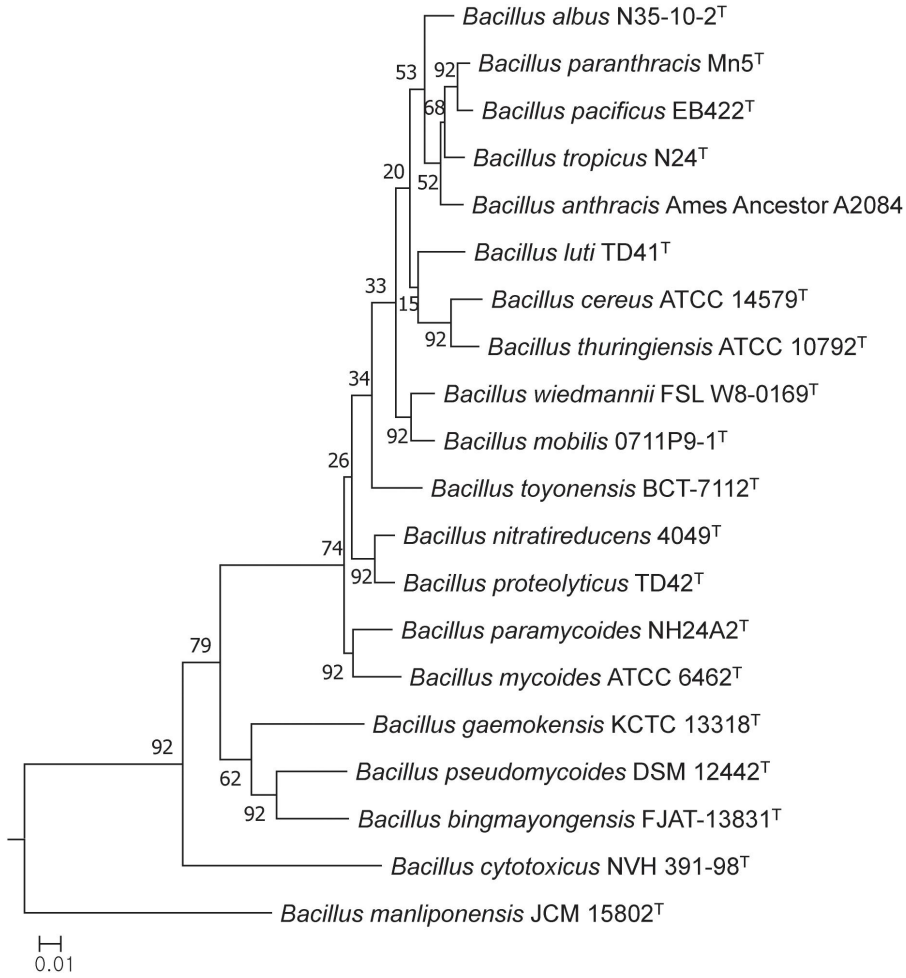

Figure 2. 1* Phylogenetic tree showing relationships between *B. cereus s.l.* species (Baek et al., 2019).

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B. cereus is a Gram-positive spore-former, ubiquitous in the environment and a foodborne pathogen causing human gastrointestinal diseases, diarrhoea and emesis (Schoeni and Lee Wong, 2005; Ceuppens et al., 2013). *B. cereus* can grow in the temperature range of 10 - 50°C, but optimally between 28-35 °C and pH of 4.9-9.3 and can tolerate NaCl levels of 7.5% (Wijnands et al., 2006a). *B. cereus* is unable to grow at temperatures below 4 °C, however, psychrotolerant strains can grow down to 6 °C (Beno et al., 2019). *B. cereus* is found frequently in raw materials as well as processed food, such as cooked chilled food, Refrigerated Processed Foods of Extended Durability (REPFED), sauces, rice and dairy products (such as dried milk powder and infant milk products), although usually at low levels (< 10^3 CFU/g) (Wijnands et al., 2006a; Bartoszewicz et al., 2008; Park et al., 2009; Samapundo et al., 2011). These processed foods are especially high-risk foods for *B. cereus* poisoning, as other non-spore-forming competitive microbiotas are inactivated by drying or heat treatment (pasteurization) leaving *B. cereus* spores.

The numbers and types of *B. cereus* are related to the inherent properties of specific food products and food processing environments. These include pH, water activity and thermal processing. High *B. cereus* counts are associated with dairy products containing high-fat content, as they seem to be protected by fat (Saleh-Lakha et al., 2017; Riol et al., 2018). Milk products are associated with psychrotolerant *B. cereus* strains, as milk products are normally stored at refrigerated temperatures to maintain the quality where the growth of other bacteria are inhibited but *B. cereus* spores survive and psychrotolerant strains can still grow (Wijnands et al., 2006a; Beno et al., 2019). The level of contamination in raw milk varies with seasonal changes, with higher levels of *B. cereus* found during the spring and summer months due to the reduced exposure of cows to the environment when fed in sheds during late autumn and winter in European countries (Svensson et al., 1999; Bartoszewicz et al., 2008). In addition, the level of contamination may be caused by the warmer temperatures or higher moisture during the spring and summer seasons. Rice dishes and other farinaceous foods such as pasta and noodles are commonly associated with emetic *B. cereus* strains with CFU counts of 10^4 to 10^5 per gram which is enough to cause disease (Chang et al., 2011; Delbrassinne et al., 2012). Rice containing 79% of carbohydrates, 7% proteins and a pH close to 7, is a favourable medium for *B. cereus* growth

and toxin production (Rodrigo et al., 2021). This is a concern in cooked or fried rice, as the cooking germinates the *B. cereus* spores and unrefrigerated storage allows the bacteria to grow and produce emetic toxin (Little et al., 2002; Rodrigo et al., 2021).

The storage temperature is the major factor influencing the numbers of *B. cereus* in food products and this, in turn, influences the shelf life of food products. There was less than 50 CFU/g of *B. cereus* found in the zucchini puree stored at 4 °C for 21 days, while 4.0×10^4 CFU/g were found after storage at 10 °C for 21 days (Guinebretiere et al., 2003), suggesting even small temperature changes may affect *B. cereus* counts. Saleh-Lakha et al., (2017) showed that more than 5.5% of milk products stored at 7 °C contained over 10^5 CFU/mL *B. cereus* and more than 31% of the products contained over 10^5 CFU/mL when stored at 10 °C.

The scientific convention is that for *B. cereus* to be able to cause foodborne illness, at least 10^5 to 10^8 *B. cereus* cells/spores must be consumed (Granum and Lund, 1997). However, food poisoning caused by *B. cereus* is not always linked to the number of cells present in the contaminated foods but is also affected by *B. cereus* strains, the presence of toxins, host physiology and the food matrix (Ceuppens et al., 2013). Table 2. 1 gives some examples of food poisoning caused by *B. cereus*. Six diarrhoeal cases were reported out of 34 healthy volunteers aged between 20 to 60 years old who consumed two milk samples containing either no *B. cereus* or 10^8 cells per sample. The diarrhoeal enterotoxin concentration was very low in the samples, even in those samples with high numbers of *B. cereus* cells (Langeveld et al., 1996), indicating diarrhoea is not always associated with cell numbers. Three people died after the consumption of vegetable puree containing 3.2×10^5 CFU/g *B. cereus*, believed to be caused by CytK toxin (Lund et al., 2000). Outbreaks caused by *B. cereus* with 45 and 209 cases were reported in Australia and China, respectively, after consuming various food products such as beef and rice (Chen et al., 2019; Thirkell et al., 2019). Dose-response studies are difficult to compare since there is a time delay between consumption, the development of food poisoning symptoms and the analysis of food samples and differences in susceptibility between individuals. Foodborne diseases caused by *B. cereus* are diarrhoea and emesis, which have an incubation period of 8-16 hours and 0.5-5 hours, respectively (Granum, 1994), causing difficulty in tracing the source of the contamination.

Table 2. 1 Some examples of cases caused by *B. cereus* in recent years.

Number of cases	Symptoms(s)	Related food products	Number of cells contaminated	Country	Reference
6	Diarrhoea	Milk	10^8	The Netherlands	Langeveld et al., (1996)
3	Poisoning (died)	Vegetable puree	3.2×10^5 CFU/g	France	Lund et al., (2000)
45	Diarrhoea and potential emesis	Beef, arancini etc.	1.9×10^4 CFU/g (beef)	Australia	Thirkell et al., (2019)
209	Diarrhoea and vomiting	Drink, rice etc.	10 to 1.6×10^5 CFU/g	China	Chen et al., (2019)

In USA, out of 347 retail seafood samples, 62 were found to contain *B. cereus* (Rahmati and Labbe, 2008). In Belgium, *B. cereus* was presented in 100% of the raw rice, 81% of the béchamel sauce, 77% of the Bolognese sauce samples, 70% of the retail lasagne, 40% of the carrots, 20% of the cooked pasta, 15% of the fresh minced beef, and 5% of the bell pepper (Samapundo et al., 2011). In the Netherlands, a wide variety of food products, including milk and milk products, vegetables and ready-to-eat foods, were contaminated with *B. cereus* (Wijnands et al., 2006a). In Australia, the overall prevalence of *B. cereus* in retail food samples was low, with no detectable *B. cereus* (less than 10^2 CFU/g) in 98% of samples (Eglezos et al., 2010). These results indicate worldwide food safety issues associated *B. cereus* over a wide range of foods.

Due to the variation in numbers and types of *B. cereus* cells in products associated with food poisoning, the maximum allowed levels vary between countries and foods, summarized in Table 2. 2. *B. cereus* levels above 500 CFU/g in dried infant formula are regarded as unsatisfactory based on the European Food Safety Authority requirement (EFSA) (https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=celex:32007R1441), while the limit is set below 100 CFU/g for powder for infants in New Zealand (https://www.mpi.govt.nz/dmsdocument/21185-Microbiological-reference-criteria-for-food) which is the same criteria in the Food and Drug Administration (FDA, US) (https://www.govinfo.gov/content/pkg/FR-1996-07-09/html/96-17058.htm). In New Zealand, the limit of *B. cereus* varies between different food products. In general, the limits for ready-to-eat, dried

and instant food are set below 10^3 CFU/g, while those foods that required further cooking should be below 10^4 CFU/g (https://www.mpi.govt.nz/dmsdocument/21185-Microbiological-reference-criteria-for-food).

Table 2. 2 Examples of maximum allowed levels in different countries and related food products.

Countries	Maximum allowed levels	Food products
European countries (EFSA)	500 CFU/g	Dried infant formula related dairy products
The United States (FDA)	100 CFU/g	Infant formula
New Zealand	100 CFU/g	Infant formula
	10^3 CFU/g	Ready-to-eat, dried and instant food
	10^4 CFU/g	Foods that required further cooking

2.2 Sporulation of *B. cereus*

B. cereus is considered as a problem in the food industry, due to its spore-forming ability and the ubiquity of *B. cereus* spores in the general environment (Andersson et al., 1995). The metabolically dormant *B. cereus* spores enable survival in many different environments, including those unfavourable to microbial growth such as heat, freezing, drying and radiation (Nicholson et al., 2000; Divanac'h et al., 2012). Pasteurization (e.g., 72 °C for 15 s) of raw products is efficient to destroy heat-sensitive pathogenic bacteria, however, thermoduric *B. cereus* spores can survive the process and are difficult to eliminate either in food products or processing lines (Flint et al., 1997; García-Armesto and Sutherland, 1997; Larsen and Jørgensen, 1997). Strain diversity and sporulation conditions such as medium composition, temperature, pH and water activity can affect spore production and heat resistance of the spores, although this varies between studies (Andersson et al., 1995; Faille et al., 2002; Vanasselt and Zwietering, 2006; Abee et al., 2011; Hayrapetyan et al., 2016; Reich et al., 2017). Incubation temperature is an important factor influencing the heat resistance of *B. cereus* spores, with spores produced at 15°C being more susceptible to heat than spores produced at 37°C (Gounina-Allouane et al., 2008). The structure of spores contributes to resistance, with several

protective layers including the inner membrane, cortex, and inner and outer coats (Abee et al., 2011). Dipicolinic acid (pyridine-2,6-dicarboxylic acid or PDC and DPA) that comprises about 5% to 15% dry weight of bacterial spores and is chelated with divalent cations (mainly Ca^{2+}), is implicated as being responsible for the heat resistance of the spores (Slieman and Nicholson, 2001; Setlow, 2006).

The hydrophobicity of the spores is thought to contribute to the greater adherence of *B. cereus* spores to food and food processing equipment surfaces (Wiencek et al., 1990; Husmark and Rönner, 1992). Rönner et al., (1990) and Husmark and Rönner (1992) reported that spores of *B. cereus* demonstrated an ability to attach to stainless-steel surfaces and concluded that the hydrophobic nature of the stainless-steel and spores played a key role in the attachment of spores. The long appendages (an example is shown in Fig 2. 2) which have distinct tubular morphology comprised of proteins cover *B. cereus* spores and are thought to play a role in the adhesion of spores to surfaces (Andersson et al., 1995; Stalheim and Granum, 2001; Tauveron et al., 2006). Strong adhesion can result in contamination for the whole processing line in the food industry (Shaheen et al., 2010).

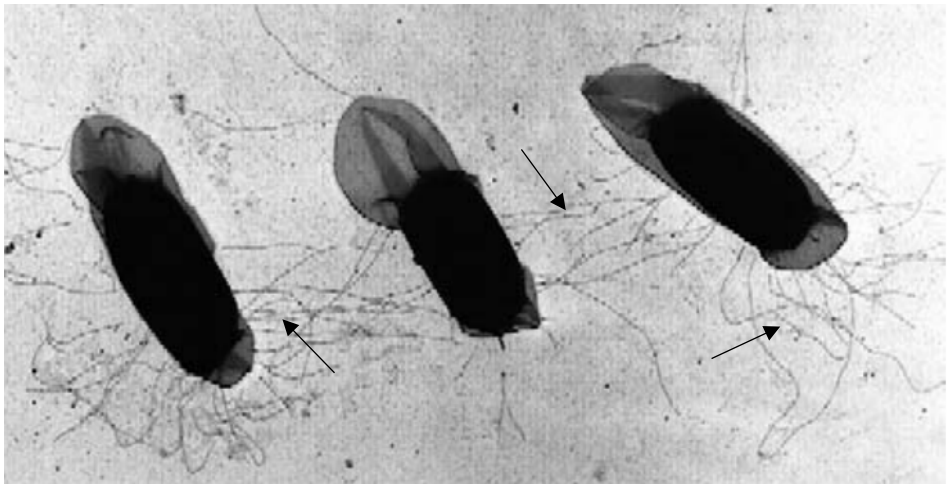

Figure 2. 2* Transmission electron microscopy of stained *B. cereus* spores covered by long appendage (length: 2 μm, arrows pointed) reported by Tauveron et al., (2006).

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Spore germination is an irreversible process to reactivate spores into vegetative cells (Setlow, 2003; Abee et al., 2011). This is induced by nutrients through germinant receptors located in the cell membrane, and these receptors are thought to vary in different strains of *B. cereus* (Abee et al., 2011). Hornstra et al., (2006) reported that germination was not affected by the loss of the GerL receptor in *B. cereus* ATCC 14579, while Barlass et al., (2002) showed that the GerL receptor was L-alanine specific for *B. cereus* ATCC 10876. Non-nutrient pathways and environmental conditions can induce germination as well. For example, lysozyme can degrade the cortex of spores and is a potential pathway for spore germination (Setlow, 2003); high pressure (500 – 600 MPa) triggers germination by opening the spore's Ca^{2+} -DPA channels (Paidhungat et al., 2002); spores produced in liquid culture germinate more readily than those produced in agar plates (Rose et al., 2007).

2.3 Toxin production of *B. cereus*

B. cereus can produce the enterotoxins haemolysin BL (Hbl), non-haemolytic enterotoxin (Nhe), cytotoxin K (CytK) and an emesis-inducing toxin cereulide, causing foodborne illnesses including diarrhoea and emesis (Agata et al., 1994; Beecher et al., 1995; Lund et al., 2000). *B. cereus* induced diarrhoea is caused by enterotoxins produced in the intestine where outgrowth of spores occurs after consuming contaminated foods (Ceuppens et al., 2012b), while emesis is caused by a preformed emetic toxin in contaminated foods, before ingestion (Ehling-Schulz et al., 2004). The main features of these enterotoxins and the emetic toxin produced by *B. cereus* are listed in Table 2. 3. Other virulence factors/toxins, including haemolysins, phospholipases, sphingomyelinase, enterotoxin T (BcET), enterotoxin FM (EntFM), have been reported to support the pathogenicity of *B. cereus* (Agata et al., 1994; Bhunia, 2007; Doll et al., 2013). The pathogenicity of *B. cereus* or the toxicity induced by Hbl and Nhe toxin is normally produced in the early stationary phase and accumulates when high bacterial densities (over 10^5 cells) are reached (Stenfors Arnesen et al., 2008; Ramarao and Sanchis, 2013). The genetic information and enterotoxigenic potential of *B. cereus* are diverse with different prevalence and distribution of enterotoxin and/or emetic toxin genes (Carter et al., 2018), and the production of toxin depends on strain diversity and environmental factors such as temperature,

carbon sources and oxygen availability (van Netten et al., 1990; Fermanian et al., 1997; Ouhib-Jacobs et al., 2009; Van Der Voort and Abee, 2009; Jeßberger et al., 2015).

Table 2. 3* Main features of enterotoxins and emetic toxin produced by *B. cereus*, modified from Bhunia (2007).

Toxins	Genes	Molecular weight (kDa)	Activity
Hemolysin -Hbl			Haemolytic, enterotoxic, dermonecrotic; vascular permeability (Beecher and Wong, 1994b; Beecher and Wong, 2000)
B-component	*hblA*	37.8	
L1-component	*hblD*	38.5	
L2-component	*hblC*	43.5	
Nonhemolytic enterotoxin -Nhe			Enterotoxic, cytotoxic (Lund and Granum, 1996)
NheA	*nheA*	41	
NheB	*nheB*	38.9	
NheC	*nheC*	36.5	
Emetic toxin	*ces*	1.2	Emesis (vomiting)
Cytotoxin K (CytK)	*cytK*	34	β-barrel pore-forming toxin; necrotic dermatitis (Hardy et al., 2001)

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2.3.1 Enterotoxins

Diarrhoea caused by *B. cereus* is associated with three known protein enterotoxins: Hbl (Beecher et al., 1995), Nhe (Granum et al., 1999) and CytK (Lund et al., 2000), with molecular weights shown in Table 2. 3. These diarrhoeal enterotoxins are sensitive to heating (55°C for 20 min) and protease activity (trypsin, pepsin, and chymotrypsin) (Turnbull et al., 1979; Fermanian et al., 1996; Bhunia, 2007). Therefore, pre-formed enterotoxin in food is unlikely to survive food processing and gastrointestinal passage. Ceuppens et al., (2012a) showed that enterotoxin production was absent or impaired at acidic pH (i.e., pH 5.0 of gastric medium). However, the presence of enterotoxin, produced by *B. cereus*, in food products cannot be ignored and is indicative of a product that is generally unsafe for human consumption. A hypothesis for diarrheal food poisoning for *B. cereus* enterotoxins has been suggested by Ceuppens et al., (2013). Briefly, the host ingests the food contaminated with *B. cereus* vegetative cells and/or spores followed by gastric passage. Most of the

vegetative cells are inactivated by gastric acid or other digestive secretions in the small intestine, while the spores remain viable (Clavel et al., 2004; Ceuppens et al., 2012b). It is assumed that only cells, most likely spores, that reach the mucus layer on arrival in the small intestine can survive and adhere to the host cells followed by germinating and producing enterotoxin (Ceuppens et al., 2012a). This was supported by Wijnands et al., (2007) who showed model epithelial cells were able to germinate *B. cereus* spores. The enterotoxins may form pores in the nearby epithelial cells resulting in crovilli damage and osmotic lysis of the epithelial cells, eventually causing diarrhoea (Hardy et al., 2001; Minnaard et al., 2001; Ramarao and Lereclus, 2006; Fagerlund et al., 2008).

Both Hbl and Nhe consist of three individual protein subunits (Table 2. 3) transcribed from *hbl* and *nhe* genes, respectively, which show similarities in gene structure although they are in two independent chromosomal operons (Granum and Lund, 1997; Granum et al., 1999; Beecher and Wong, 2000). The *hbl* operon (*hblCDA*) includes the three subunits: *hblC* encoding L_2 protein (lytic protein), *hblD* encoding L_1 protein (lytic protein), and *hblA* encoding B protein (binding protein) (Beecher and MacMillan, 1991; Beecher and Wong, 2000). The putative B' protein encoded by *hblB* was sequenced with 73% identity to B protein, and it was hypothesized to act as a substitute for B protein (Granum and Lund, 1997). At least two haemolytic components are required to maximize the haemolytic, cytotoxic and dermo-necrotic activities of the Hbl toxin (Beecher and MacMillan, 1991; Beecher et al., 1995). Nhe encoded by the *nheABC* operon consists of three proteins, NheA, NheB and NheC, encoded by genes *nheA*, *nheB* and *nheC*, respectively (Granum et al., 1999). NheB and NheC are required for membrane binding, while NheA triggers cytotoxicity (Lindback et al., 2010). The Nhe toxin requires all those elements for its enterotoxin activity (Bhunia, 2007). Lindbäck et al., (2004) reported that the maximum cytotoxic activity of the Nhe enterotoxin was obtained when the NheA: NheB: NheC molar ratio was 10:10:1, and postulated that, *B. cereus* strains express only small amounts of NheC compared to NheA and NheB.

The cytotoxicity of *B. cereus* strains is thought to be dominated by the Nhe toxin, as Moravek et al., (2006) reported that the toxic activity of *B. cereus* strains producing both Hbl and Nhe was like a sole Nhe producer. Overall, 42% of the strains harbour the *hbl* genes based on molecular characterization

(Moravek et al., 2006). Similarly, Dietrich et al., (1999) indicated that only 50% of the strains tested could produce Hbl toxin, and Andersen Borge et al., (2001) showed that only six of eleven *B. cereus* strains isolated from milk and meat products contained the *hbl* gene while all eleven strains contained the *nhe*, highlighting the importance of Nhe in the pathogenicity of *B. cereus*. However, Carter et al., (2018) found that the *hbl* was the most prevalent toxin gene observed in their isolates, indicating the strain dependence of toxin genes. Cadirci et al., (2018) showed that 31.9% of isolates of *B. cereus* from ice cream had all three Hbl complex encoding genes, 10.6% had two *hbl* genes and 6.3% contained only one *hbl* gene. They also showed that 15.9% of the isolates contained three Nhe complex encoding genes, 31.9% had two *nhe* genes and 20.2% contained only one *nhe* gene, suggesting that toxin gene prevalence and distribution among *B. cereus* strains varies.

Cytotoxin K (CytK) is a protein of 34 kDa with high cytotoxic, necrotic and haemolytic activity (against bovine and rabbit red cells), which belongs to the family of β-barrel pore-forming toxins, is widely distributed among *B. cereus* strains (Lund et al., 2000; Stenfors Arnesen et al., 2008; Ramarao and Sanchis, 2013). CytK is encoded by genes including *cytK1* and *cytK2* (Fagerlund et al., 2004), and the presence of the *cytK* genes varies in different strains from different sources with positive results found in samples from spices, powdered infant formula (PIF), dietary supplements and medicated fish feeds (Carter et al., 2018), but not in dairy desserts (Çadirci et al., 2013).

2.3.2 Emetic toxin (cereulide)

Emetic food poisoning is usually caused by the emetic toxin, “cereulide”, which is a plasmid-encoded cyclic peptide and ingested as a toxin. Nausea and vomiting caused by the cereulide normally happen within 1-5 h of ingestion and the symptoms are usually mild with durations of 6-24 h (Granum and Lund, 1997; Schoeni and Lee Wong, 2005). However, Dierick et al., (2005) reported that cereulide-induced emesis has been implicated in at least one case of child mortality, and the health concern caused by cereulide is due to its high liver toxicity, mitochondrial toxicity, lipophilicity and immunotoxicity (Agata et al., 1994; Mikkola et al., 1999; Paananen et al., 2002). Ceuppens et al., (2011) suggested that the dose of cereulide causing gastrointestinal illness is between 0.02 and 1.83

µg per kg body weight, together with the evidence that emetic strains could produce 0.004 to 0.130 µg cereulide per 10^6 cells. It can be concluded that ingestion of 10^5 to 10^8 cells in total, could produce enough cereulide to cause illness (Häggblom et al., 2002; Jääskeläinen et al., 2004).

Unlike enterotoxins, cereulide is highly resistant to heat (120 min at 90 °C), acid and protease activity (Shinagawa et al., 1996; Agata et al., 2002; Carlin et al., 2006; Rajkovic et al., 2008). Consequently, cereulide is not inactivated during gastrointestinal passage and normal food processing (such as pasteurization), and preformed cereulide in food is extremely difficult to eliminate from the manufacturing line. Emetic toxin-producing strains of *B. cereus* have distinct characteristics from other *B. cereus* strains, generally showing slow spore germination in rich medium and higher resistance of spores than diarrhoeal strains at 90 °C (Carlin et al., 2006; Ehling-Schulz et al., 2015). This increased heat resistance results in a greater risk of *B. cereus* in heat-processed foods. Although the overall prevalence of emetic strains among *B. cereus* isolates is low with around 1.5% on average (Altayar and Sutherland, 2006; Dobrynin et al., 2010), the strains are distributed in various food products such as ice cream, fish products, ready-to-eat foods, and bovine milk as well as the environment (soil) (Altayar and Sutherland, 2006; Svensson et al., 2006; Wijnands et al., 2006a; Messelha¨ Usser, 2010). Emetic food poisoning is usually related to food products with high starch content, such as pasta, rice and mashed potatoes which stimulate the production and accumulation of the emetic toxin cereulide (Ehling-Schulz et al., 2015). Most of the cereulide-producing strains are mesophilic, but some strains can grow at 8 °C (Hoton et al., 2009). Carlin et al., (2006) showed that no emetic strains were able to grow below 10 °C but all of them could grow at 48 °C, while diarrhoeal strains, could grow at 4 °C and/or 7 °C but only a small percentage of the strains could grow at 48 °C, highlighting different growth characteristics for emetic and diarrhoeal strains.

Cereulide is a small, heat-stable circular dodecadepsipeptide with a ring-shaped structure of three repeats of four amino acids and/or oxyacid $[D\text{-}O\text{-}Leu\text{-}D\text{-}Ala\text{-}L\text{-}O\text{-}Val\text{-}L\text{-}Val]_3$. This ring structure has a molecular mass of 1.2 kDa and is chemically closely related to the potassium ionophore valinomycin (Agata et al., 1994; Granum and Lund, 1997). The emetic *B. cereus* possesses the plasmid-encoded cereulide synthase gene cluster *cesHPTABCD* (*ces* gene), which is a non-ribosomal

peptide (NRPS: non-ribosomal peptide synthetase) located on a pOX1-like mega-plasmid of 200-270 kb (Ehling-Schulz et al., 2005; Ehling-Schulz et al., 2006a). The presence of the *ces* gene is used to determine whether an isolate is an emetic *B. cereus* strain, as the *ces* gene is essential for cereulide synthesis and is capable of horizontal gene transfer among *B. cereus* isolates in various substrates including food (Van der Auwera et al., 2007).

2.3.3 Detection and quantification of toxins

The use of PCR in detecting toxin genes is widely used in studies to investigate the presence of diarrhoea and or emetic *B. cereus* strains within contaminated samples. Recently, Carter et al., (2018) demonstrated an integrated approach using PCR with primers of toxin genes and whole-genome sequencing (WGS) to study the enterotoxin producing potential and genomic diversity in parallel. However, the molecular-based assay only allows an assessment of the enterotoxic potential of the strain but not necessarily the toxin production and its pathogenicity (Wehrle et al., 2009). Immunological assays were introduced to quantify Hbl and Nhe toxin production using high-affinity antibodies targeted at specific subunit proteins in these enterotoxin complexes (Dietrich et al., 2005; Wehrle et al., 2009; Ceuppens et al., 2012a). Immunoassay-based commercial detection kits, *Bacillus cereus* Enterotoxin Reverses Passive Latex Agglutination test (BCET-RPLA; Oxoid), Diarrheal Enterotoxin Visual Immuno Assay (TECRA-BDE; ELISA immunoassays kits) and Duopath® Cereus Enterotoxins (Merck) were designed and used for detecting or semi-quantifying enterotoxin production (Beecher and Wong, 1994a; Krause et al., 2010; Ceuppens et al., 2012a). However, the detection of enterotoxin does not necessarily imply the presence of biologically active enterotoxins (Buchanan and Schultz, 1994), therefore, the cytotoxicity assay is widely used for investigating the biological activity of toxins (both enterotoxin and emetic toxin) produced by *B. cereus* (Beattie and Williams, 1999). The presence of toxins is detected by measurement with tetrazolium salt MTT (metallization test; 3-(4,5,-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) viability assay, as the toxin adversely affects the metabolic status of cultured live tissue cells, such as CHO (Chinese hamster ovary) and Vero cells (Beattie and Williams, 1999; Finlay et al., 1999; Moravek et al., 2006). Other *in vitro* assays were introduced for detecting emetic toxin produced by *B. cereus* using cell lines

including HEp-2 (human carcinoma of the larynx), Int 407 (intestine 407, embryonic intestine) and CHO, because of the morphological changes like granulation, cell rounding, acid production and arrested cell multiplication caused by emetic toxin (Hughes et al., 1988; Szabo et al., 1991; Agata et al., 1994). The sperm microassay was introduced as a semi-quantitative estimation of cereulide by the cessation of motility of boar sperms, which was used as a "gold standard" for cereulide toxin detection (Andersson et al., 2004), but currently, a quantitative and sensitive chemical assay based on high-performance liquid chromatography (HPLC) connected to mass spectrometry has been developed and is commonly used to quantify emetic toxin production (Häggblom et al., 2002; Rønning et al., 2015; Hiroshi Koike, 2018; In 'T Veld et al., 2019).

2.4 Biofilm formation of *B. cereus*

B. cereus is known to produce biofilms which are surfaced-based microbial communities and often described as a survival strategy for bacteria facing adverse environmental stresses (Shaheen et al., 2010; Flemming et al., 2016). Biofilm formation is a very complex and dynamic process involving five stages, initial attachment, irreversible attachment, early biofilm structure establishment, three-dimensional structure maturation and dispersion (Monroe, 2007). The biofilm matrix acts as a protective layer for *B. cereus* and contains proteins, carbohydrates, and extracellular DNA (Whitchurch et al., 2002; Vilain et al., 2009; Karunakaran and Biggs, 2011). Biofilm formation confers advantages for bacteria compared to free-floating planktonic cells. These include higher survival rates under adverse environmental conditions such as physical and chemical stress, which may be due to the reduced metabolic and growth rates in biofilm cells, protection by extracellular polymeric substances (EPS) in the biofilm complex and the higher rates of DNA transfer between cells imbedded within the biofilm structure (Chmielewski and Frank, 2003; Davies, 2003; Flemming et al., 2016).

B. cereus can form different types of biofilms including submerged biofilms, pellicles (floating biofilm) and air-liquid interface biofilms, under static and flow conditions. The air-liquid biofilm is regarded as a typical biofilm for *B. cereus* strains possibility caused by aerotaxis (migration towards

oxygen) of cells (Daniel, 1984; Wijman et al., 2007; Hayrapetyan et al., 2015a; Hussain and Oh, 2017). The type of biofilm and biofilm-forming ability is affected by strains, the origin of isolates and environmental conditions and it was concluded that food isolates prefer to attach at the air-liquid interface during the early stage of biofilm development (Wijman et al., 2007; Gao et al., 2015; Kwon et al., 2017; Hussain and Oh, 2018). The composition of the growth medium can play a vital role in the biofilm formation of *B. cereus*, as Elhariry (2011) showed that biofilm formation of *B. cereus* is enhanced under nutrient starvation (diluted TSB). Kwon et al., (2017) indicated that a low concentration of glucose (less than 1% w/v) in the combination of NaCl (less than 2% w/v) may increase biofilm formation of *B. cereus*. Bragadeeswaran (2011) showed that carbon, nitrogen and phosphate concentrations in the growth medium can affect EPS production. For example, the production of EPS was increased with increased sucrose concentration (from 0.25% to 3%). The properties of the substratum (surface hydrophobicity, free energy and electrostatic charges) can influence initial bacterial attachment and consequently biofilm formation (Peng et al., 2001; Palmer et al., 2007). Kwon et al., (2017) showed higher biofilm biomass (OD_{595nm} measured after crystal violet staining) was formed on stainless-steel (SS) compared to plastic and glass surfaces (PS & GS) which could be linked to iron availability that affects *B. cereus* biofilm formation showing a possible role in surface-associated behaviour such as cell-cell interaction (Hayrapetyan et al., 2015a). Some signalling molecules present in the food environment may trigger *B. cereus* biofilm formation (Petrova and Sauer, 2012). These include Mn^{2+} and heme which play a role in swarming mobility and cause strain-dependent biofilm formation in *B. cereus* (Hussain et al., 2018). Yan et al., (2017) showed that some small volatile chemicals, such as ethanol and acetoin derived from metabolic shifts, can act as stimulating signals for biofilm formation in *B. cereus*. Biofilm formation in *B. cereus* is influenced by many factors ranging from the nutrients in the environment to the type of surface to which the bacteria are attached and even the presence of small volatile chemicals within the suspending medium.

B. cereus is frequently identified in a range of dairy products due to the availability of nutrients and organic components and the inability of pasteurization to eliminate *B. cereus* spores (Parkar et al., 2001a; Murphy et al., 2007; Shaheen et al., 2010). *B. cereus* is a common contaminant in raw milk,

with an ability to survive pasteurization by forming spores and the ability to form biofilm on the whole milk processing continuum. *B. cereus* can produce highly hydrophobic spores that can firmly adhere to inert materials, such as stainless-steel and polymers used in food processing to form biofilms (Wiencek et al., 1991). However, monitoring biofilm formation in an industrial environment is difficult. The control methods include chemical treatment, enzymatic disruption, steel coating, biosurfactants, bacteriophages and non-thermal plasma (Galié et al., 2018). Chemical sanitizers that are commonly used in industry include oxidizing agents and chlorine-based detergents (sodium hypochlorite), hydrogen peroxide, ozone and peracetic acid and surface-active compounds including quaternary ammonium compounds (QACs) (Van Houdt and Michiels, 2010; Srey et al., 2013; Tachikawa and Yamanaka, 2014). However, sanitation alone is not enough to eliminate all bacterial biofilms as they can be resistant to sanitizers and a biofilm "footprint" of cells and EPS may survive a sanitation programme (Pontefract, 2013; Galié et al., 2018), therefore, chemicals coupled with mechanical methods have been suggested to disrupt and break down the matrix, thereby reducing bacterial adherence to surfaces and increasing the effects of chemical biocides (Simões et al., 2010; Gopal et al., 2015). More environmentally friendly strategies such as the use of enzymes, bacteriophages, plant-based essential oils (Simões et al., 2010; Gutiérrez et al., 2016) and other advanced technologies including high-intensity focused ultrasound (Xu et al., 2012) and non-thermal plasma (Scholtz et al., 2015), have been introduced in the past decade. However, in many cases, these methods are restricted to laboratory application due to the high cost and some of the approaches developed in the laboratory cannot directly be transferred to industry (Janknecht and Melo, 2003).

2.5 Genetic links for spores, toxin, and biofilm production

Sporulation, toxin production and biofilm formation for *B. cereus* have been intensively studied, however, there is a lack of information on how each of these are linked. Toxin production and spore formation are most frequently reported for the planktonic growth of *B. cereus* but there are no/few reports of toxin production and spore formation from biofilm cells. Table 2. 4 and Figure 2. 3 below summarise the current knowledge of genetic determinants and related pathways involved in spore,

toxin and biofilm formation of *B. cereus* and/or *Bacillus subtilis* (*B. subtilis*) based on a literature review.

Table 2. 4 Summary of genetic determinants involved in spore, toxin, and biofilm formation of *B. cereus* and/or *B. subtilis*.

Genetic determinants	Effect on sporulation	Effect on enterotoxin production	Effect on emetic toxin production	Effect on biofilm development	References
spo0A	√			√	Hamon and Lazazzera, (2001)
comER	√			√	Yan et al., (2016)
plcR		√		√	Agaisse et al., (1999); Gohar et al., (2008); Hsueh et al., (2006)
codY		√	√	√	Ehling-Schulz et al., (2015); Lindbäck et al., (2012)
abrB			√	√	Ehling-Schulz et al., (2015); Lucking et al., (2009)
rpoN (Sigma 54)	√	√		√	Hayrapetyan et al., (2015b)
flhA (Flagella)		√		√	Bouillaut et al., (2005); Ghelardi et al., (2002); Ramarao and Lereclus, (2006)

Intertwined regulatory pathways between biofilm formation and sporulation have been proposed for *B. subtilis* (Vlamakis et al., 2013), which could also be similar in *B. cereus*, but this needs to be confirmed. A *spo0A* deletion mutant of *B. subtilis* shows lower biofilm biomass, however, *spo0A* is also a well-known transcriptional factor required for early sporulation (Hamon and Lazazzera, 2001), indicating that sporulation and biofilm development may be intrinsically linked in *Bacillus* cells. Hamon and Lazazzera (2001) also indicated that the role of *spo0A* in biofilm formation is to negatively regulate *abrB* which is another transcription factor that negatively regulates biofilm formation of *B. subtilis*. Hamon and Lazazzera (2001) also suggest that the cells had the option of taking either the spore formation or biofilm development pathways upon activation of *spo0A*, indicating two distinct pathways from a single gene. However, Aguilar et al., (2010) showed a link between sporulation and biofilm matrix production by showing a *spo0A* mutant that was unable to

produce matrix also showed delayed sporulation in *B. subtilis*. Therefore, there is still the need to elucidate how *spo0A* regulates biofilm formation and sporulation. The *comER* plays a positive role in biofilm formation as well as early sporulation in both *B. subtilis* and *B. cereus*, which may act like *sda* encoding a checkpoint protein for both sporulation and biofilm formation, which may be part of the regulatory pathway involved in hindering the activation of *spo0A* by blocking a phosphor-relay (Yan et al., 2016). A multifunctional protein in *B. subtilis*, YabA, may lead to increased sporulation efficiency and inhibition of biofilm formation when the protein is hyper-phosphorylated (Galié et al., 2018). The importance of phosphorylation in the linkage between sporulation and biofilms could be considered by those genetic determinants mentioned above. The onset of sporulation is dependent on cell-to-cell communication (van Gestel et al., 2012), however, cell to cell communication also regulates a multitude of other bacterial processes including biofilm formation through such communication systems as quorum-sensing signalling (Davies et al., 1998). In summary, the information regarding the mechanisms and regulatory pathways involved in sporulation and biofilm formation in *B. cereus* is fragmented with many unanswered questions.

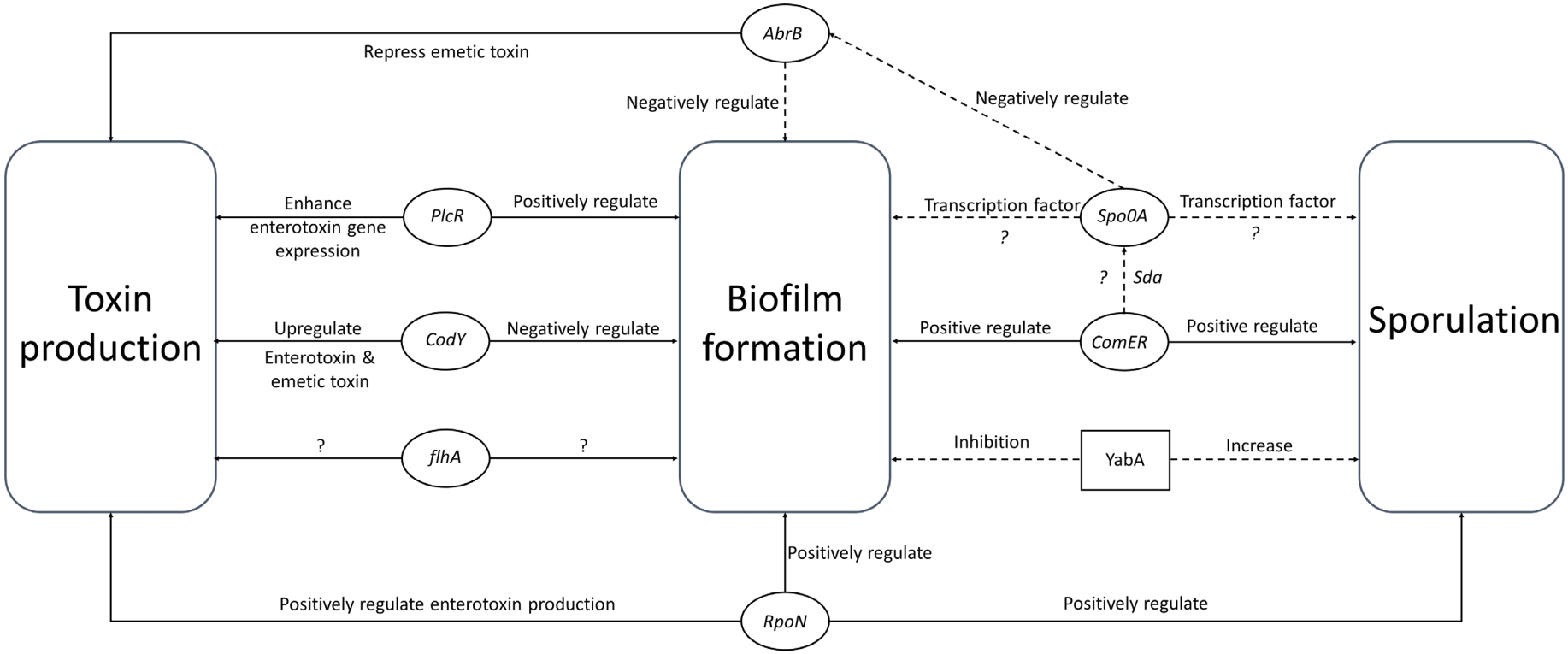


Figure 2. 3 Summary of genetic pathways related to toxin production, biofilm formation and sporulation; dashed lines mean the studies were done for *Bacillus subtilis*; solid lines mean the studies related to *B. cereus*; regulatory genes are described in circles; the related protein is described in the square.

Similarly, a complete picture of the regulatory pathway for toxin production and biofilm development of *B. cereus* is not fully understood (Vlamakis et al., 2013; Okshevsky et al., 2018). *PlcR* is a pleiotropic transcriptional regulator for cell-to-cell communication that may directly or indirectly repress bio-surfactant production which is required for biofilm formation (Hsueh et al., 2006). *PlcR* may also enhance the transcription of genes related to enterotoxin production and other virulence factors such as haemolysins and phospholipase C, however not the emetic toxin (Agaisse et al., 1999; Gohar et al., 2008; Lucking et al., 2009). *CodY*, another global regulator, represses biofilm formation, however, it may indirectly upregulate enterotoxin production in *B. cereus* (Lindbäck et al., 2012). On the other hand, it also has a major role in emetic *B. cereus* pathogenesis by directly repressing *ces* gene transcription (Ehling-Schulz et al., 2015). *AbrB* is a transcriptional factor affecting the biofilm formation together with *spo0A* explained previously, however *abrB* also acts as a repressor of cereulide production (Lucking et al., 2009). *RpoN* (Sigma 54) regulates multiple functions in *B. cereus*, with a deletion mutant showing impaired biofilm formation, sporulation and enterotoxin production (Hayrapetyan et al., 2015a). Flagella are involved in cell adhesion for many bacteria. *FlhA* encodes a component of the flagellum-apparatus formation and thus is associated with cell adhesion, which is also a factor for the secretion of virulence-associated proteins of *B. cereus* (Ghelardi et al., 2002; Bouillaut et al., 2005; Ramarao and Lereclus, 2006). A correlation between virulence factors and biofilm formation has been reported in other bacteria. The regulatory circuits of quorum sensing for *Pseudomonas aeruginosa* act to control the expression of virulence factors, showing that in high cell density, the gene *toxA* encoding for exotoxin A could be promoted by quorum sensing regulators of *P. aeruginosa* (Jones et al., 1993; Bassler, 2001; Passador et al., 2011). The *hapR* gene regulates the expression of the extracellular polysaccharide within biofilms for *Vibrio cholera*, which encodes a transcription factor that inhibits *aphA* expression (a negative regulator of virulence) (Branda et al., 2006). *σB* regulates virulence functions and stress response such as biofilm formation for *Listeria monocytogenes* and *Staphylococcus aureus* (Rachid et al., 2000; Kazmierczak et al., 2003; Patiño-Navarrete and Sanchis, 2017). These studies reveal a possible link between virulence factors and biofilms at the molecular level, however, whether the biofilm mode of growth is a favourable

condition for toxin production, and or whether toxin production leads to biofilm formation is unknown. Similarly, the involvement of biofilm formation on spore formation is unclear.

2.6 Spores in biofilm formation

2.6.1 Biofilm: a supportive reservoir for sporulation?

B. cereus biofilms are mainly comprised of vegetative cells, however, during maturation and ageing, *B. cereus* can form spores within the established biofilm structure, providing a source of contamination in manufacturing lines and food products (Ryu and Beuchat, 2005; Wijman et al., 2007; Faille et al., 2014). However, there are some doubts about how biofilm and planktonic growth compare for the sporulation of *B. cereus*. Some studies indicate that spores within the attached biofilms may be 0.01-10% of the total cells, but this may vary from strain to strain (Ren et al., 2004; Lindsay et al., 2005). Ryu and Beuchat (2005) showed in their work that only 10% of the total *B. cereus* cells in the biofilm were spores after 6 to 12 days of incubation. However, air-liquid biofilms formed by *B. cereus* have been reported to contain up to 90% spores and such interfaces in food processing lines may be an important source of food contamination (Wijman et al., 2007). Faille et al., (2014) showed high levels (over 50%) of sporulation within 48 h in submerged biofilms of different *B. cereus* strains on stainless-steel slides and Hussain et al., (2018) reported that the biofilm formation of *B. cereus* food isolates is linearly correlated with the number of spores in Brain Heart Infusion (BHI) incubated for three days. The high sporulation efficiency in biofilms may be explained by the high cell density and nutrient limitation in the biofilm (van Gestel et al., 2012). Ryu and Beuchat (2005) showed the exposure of the biofilm formed by *B. cereus* to air at high relative humidity (> 97%, at 22 °C) promotes sporulation, while exposure to air at low relative humidity (85%, at 22 °C) did not affect sporulation. Hayrapetyan et al., (2016) showed that drying/air exposure (relative humidity 73 ± 4%) accelerated spore formation within a preformed 24 h - old biofilm resulting in a high percentage of spores, supporting the air/liquid interface effect on sporulation but contradicting the effect of water activity in earlier research. In summary, it appears that there are some contradictory observations from very low to high sporulation within *B. cereus* biofilms. The air-liquid

interface seems to stimulate sporulation and many other factors including strain differences, oxygen availability, high relative humidity, and temperatures, could also influence the sporulation within biofilms but these need investigation.

Spores of *B. cereus* derived from air-liquid biofilm are larger and have higher heat resistance and lower germination capacities compared with spores from liquid culture (van der Voort and Abee, 2013), suggesting that spores derived from biofilms show distinct characteristics from those derived from planktonic cells. However, the underlying mechanisms contributing to these spore characteristics remain to be elucidated. The slower germination of spores in biofilms may contribute to the increased resistance of both *B. cereus* and *B. subtilis* biofilms to cleaning and sanitation regimes (Lindsay et al., 2006), which was also found for *Clostridium difficile* (Semenyuk et al., 2014). Hayrapetyan et al., (2016) showed that *B. cereus* spores harvested from the wet biofilms on stainless-steel (SS) displayed a higher heat resistance compared to those grown in liquid, on agar plates and polystyrene (PS), which could be explained by the enhanced metal availability during sporulation, especially iron (>70% of SS composition) and manganese (1 % of SS composition) (Rajasekar and Ting, 2011; Bragadeeswaran, 2011). A typical dairy thermophile *Anoxybacillus flavithermus*, studied by Burgess et al., (2009), shows that the presence of spores within the biofilm population could increase its resistance to cleaning and high temperatures in the dairy factory, and the numbers of spores were influenced by temperature, indicating the importance of substrates and the environments on the sporulation and heat resistance of spores within biofilm populations. Once released from the biofilm, when the biofilm reaches maturity, the *B. cereus* spores can directly contaminate food or attach at another location on the surface of equipment where conditions are favourable for germination and biofilm growth (Lindsay et al., 2006; Hornstra et al., 2007). It has been suggested that biofilms would be of particular concern as a potential source of *B. cereus* spore contamination of food (Faille et al., 2014), however, how biofilms play a role in supporting sporulation is uncertain, and factors that favour biofilm sporulation during food processing need to be investigated.

2.6.2 Spores: potentially easier than vegetative cells to form a biofilm

B. cereus biofilms are particularly problematic for the food industry, mainly due to their hydrophobic, highly resistant spores that strongly adhere to inert materials such as stainless-steel and polymers used in food processing followed by biofilm development leading to the contamination of food products in contact with bacteria on surfaces (Wiencek et al., 1991). *Bacillus* spores more readily attach to stainless-steel than vegetative cells as they have high hydrophobicity and are covered with long appendages (Rönner et al., 1990; Husmark and Rönner, 1992; Andersson et al., 1995; Tauveron et al., 2006) which promote the adhesion of spores on the surfaces. The adhesion of *B. cereus* spores presumably increases the probability of forming biofilms on the substratum. Besides, this strong adhesion is also an advantage for the spreading of spores with rinse water through the processing line, which is a strategy for *B. cereus* spore survival (Shaheen et al., 2010).

2.7 Toxins in/by biofilms

2.7.1 Biofilm: a microbial community that favours toxin production?

The expression of toxin genes and toxin production can be influenced by environmental factors and can vary between strains of *B. cereus* (Jeßberger et al., 2015), making studies difficult. Besides, there is little knowledge about toxin production within biofilms cells compared to planktonic grown cells. It is commonly accepted that *B. cereus*-induced diarrhoea is caused by enterotoxin produced in the intestine (Ceuppens et al., 2012a), therefore, the biofilm formed in the intestine should be considered. The emetic toxin is produced in food before consumption, so the biofilm formed in food or food processing conditions may be important. Studies have shown that *B. cereus* cells adhere to epithelial cells (Andersson et al., 1998; Ramarao and Lereclus, 2006; Tran et al., 2010), however, the biofilm colonization of *B. cereus* in the intestine or under the gastrointestinal environment is poorly studied. *B. cereus* can form biofilms and secrete metabolites, enzymes and toxins within the biofilm as suggested by Majed et al., (2016), however it is unknown which toxins can be produced by biofilm cells of *B. cereus*. EPS from both biofilm and planktonic suspensions of *B. cereus* strains contain the

enterotoxins (Hbl and Nhe), together with other virulence factors (Karunakaran and Biggs, 2011), but the comparison between biofilm and planktonic cells in terms of toxin production is still uncertain. For *Bacillus thuringiensis*, one study indicated that the transition phase regulator, *sinR*, controls both biofilm formation and Hbl toxin expression, showing that a *sinR* mutant enhanced both biofilm and Hbl production compared with the parental strain (Fagerlund et al., 2014). This study also shows a lower expression of *hbl* in a biofilm population while expression was continuously expressed in all planktonic populations (Fagerlund et al., 2014). However, there is little knowledge about toxin gene expression in biofilms of *B. cereus*. Moreover, it is unclear if there is any toxin produced by biofilm cells or those associated with biofilms of *B. cereus*. If enterotoxins/emetic toxins are produced and accumulate within the biofilm matrix, this may increase the risk posed by *B. cereus*. Biofilms provide a protective environment for bacteria in the face of outside stress, which could be a favourable niche for toxins to accumulate within the biofilm matrix. For instance, toxins A and B are major virulence factors for *Clostridium difficile*, which accumulate in mature biofilms (Semenyuk et al., 2014). EPS include carbohydrate-rich polymers and proteins as important components in the biofilm matrix and they affect bacterial virulence (Branda et al., 2005), indicating the correlation between biofilm and virulence. This is particularly interesting since those enterotoxins produced in the intestine by *B. cereus* are relatively sensitive to protease from the digestive tract compared with the highly resistant emetic toxin and could be protected by biofilms enhancing toxicity.

2.7.2 Toxins: how may they influence biofilm development?

Toxins may have a functional role within the biofilm community. A poorly studied enterotoxin called EntFM produced by *B. cereus* related to cell wall peptidases (called CwpFM) was thought to be involved in biofilm formation (Tran et al., 2010), but this needs to be confirmed and whether toxins have a functional role within biofilm community is uncertain. Alpha-toxin is a haemolytic toxin secreted by *Staphylococcus aureus*, required for cell-to-cell communication during biofilm development (Caiazza and O'Toole, 2003), however, it is unknown if toxins perform a similar specific function within biofilms of *B. cereus*. Toxins may function as regulators within the microbial community by mediating quorum sensing (Bassler, 2001; Riley and Wertz, 2002), as Gram-positive

bacteria use secreted peptides as auto-inducers for cell-to-cell communication in response to high cell-population density as in biofilms (Bassler, 2001). The peptide toxin, cereulide, may serve as an auto-inducer for quorum sensing of *B. cereus* and more specifically, may have a function in biofilm development. Riley and Wertz (2002) presumed that toxins act as an anti-competitor or a defensive role to protect an occupied niche by inhibiting the invasion of other species. Cereulide-producing *B. cereus* demonstrated antifungal activity and the role of the cereulide toxin was confirmed using a non-cereulide-producing mutant (Ladeuze et al., 2011). Multispecies biofilms lead to competition between species, due to the high cell density and scarce nutrient availability within the biofilm matrix. Many bacteria secrete molecules to help them to exclude competing bacteria in biofilms (Rendueles and Ghigo, 2012). Kobayashi and Ikemoto (2019) reported that *B. subtilis* can produce a biofilm-associated toxin called YIT, to attack competitors in biofilms. As reported, the great number of studies describing the molecules and molecular mechanisms of *Bacillus* biofilm formation and toxicity are based on studies on *B. subtilis*, leaving these important features understudied in pathogen, *B. cereus*.

2.8 Conclusions

B. cereus is widely represented in the environment and a common contaminant in food products causing foodborne diseases including diarrhoea and emesis which are due to toxin production. The production of spores and biofilms contributes to the difficulties of dealing with *B. cereus* in the food industry. Toxin production is important for a foodborne pathogen and any sporulation and biofilm formation are likely to exacerbate the risk of food poisoning. However, the link between spores, toxin production and biofilm formation for *B. cereus* is still unclear. A study on the link between these three features could help the food industry to better monitor and control the safety issues caused by *B. cereus*.

2.9 Copyright information

Parts of contents in this chapter have been published in Food Microbiology, and the Online Statement of Contribution form is attached in Appendix I.

Chapter 3 Materials and methods

3.1 Bacterial isolates and culture conditions

3.1.1 Sources of isolates and initial identification

Isolates were provided to us from a testing lab that had tested dairy and potato-based products, and these isolates were from potential commercial products. The isolates were detected on MYP (mannitol yolk polymyxin) agar plates, a selective agar plate for *B. cereus*. Around 20 colonies were randomly picked from these MYP plates from each source. Two reference strains, ATCC 14579 that is a non-emetic strain and F4810/72 (also DSM 4312 or NCTC11143) that is the emetic producer (DSMZ, Germany), were bought and included in this study. Confirmed identifications used PCR, phylogenetic marker 16S rRNA sequencing and whole-genome sequencing (WGS) are explained in section 3. 9. Eight food isolates, including potato isolates (P2, P4 and P5) and dairy isolates (M1, M2, M3, M4 and M5) were selected and used in this study.

3.1.2 Culture conditions

Stock cultures of isolates were prepared by streaking bacteria on Tryptic soy agar (TSA, Difco™, Becton, Dickinson and Company, USA) plates and growing for 24 h at 30°C. A single colony was inoculated into the preservation tube (Protect Microorganism Preservation system, UK) and stored at -80°C. Overnight cultures were obtained by streaking a stock culture on TSA and incubating for 24 h at 30 °C to obtain single colonies followed by inoculating a single colony into Tryptic soy broth (TSB, Difco™, Becton, Dickinson and Company, USA) and incubating overnight (17 - 18 h) at 30 °C with 120 rpm shaking. The overnight culture was used for subsequent experiments.

3.1.3 Dilutions and plating method

The number of viable cells was counted using standard spread plating. Cultures or cell resuspensions were diluted in serial 10-fold dilutions using sterile saline (0.85% NaCl solution) and the desired dilutions were spread onto TSA followed by incubating at 30 °C for at least 24 h before counting.

3.2 Planktonic and biofilm growth

3.2.1 Planktonic growth

The planktonic culture was obtained from a 500 mL shaking flask filled with 100 mL media and inoculated with 1% (vol/vol) overnight culture. The planktonic culture was incubated at 30° C shaken at 120 rpm to avoid sedimentation of cells. Different periods of incubation time were used depending on each experiment.

3.2.2 Pellicle formation

Pellicles of these isolates were formed in a 48-well plate (Costar®, Corning, USA) filled with 0.5 mL TSB and inoculated with 1% (vol/vol) overnight culture. Wells containing only medium was included as the negative controls. The pellicle formation was compared between isolates visually and photographing the wells after one-, two-, and three-day incubation. The plates were incubated at 30 °C either in static or shaken at 120 rpm.

3.2.3 Biofilm grown on stainless-steel coupon

Biofilms were developed on stainless-steel (SS) coupons (10 mm x 10 mm x 1 mm; 304-2B stainless-steel; Advanced Sheetmetals Ltd., Palmerston North) treated with nitric acid (50% nitric acid, heated at 70°C for 30 min to clean and generate an oxide coat on the surface, as is done in the food industry for new stainless-steel– a process called passivation), rinsed with deionised water and sterilized (autoclaved at 121°C for 15 min). SS is the most common material used in food manufacturing plants. These coupons were placed vertically into the wells in a 48-well plate containing 0.5 mL media and 1% (vol/vol) overnight-grown culture. Around half (100 mm^2 surfaces in total, 50 mm^2 on each side) of the coupons were submerged vertically into the liquid medium to create an air-liquid interface. Plates were wrapped with parafilm (Bemis®, USA) to prevent evaporation and then incubated for the required days at 30 °C to allow the growth of biofilm cells.

3.2.3.1 Crystal Violet (CV) assay of biofilm formed on SS coupons

The total biofilm biomass grown on SS coupons was quantified according to protocols described by Castelijn et al., (2012) using Crystal Violet (CV) with modification. Briefly, after incubation, coupons containing biofilms were gently washed by dipping three times in sterile saline and inserting into a new 48-well plate filled with 0.1% CV (Acros Oranics, USA) to stain for 30 min. After staining, the coupons were washed 3 times again to remove background dye and subsequently de-stained in new plates filled with 70% ethanol for 45 min. The absorbance of the CV solution was measured by spectrophotometer (SpectrostarNano, BMG Labtech, New Zealand) at 595 nm.

3.2.3.2 Biofilm cell detachment and enumeration

Glass beads (Diameters =6.35 mm, Paul Marienfeld GmbH & Co., Czech Republic) were used to detach biofilms from the SS coupon. Each coupon was washed by dipping three times in sterile saline and placing it in a sterile glass bottle containing 5 mL sterile saline with 5 g sterile glass beads. Bottles were mixed by vortex (Scilogex, Germany) at maximum speed for 1 min to detach the biofilm cells from the coupon. Hayrapetyan et al., (2015a) indicated that this method can effectively separate cells from the substratum without affecting cell viability. The detached biofilm resuspension was diluted and spread plated to determine the amounts of biofilm cells as described in section 3. 1. 3.

Another detachment method used sterile cotton swabs (COPAN®, USA) to swab biofilm cells from SS coupons after dipping to wash three times in saline, and the swabbed biofilm cells were resuspended in saline followed by different experimental procedures.

3.2.4 Biofilm grown on glass wool and stainless-steel wool

Glass wool (GW) and stainless-steel wool (SSW; “Stainless-steelo”, Steelo Scourer No Rust Stainless-steel Pads, New Zealand) provided the larger surface for bacterial attachment and biofilm growth. Like the SS coupon, SSW was treated with nitric acid and sterilized. A 500 mL shaking flask containing 100 mL TSB and different amounts of SSW or GW were inoculated with 1% (vol/vol) of overnight-grown culture and incubated at 30 °C with 120 rpm shaking for 24 h to allow biofilm

growth on the wools. The biofilm cells on the GW or SSW were detached using 30 g glass beads followed by strong manual shaking for 5 min after washing twice with saline (Lindsay et al., 2006). The attachment (one-hour incubation), growth (24 h incubation) and detachment (after using glass beads) on the wool surfaces were viewed by DIC microscope (Differential Interface Phase Contrast, Olympus BX53).

3.2.5 Independently grown biofilms on GW

To study the toxin produced by biofilm cells and minimize the presence of planktonic culture, an independently grown biofilm system was designed for this study (Fig. 3. 1). Briefly, a 500 mL flask containing 100 mL TSB with 0.5 g GW was inoculated with 1% (vol/vol) overnight-grown culture and incubated at 30 °C with 120 rpm shaking for one hour to allow cells attachment on the GW. The GW was aseptically removed and put into a new sterile empty bottle for further incubation for 24 h at 30 °C with 120 rpm shaking without additional medium added. The biofilm cells on GW were detached using 30 g glass beads as described previously (section 3. 2. 4).

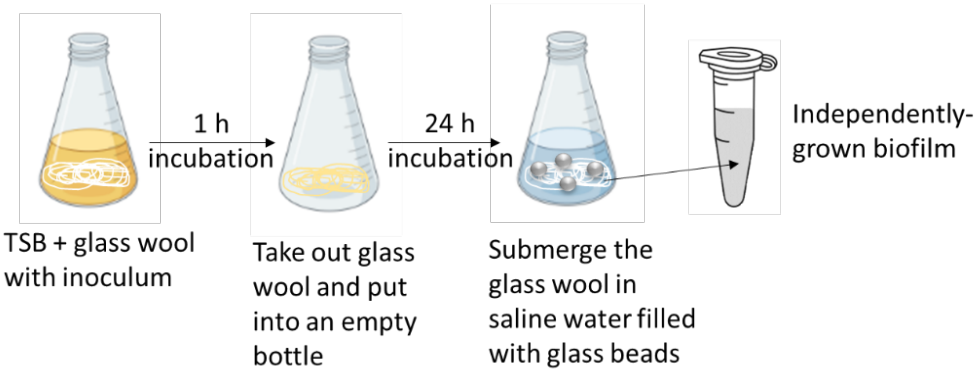


Figure 3. 1 The experimental design of independently grown biofilms on glass wool.

3.3 Spore culturing and counts

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) or thermo-cycle (ProFlex PCR system, USA) to inactivate all vegetative cells. The number of total cells and their spores were determined by

standard spread plate counting grown on TSA after 24 h incubation at 30°C. The spore counts were incubated for up to three days followed by counting. The sporulation percentages within either planktonic or biofilm populations were calculated based on the equation below:

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

3.4 Heat resistance of spores

3.4.1 Harvest spores

Spores were harvested after three days of incubation at 30°C grown in either TSB or homogenised pasteurised whole milk (Anchor, New Zealand). To harvest sufficient spores, the biofilm cells were harvested by swabbing from 20 SS coupons using sterile cotton swabs (refer to section 3. 2. 3. 2) and 4 mL of planktonic cultures were harvested. Spores were harvested by centrifuging planktonic and biofilm resuspensions at 11,000 × g for 15 min at 4°C. The pellets were washed twice with sterile saline (11,000 × g for 2 min at 4°C) and the crude spore suspension was suspended in 1 mL sterile saline and stored at -20°C until testing.

3.4.2 Heat treatment

Fifty microliters of crude spore suspension of either planktonic or biofilm culture with 10^6-10^7 CFU/mL were heated at 90°C at regular intervals from 5 to 20 min in the thermocycler (ProFlex PCR system). An additional tube of the sample was heated at 80°C for 10 min as a control and the counts of this sample were taken as initial spore count. Serial 10- fold dilutions of the heated sample were spread plated on TSA, incubated at 30°C for up to three days followed by counting. Decimal reduction times ($D_{90°C}$ value) were calculated through the negative inverses of the slope of the regression line plot of the survivors over time. All of the experiments were conducted with three biological replicates.

3.5 Dipicolinic acid (DPA) content measurement

3.5.1 DPA content standard curve and measurement

The DPA (Dipicolinic acid, pyridine-2,6-dicarboxylic acid or PDC) concentration of spores was determined with fluorescence emitted by the binding of terbium ions (Tb^{3+}) to DPA, described by Jamroskovic et al., (2016). A standard curve was drawn by adding known concentrations of DPA (0 – 0.3 ppm; Sigma-Aldrich, USA) to sterile distilled water containing terbium chloride ($TbCl_3$; Sigma-Aldrich, USA) at a final concentration of 50 μM (Fig. 3. 2). The best lines are fitted with 95% confidence. The fluorescence was measured using a Spectro fluorimeter (Perkin Elmer LS55, USA) with the following settings: excitation wavelength: 270 nm, emission wavelength: 545 nm, slit width: 15 nm, scan speed: 500 nm/min and a 420 nm long-pass filter.

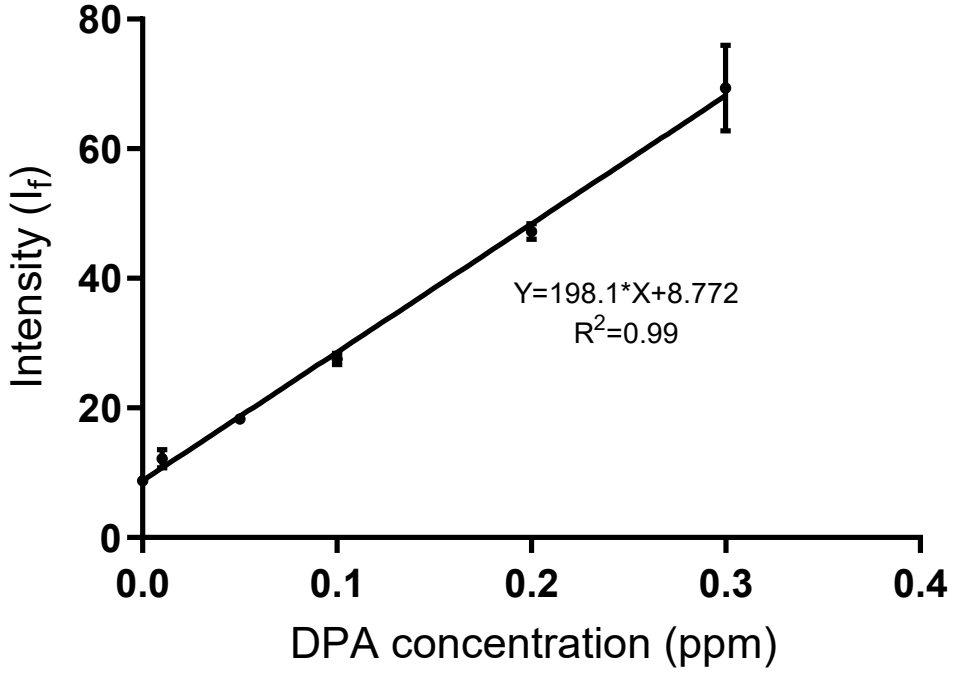


Figure 3. 2 The standard curve of DPA measurement using a spectro fluorimeter.

3.5.2 DPA release from spores

A crude spore suspension (in sterile saline) prepared as described in section 3. 4. 1, was autoclaved (121°C for 15 min) to release DPA. After autoclaving, the suspensions were centrifuged (13,000 × g for 5 min) and the supernatants were added to $TbCl_3$ followed by measuring fluorescence intensity as described above. The DPA measurements were performed with three biological replicates.

3.6 Transmission electron microscopy (TEM)

The structure of spores harvested from planktonic culture and biofilms were studied using transmission electron microscopy (TEM). The preparation of samples was conducted by Yanyu He and Raoul Solomon at the Manawatu Microscopy and Imaging Centre (Palmerston North, New Zealand). Briefly, undiluted samples were injected and sealed in agarose tubes. Primary fixation and post-fixation were performed using 3% glutaraldehyde and 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2), with buffer washings between the two fixations. Dehydration of the samples was achieved using an acetone series, before infiltration and embedding in fresh resin (Procure 812; ProSciTech Pty Ltd., Thuringowa Central, Queensland, Australia). Ultra-thin sections (100 nm thick) were cut using a diamond knife (Diatome, Switzerland) and an ultramicrotome (Leica, Vienna, Austria). Samples were stained for 4 min with saturated uranyl acetate and lead citrate, 50% ethanol and MilliQ water washing steps were included. Samples were viewed in an FEI Tecnai G2 Spirit BioTWIN (Czech Republic) (Camera: Veleta, Olympus SIS Germany).

3.7 Haemolytic toxin measurement

3.7.1 Types of cultures tested

3.7.1.1 Planktonic cultures and cells grown on plate

The planktonic culture was grown as described in section 3. 2. 1. To investigate if Hbl toxin produced is associated with cells or released into media, the planktonic culture was centrifuged at 11,000 × g for 30 sec, and Hbl toxin was measured in total planktonic culture, supernatant of planktonic culture after

centrifuging and resuspension of planktonic cell pellets in saline. The experimental design is illustrated in Fig. 3. 3A.

The Hbl toxin in cells grown in TSA was also measured. Approximately 5 µL overnight culture was inoculated in the centre of a TSA, followed by 24 h-incubation at 30°C and swabbed the colony and resuspended in saline. The experimental design is illustrated in Fig. 3. 3B.

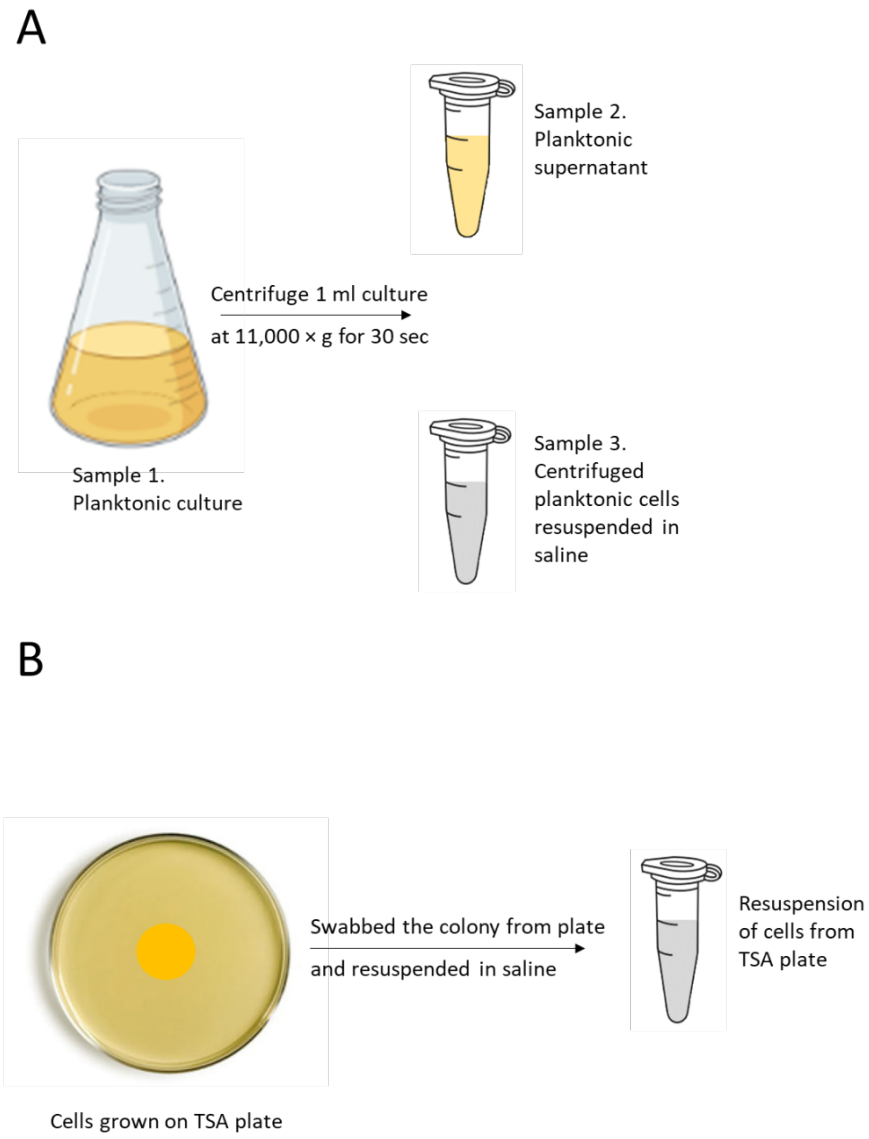


Figure 3. 3 Pictorial flow diagrams of the experimental setup for measuring toxins in planktonic growth (A) and cells grown on TSA (B).

3.7.1.2 Biofilm grown on stainless-steel coupon

The biofilm grown on SS coupon is described in section 3. 2. 3 and the types of cultures in the presence of SS coupon are explained in Fig. 3. 4. Three types of culture were assessed for toxin production in the presence of biofilms grown on SS coupons (Fig. 3. 4): 1. The planktonic culture was obtained from a well without SS coupon insertion (Fig. 3. 4A); 2. Planktonic culture from the medium surrounding SS coupons growing biofilm (Fig. 3. 4B); 3. Biofilm was obtained by swabbing biofilm cells from SS coupon and resuspending in 1 mL saline (Fig. 3. 4B).

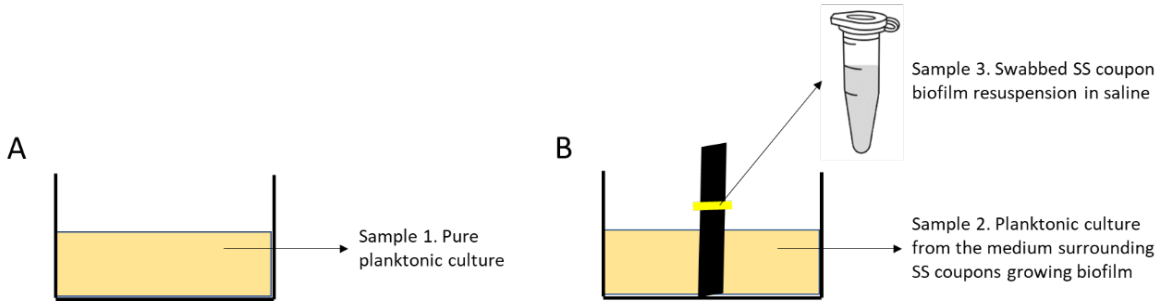


Figure 3. 4 Pictorial flow diagrams of the experimental setup for growing biofilm on stainless-steel coupons inserted into a 48-well plate. "A" shows planktonic culture obtained from the well without an inserted stainless-steel coupon; "B" shows the stainless-steel coupon biofilm growth; Sample 2 is the planktonic culture from medium surrounding stainless-steel coupon growing biofilm; Sample 3 is the swabbed stainless-steel coupon growing biofilm resuspension in saline.

3.7.1.3 Biofilm grown on glass wool and stainless-steel wool

The biofilms grown on GW or SSW are described in section 3. 2. 4. A preliminary experiment tested biofilm cells grown on different amounts of GW and SSW. P5 was used to develop biofilms on two substrates. The resuspension of detached biofilm from 0.5 g, 1 g and 3 g GW all contained approximately 10^8CFU/mL cells, therefore, 0.5 g GW was used for biofilm development. As SS material is heavier than GW, biofilm cells detached from 1 g, 2 g and 5 g SS were counted. The resuspensions of detached biofilm from 1 g SSW contained $10^{7.6}$ CFU/mL while it contained 10^8 CFU/mL from both 2 g and 5 g SSW. Therefore, 2 g SSW was used for biofilm growth.

The toxin production experimental setup in biofilm grown on GW and SSW is illustrated in Fig. 3. 5. The Hbl toxin was detected in the four types of culture. Planktonic culture (Fig. 3. 5A) and planktonic

cells surrounding the biofilms (Fig. 3. 5B) represented the Hbl toxin in the presence of biofilms. The effect of substratum on Hbl toxin production was studied by comparing the mixed culture of planktonic and biofilm cells (detaching biofilms into planktonic culture) (Fig. 3. 5C) from wool, and resuspension of detached biofilm cells from two substrates in saline (Fig. 3. 5D). The biofilm cells were detached from the wool by using glass beads (approximately 30 g) for 5 min as described in section 3. 2. 4.

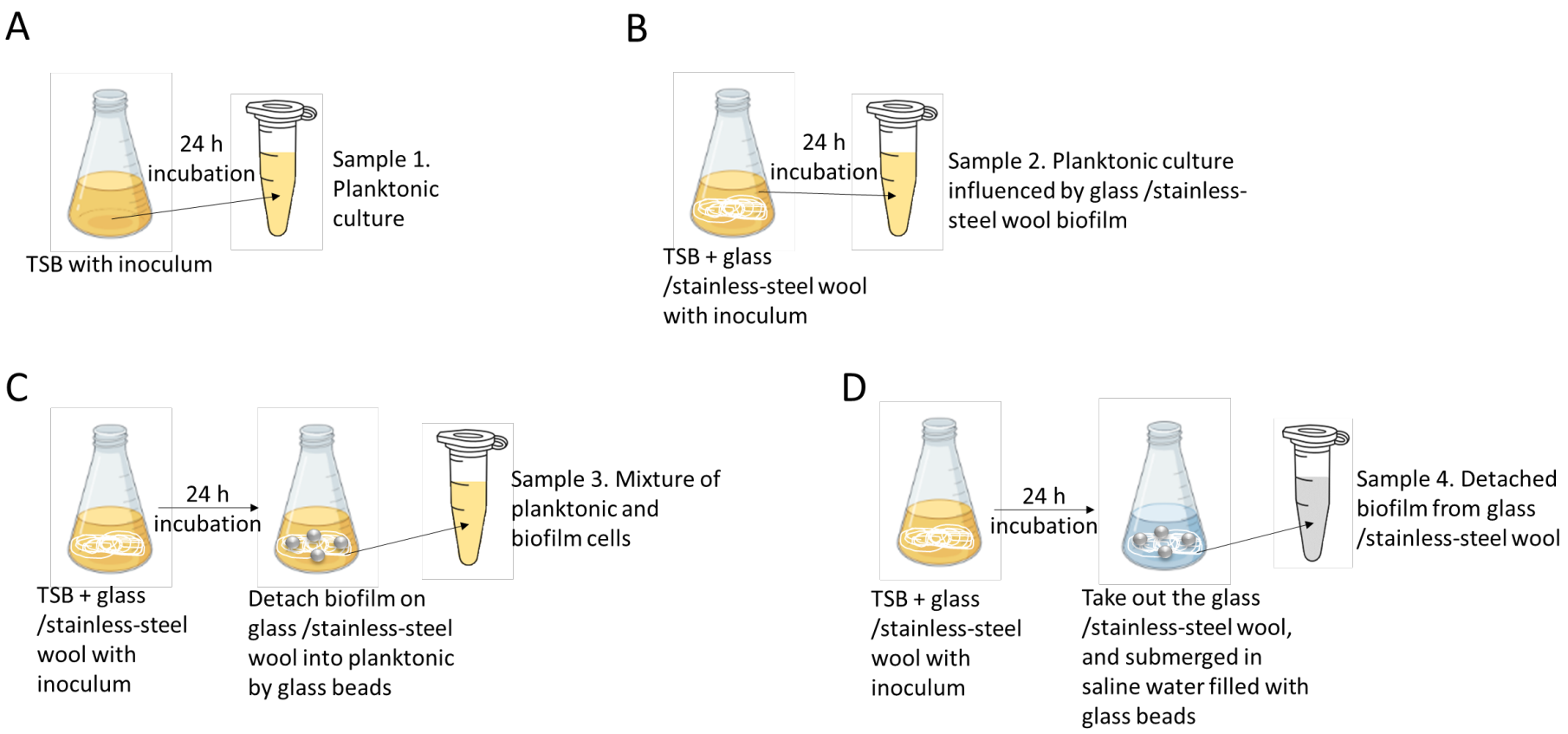


Figure 3. 5 Pictural flow diagram of the experimental setup for measuring toxin in the presence of wools. "A" shows planktonic culture obtained from a 24 h-grown culture without biofilms; "B" shows planktonic culture surrounding biofilms obtained from cultures grown in the presence of biofilms; "C" shows the mixture of planktonic and biofilm cells, where combined cells growing planktonically and those detaching from the biofilm; "D" shows detached biofilm cells obtained by removing wools after 24 h growth and washed twice with saline followed by detaching biofilm cells into 50 mL saline using 30 g glass beads.

3.7.2 BCET-RPLA (*Bacillus cereus* enterotoxin test-reverses passive latex agglutination) detection kit measurement

Before toxin detection, the cultures were diluted to the same number of cells by adding saline. One mL of each culture was assessed for Hbl production using the BCET-RPLA kit (Oxoid ™ Thermo Fisher, Japan) according to the instructions provided by the manufacturer. Briefly, the cultures after normalizing to the same amount of cells (10^7 CFU/mL) were centrifuged at 900 × g for 20 min at 4°C, and the supernatant was added to a well in a 96 V-well microtiter plate (Corning®, USA). The toxin production results are interpreted based on agglutination patterns (Fig 3. 6). To compare the toxin production between cultures, two-fold dilutions of the supernatant from normalized cultures were tested. The Hbl toxin measurements were conducted with at least three biological replicates.

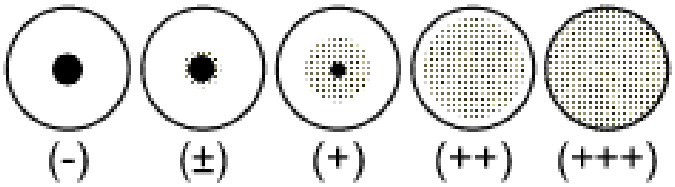


Figure 3. 6 Interpretation of Hbl toxin results using the BCET-RPLA kit; "+", "++" and "+++" indicates the presence of Hbl toxin while "-" denotes the absence of Hbl toxin.

3.8 Emetic toxin (cereulide) measurement

3.8.1 Types of culture tested

B. cereus emetic reference strain F4810/72 (DSMZ, Germany) was used in the emetic toxin (cereulide) study. The planktonic and biofilm cells were cultured as described in section 3. 2. Biofilm grown on SS coupons (refer to section 3. 2. 3), GW and SSW (refer to section 3. 2. 4) was studied. The types of culture tested for cereulide quantification were the same as those used in the Hbl toxin study as described in section 3. 7. 1, however, different amounts of GW (0.5 g, 1 g and 3 g) and SSW (1 g, 3 g and 5 g) were used in the cereulide toxin study.

3.8.2 Toxin attachment

The planktonic culture was centrifuged at 11,000 × g for 2 min at 4°C and the supernatant was used as the toxin-containing solution to minimize the effect of cells. Either 0.5 g or 3 g of GW or SSW was added into 30 mL supernatant and shaken at 120 rpm for 30 min to allow the attachment of toxin, followed by extracting and quantifying the toxin in the supernatant after attachment to detect the residual toxin after attachment to the wools. Planktonic supernatant without adding GW or SSW was used as a control. The experiment is illustrated in Fig. 3. 7.

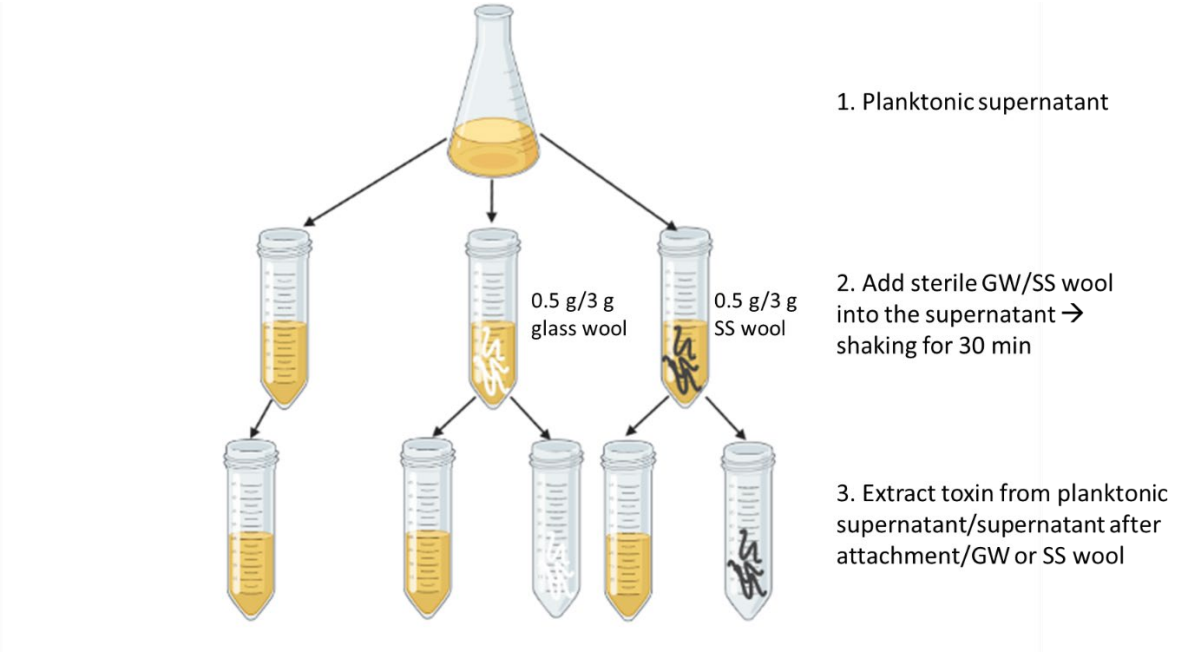


Figure 3. 7 The illustration of the cereulide toxin attachment experiment.

3.8.3 Extraction method

Cereulide in bacterial culture or on wools (GW or SSW) was extracted by acetonitrile to optimize the recovery of the toxin (Hiroshi Koike et al., 2018). One mL of culture was added to 9 mL acetonitrile and shaken for one hour at 150 rpm, followed by centrifugation at 15,000 × g at 4°C using a high-speed refrigerated centrifuge (Himac CR22GII, Japan). The upper clear liquid was collected into an Eppendorf tube and centrifuged again at 4°C. The upper one mL of liquid was used for cereulide

quantification. All the tested bacterial cultures had 25 ng/mL valinomycin added as an internal standard before extraction.

3.8.4 LC-MS/MS

3.8.4.1 LC-MS/MS conditions

The LC-MS/MS settings were developed with the assistance of Trevor Loo from the School of Fundamental Science, Massey University. Liquid chromatographic separation was performed using an UltiMate 3000 UHPLC system (Thermo Fisher Scientific, USA) equipped with an Accucore™ 150-C18 column (100 x 2.1mm, 2.6 µm, 150 Å) and a matching Accucore™ Defender Guard Column (Thermo Fisher Scientific, Lithuania) maintained at 40°C. The gradient used was according to Hiroshi Koike et al., (2018). Briefly, the water phase (buffer A) was applied using 1 mmol/L ammonium formate in water containing 0.1% formic acid and the organic phase was methanol containing 0.1% formic acid (buffer B). The detailed gradient elution conditions are shown in Table 3. 1. The flow rate was 0.35 mL/min and the injection volume was 2 µL.

Table 3. 1 LC (gradient elution) conditions for the quantification of cereulide in this study.

Time	Flow rate (mL/min)	A (%)	B (%)
0.00	0.35	85	15
2.00	0.35	5	95
4.00	0.35	5	95
6.00	0.35	85	15
10.00	Stop Run		

The mass spectrometric detection was performed using a Q Exactive™ Focus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Fisher Scientific, USA). The mass spectrometer was operated according to the parameters listed and the expected retention times for cereulide and valinomycin detection are listed in Table 3. 2. The product ions for peak integration (quantification) used were 1125.7 (m/z) and 1083.6 (m/z) for cereulide and valinomycin, respectively (Hiroshi Koike et al., 2018).

Table 3. 2 Parameters of MS/MS detector.

Scan parameters	
Survey scan range	350 to 1500 *m/z*
Resolution	70,000 (MS1), 35,000 (MS2)
Polarity	Positive
MS2 isolation window	1.0 *m/z*
Default charge	1
AGC target	1e6 (MS1), 5e4 (MS2)
Max IT (ms)	auto
Microscans	1
Spectrum data type	Profile

HESI source	
Sheath gas flow rate	35 psi
Aux gas flow rate	6 psi
Spray voltage	4.5 kV
Capillary temperature	350°C
S-lens RF level	85%
Aux gas heater temperature	275°C

Inclusion list

Chemical	Mass (*m/z)*	CS (z)	Start-End (min)	CE
Cereulide	1170.712	1	6.3-7.8	53
Valinomycin	1128.665	1	6.5-8.0	50

3.8.4.2 Standard curve of synthetic cereulide and valinomycin

The synthetic cereulide standard was purchased from Chiralix B.V. (Nijmegen, the Netherlands) in the form of powder. The valinomycin (HPLC grade; ≥ 90%; Merck) was used as an internal standard. Other reagents including acetonitrile, methanol, water, formic acid and ammonium formate were all Optima™ grade purchased from Thermo Fisher Scientific (Lithuania, USA). Both the synthetic cereulide and valinomycin were dissolved in methanol to the concentrations of 0.01, 0.05, 0.1, 1, 5, 10 and 20 ng/mL. All the stocks and standard solutions were stored at -20°C until testing. The standard curves are shown in Fig 3. 8 and 3. 9. The best lines are fitted with 95% confidence.

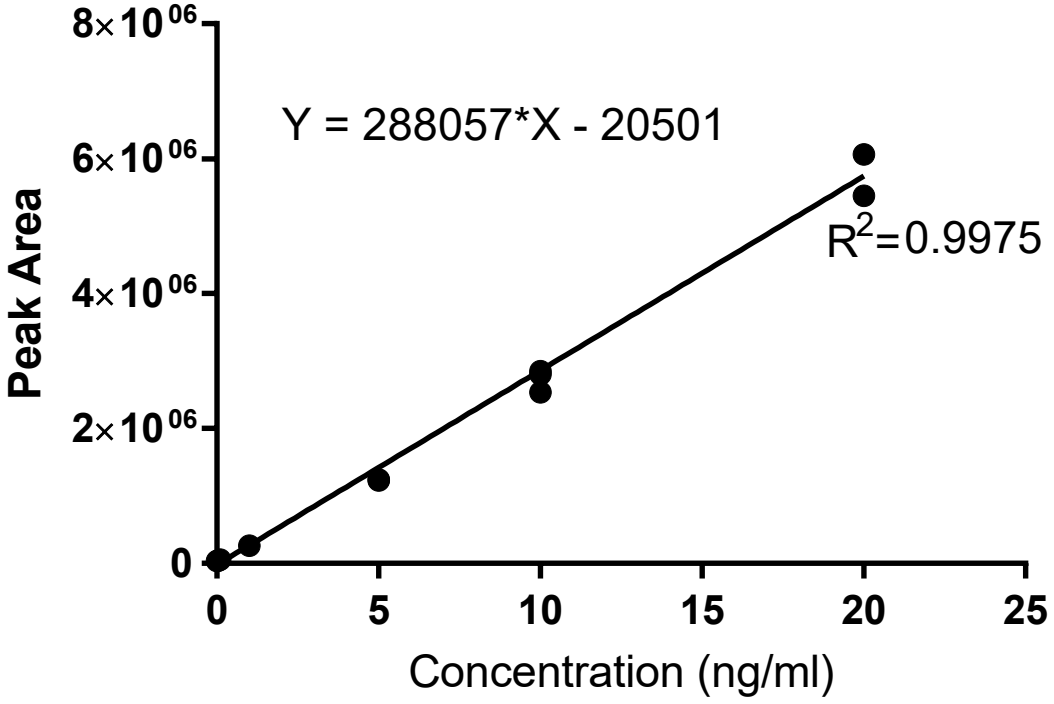


Figure 3. 8 The standard curve for quantifying cereulide standard solution with concentrations of 0.01, 0.05, 0.1, 1, 5, 10 and 20 ng/mL.

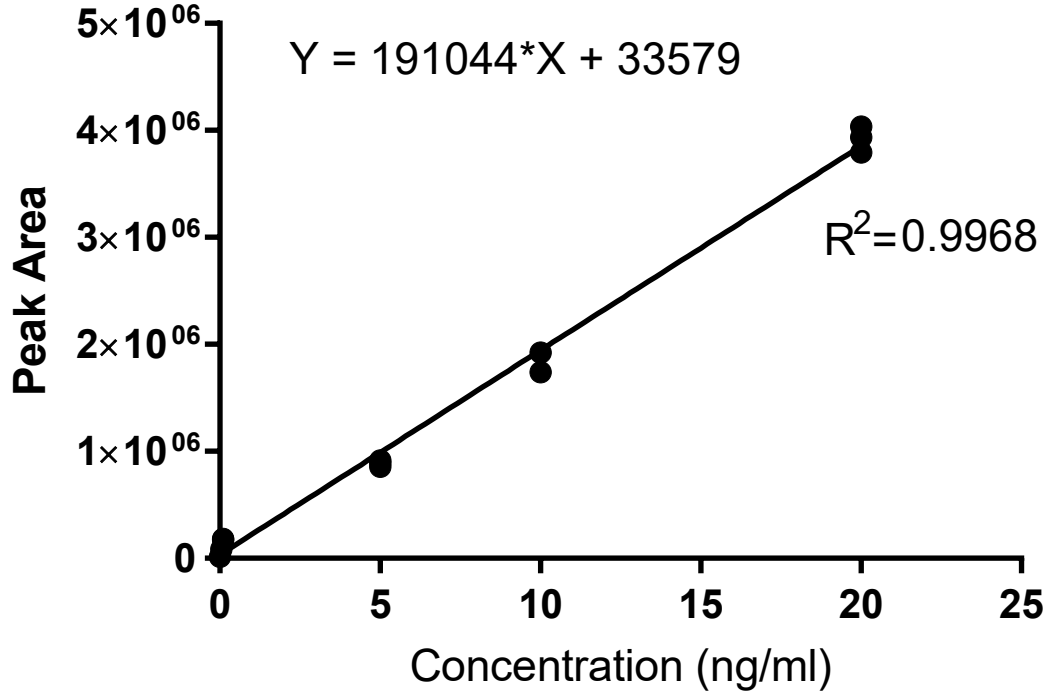


Figure 3. 9 The standard curve for quantifying valinomycin standard solution with concentrations of 0.01, 0.05, 0.1, 1, 5, 10 and 20 ng/mL.

3.8.4.3 Validation experiments

The correlation of cereulide and valinomycin was measured by combining different concentrations of the standard solution of cereulide (0.1, 1 and 10 ng/mL) and valinomycin (0.1 and 10 ng/mL), and the result is shown in Table 3. 3. This result indicates that the correlation of the two compounds was decreased when both of them were over 1 ng/mL.

Table 3. 3 Correlation of cereulide and valinomycin.

Added concentration (ng/mL)		Measured concentration (ng/mL)	
cereulide	Valinomycin	cereulide	Valinomycin
0.1	0.1	0.31 ± 0.02	0.30 ± 0.04
1	0.1	1.09 ± 0.05	0.30 ± 0.03
10	0.1	10.96 ± 0.64	0.30 ± 0.01
0.1	10	0.29 ± 0.01	11.56 ± 0.21
1	10	1.14 ± 0.3	12.40 ± 0.58
10	10	11.08 ± 0.13	11.82 ± 0.47

The matrix effect and recovery rate of cereulide and valinomycin were examined by spiking known amounts (0.1, 1, 2.5, 5 and 10 ng/mL) of cereulide and valinomycin in TSB followed by extraction and quantification with LC-MS/MS. The result is included in Table 3. 4. The recovery rate was calculated based on the equation below:

$$\text{Recovery rate (\%)} = (\text{Measured concentration} / \text{added concentration}) \times 100\%$$

The recovery rates of both cereulide and valinomycin in the concentration of 2.5 to 10 ng/mL showed a range of 100 to 120%.

Table 3. 4 The matrix effect and recovery rate of cereulide or valinomycin in TSB.

	Added concentration (ng/mL)	Measured concentration (ng/mL)	Recovery rate (%)
Cereulide	0.1	0.19 ± 0.01	192
	1	1.45 ± 0.07	145

	2.5	2.76 ± 0.54	110
	5	5.83 ± 0.46	117
	10	11.96 ± 0.60	120
	0.1	0.13 ± 0.01	130
	1	1.21 ± 0.07	121
Valinomycin	2.5	2.71 ± 0.18	108
	5	5.95 ± 0.36	119
	10	11.56 ± 0.66	116

Seven concentrations of valinomycin (0.25, 0.5, 1, 1.25, 2.5, 5 and 10 ng/mL) were spiked into the planktonic culture of *B. cereus* F4810/72 strain to determine which concentration of valinomycin is the best as the internal standard. The recovery rate is shown in Fig 3. 10. The concentration of 2.5 ng/mL of valinomycin displayed a recovery rate of around 100%, therefore 25 ng/mL valinomycin was added in all the bacterial culture samples as an internal standard before extraction taking into consideration the 10-fold dilution factor during extraction (refer to section 3. 8. 3).

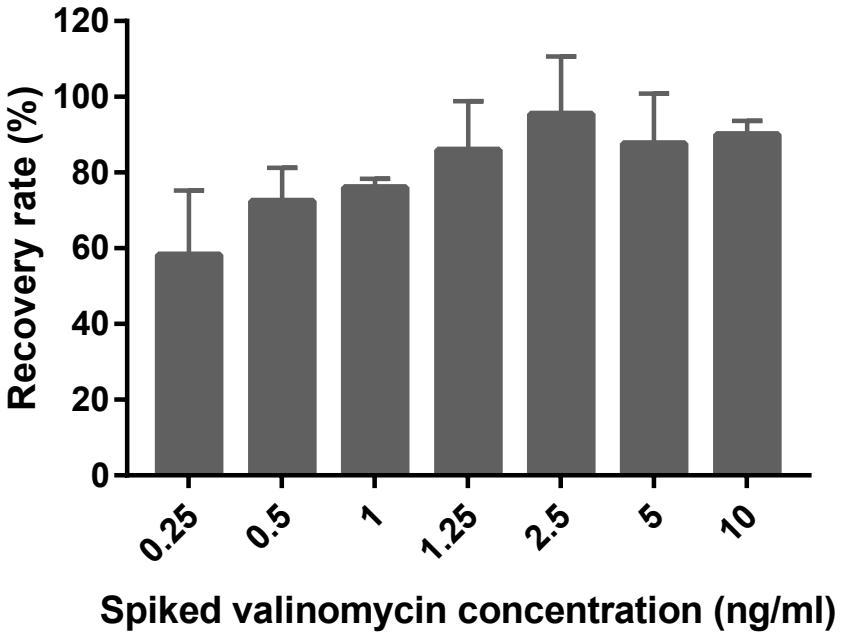


Figure 3. 10 The recovery rate of spiked valinomycin in the planktonic culture of *B. cereus* F4810/72 strain grown in TSB.

3.9 Molecular microbiology and analysis

3.9.1 DNA extraction

Bacterial cultures were grown as previously described (section 3. 1). DNA of *B. cereus* strains was extracted using QIAamp® BiOstic® Bacteremia DNA kit (QIAGEN, Germany) according to the manufacturer's instructions.

3.9.2 RNA extraction

Two RNA extraction methods were used in this study depending on different experiments:

1. Total RNA was extracted using the TRI-reagent (ThermoFisher, USA) according to the manufacturer's instructions with modifications. Briefly, planktonic cell cultures or biofilm resuspensions were centrifuged at 12000 × g for 10 sec and the pellets were immediately frozen in liquid N2 after discarding the supernatant. Approximately 0.3-0.4 g cold beads (Sigma Aldrich, USA) and 2 mL TRI-reagent were added to the pellet, followed by bead beating (FastPrep® -24 Tissue) for 4 min (1 min per time and 20 sec paused on ice). After cell lysis, 400 µL chloroform was added and the lysate was mixed by vortex for 10 sec, followed by incubation at room temperature for 2-3 min. The mixture was centrifuged at 12000 x g for 10 min at 4°C. The upper transparent phase was mixed with 1 mL ice-cold isopropanol and incubated at ambient temperature for 10 min to precipitate the RNA, and centrifuged at 12000 × g for 10 min at 4°C. The pellet was washed with 1 mL cold 75% ethanol and the pellet was air-dried for 5 min. The pellet was suspended in 50 µL RNase-free water (Invitrogen, USA) and stored at -80°C or -20°C depending on specific experiments.
2. The Nucleospin RNA Plus kit (Macherey-Nagel, Germany) was used to extract RNA for real-time quantitative PCR according to the manufacturer's instructions with modifications. Cultures were centrifuged at 12,000 × g for 30 sec at 4°C and the pellets were immediately put on ice. Approximately 0.3-0.4 g ice-cold acid-washed glass beads (Sigma Aldrich, USA) were added to the cell pellets together with lysis buffer in the extraction kit, followed by bead

beating (FastPrep® -24 Tissue) for 4 min (1 min per time and 20 sec paused on ice) to allow the maximum disruption of cells.

The RNA concentration, purity and integrity were checked by RNA Labchip® Assay (Massey Genome Service, Massey University).

3.9.3 PCR-based method

3.9.3.1 Primers

Genomic DNA was extracted as above (section 3. 9. 1). The identification of *B. cereus* group strains was performed using both an end-point PCR method with *B. cereus* specific primers (*motB*: BCFomp2/ BCRomp2) (Oliwa-Stasiak et al., 2011) and universal primers (*16s*: BAC27F/ U1492R) (Jiang et al., 2006) followed by the 16S ribosomal sequencing to identify the *B. cereus* group strains. The PCR products after amplification were purified using DNA Clean & Concentrator™ -5 kit (Zymo research, USA) following the manufacturers' instructions before sending for sequencing. These isolates were then identified as *B. cereus s.l.* based on 16S ribosomal RNA sequencing (Massey Genome Service, Massey University, New Zealand). DNA sequences were aligned and edited using the BioEdit Sequence Alignment Editor and the blast NBCI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Toxin gene profiles in this study were determined using an endpoint PCR assay and toxin gene primers based on research done by Ehling-Schulz et al., (2006b) and Guinebretière et al., (2002). The primers used (Table 3. 5) in this study were bought from Invitrogen™ (Life technologies, New Zealand Limited).

Table 3. 5 Primers used in this study.

Targeted gene	Primer name	Sequence (5'->3')	Fragment size (bp)
motB	BCFomp2 BCRomp2	CGCCTCGTTGGATGACG GATATACATTCACTTGACTAATACCG	285
16S rDNA	BAC27F U1492R	AGAGTTTGGATCMTGGCTCAG CGGTTACCTTGTTACGACTT	600-700
hbl	HD2F HA4R	GTAAATTAIGATGAICAATTTC AGAATAGGCATTCATAGATT	1091
nhe	NA2F NB1R	AAGCIGCTCTTCGIATTC ITIGTTGAAATAAGCTGTGG	766

cytK	CK F2 CK R5	ACAGATATCGGICAAAATGC CAAGTIACTTGACCIGTTGC	421
ces	CesF1 CesR2	GGTGACACATTATCATATAAGGTG GTAAGCGAACCTGTCTGTAACAACA	1271
hblA	HA F HA R	AAG CAA TGG AAT ACA ATG GG AGA ATC TAA ATC ATG CCA CTG C	1154
hblC	HC F HC R	GATAC (T, C) AATGTGGCAACTGC TTGAGACTGCTCG (T, C) TAGTTG	740
hblD	HD F HD R	ACC GGT AAC ACT ATT CAT GC GAG TCC ATA TGC TTA GAT GC	829
nheA	NA F NA R	GTTAGGATCACAATCACCGC ACGAATGTAATTTGAGTCGC	755
nheB	NB F NB R	TTTAGTAGTGGATCTGTACGC TTAATGTTCGTTAATCCTGC	743
nheC	NC F NC R	TGGATTCCAAGATGTAACG ATTACGACTTCTGCTTGTGC	683

3.9.3.2 PCR conditions

Other materials, including Green Hot Start PCR 2X Master Mix (Invitrogen™ Platinum™) and UltraPure™ Distilled Water (Invitrogen, Life Technology, USA) were used for the PCR reactions. The thermocycling conditions for the PCR reaction were dependent on the primers, listed in Table 3. 6. The gel electrophoresis for PCR products was done using the iBase™ E-gel® system (Invitrogen™) using 1.2% SYBR® Safe™ E-gel® (Invitrogen, Thermo Fisher Scientific). A 1 kb DNA ladder (BioLabs, New England) was used to estimate the size of the PCR products.

Table 3. 6 The PCR conditions for each primer used in this study.

Primer	Thermocycling conditions
BCFomp2/BCRomp2	94°C, 5 min → 30 × (94°C, 30 s/ 55°C, 1 min/72°C 1 min) → 72°C, 7 min
BAC27F/ U1492R	94°C, 5 min → 30 × (96°C, 30 s/ 50°C, 45 s/72°C, 2 min) → 72°C, 7 min
HD2F/ HA4R NA2F/ NB1R CK F2/ CK R5 CesF1/ CesR2	95°C, 15 min → 30 × (95°C, 30 s/49°C, 30 s/72°C, 1 min) → 72°C, 2 min
HA F/ HA R	94°C, 2 min → 35 × (94°C, 1 min/56°C, 1 min/72°C, 2 min) → 72°C, 5 min
HC F/ HC R	94°C, 2 min → 35 × (94°C, 1 min/58°C, 1 min/72°C, 2 min) → 72°C, 5 min
HD F/ HD R	94°C, 2 min → 35 × (94°C, 1 min/58°C, 1 min/72°C, 2 min) → 72°C, 5 min
NA F/ NA R	94°C, 2 min → 35 × (94°C, 1 min/56°C, 1 min/72°C, 2 min) → 72°C, 5 min
NB F/ NB R	94°C, 2 min → 35 × (94°C, 1 min/54°C, 1 min/72°C, 2 min) → 72°C, 5 min

NC F/ NC R	94°C, 2 min → 35 × (94°C, 1 min/54°C, 1 min/72°C, 2 min) → 72°C, 5 min

3.9.4 Multi-locus sequencing

DNA of *B. cereus* strains (food isolates and two reference strains) was extracted as described in section 3. 9. 1. Seven house-keeping genes were sequenced as described on the *B. cereus* MLST website (https://pubmlst.org/bcereus/info/primers.shtml). The PCR conditions for amplification used the protocol from Vassileva et al., (2006) and Yoo et al., (2019). Sequencing was done by the Massey Genome Service. DNA sequences were aligned using the BioEdit Sequence Alignment Editor and the allele sequences for all isolates were submitted to the *B. cereus* MLST database (http://pubmlst.org/bcereus) and the corresponding sequencing type (STs) was determined for each isolate.

3.9.5 Whole-genome sequencing

The WGS and its analysis were done by Prof. Ding and Shubo Yu from Guangdong Institute of Microbiology, China. DNA from eight *B. cereus s.l.* food isolates (P2, P4, P5, M1, M2, M3, M4 and M5) was extracted as described in section 3. 9. 1. The purity and concentration of the DNA were determined by NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) and Qubit Fluorometer 3.0 (Thermo Fisher Scientific) with Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific), respectively. The genomic DNA was used for the library construction according to the instructions of the QIAseq FX DNA Library Kit (QIAGEN, USA). 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 from sequencing were first quality trimmed using Trimmomatic v 0.39

software (Bolger et al., 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 and Rosselló-Móra, 2009). The seven house-keeping genes according to the MLST database (http://pubmlst.org/bcereus) were extracted from WGS data to determine the sequencing types (STs), which was also compared to the result from PCR-based MLST (section 3. 9. 4). Furthermore, other genetic information including the presence of some reported biofilm-related and toxin genes within those food isolates were analysed by extracting the data from WGS.

3.9.6 Real-time quantitative PCR

Real-time quantitative PCR (RT-qPCR) was performed using the Light Cycler 480 platform (Roche Diagnostics, USA) to investigate the expression of related genes for both the Hbl and emetic toxins. The Luna® Universal One-step RT-qPCR kit (BioLabs, USA) was used according to the manufacturer's instructions. The primers designed and used in this study are listed in Table 3. 7. The target Hbl toxin gene primers were designed in this study using the PrimerQuest® Tool. The cycling conditions were as follows: reverses transcription of RNA to cDNA at 55°C for 10 min, followed by initial denaturation at 95°C for 1 min; the amplification was performed with 45 cycles of denaturation at 95°C for 10 sec, annealing at specific temperatures depending on the primers used (Table 3. 7) for 20 sec and extension at 60°C for 30 sec.

The specificity of primers was assessed by using the dissociation curve method according to the instrument's recommendation. The relative gene expression analysis was performed applying the $2^{-\Delta\Delta Ct}$ (Livak) method (Livak and Schmittgen, 2001). This method is based on the following formula:

$$-\Delta\Delta Ct = -\left(\Delta Ct_{\,sample}\,(target - reference) - \Delta Ct_{\,calibrator}\,(target - reference)\right)$$

Ct denotes the cycle number of the amplification reaction that exceeds the quantification threshold of the instrument. The planktonic cells were regarded as a calibrator sample for both the Hbl and emetic toxin studies.

Table 3. 7 RT-qPCR primers used in this study.

Primer name	Sequence (5' → 3')	Annealing temperature (°C)	Reference
hblA	Forward: CTACGCAATGGGAGGATTTAG Reverses: GCTGCATTCAAGCTAGGG		This study
hblC	Forward: GTACAGTTAGAGGAAGTCACAG Reverses: CTGTGGATAGAGTTCCGATG	60	This study
hblD	Forward: GAGCAACTTCGTTCTACTCAG Reverses: CTCCTCCAATAGCTGCAATAAC		This study
16S	Forward: GGAGGAAGGTGGGGATGACG Reverses: ATGGTGTGACGGGCGGTGTG		Jeßberger et al., (2015)
cesA	Forward: GATTACGTTCGATTATTTGAAG Reverses: CGTAGTGGCAATTTCGCAT	53	
cesB	Forward: TTAGATGGTATTCTTCACTTGGC Reverses: TTGATACAAATCGCATTCTTATAACC	57	Dommel et al., (2011)
16SA	Forward: GGAGGAAGGTGGGGATGACG Reverses: ATGGTGTGACGGGCGGTGTG	63	

3.9.7 RNA sequencing and transcriptomic data analysis

The planktonic cells and biofilm grown on SS coupon were cultivated as described in sections 3. 2. 1 and 3. 2. 3, and the Trizol method was used for RNA extractions detailed in section 3. 9. 2 method 1. The RNA samples were stored in RNAstable® tube (Biomatrica, USA) to protect samples from degradation at room temperature followed by sending them to the Novogene sequencing facility (Beijing, China) for sequencing. The rRNAs in the total RNA were depleted using the Ribo-Zero rRNA Removal Kit for Gram-Positive Bacteria (Illumina, Singapore) according to the manufacturer's guidelines then strand-specific cDNA libraries were prepared using NEBNext® Ultra Directional RNA Library Prep Kit for Illumina® (Illumina). Libraries were selected for 250-300bp fragment length and sequenced on the NovaSeq 6000 using PE150 reads (Illumina), which produces 150 bp paired-end sequence data.

The obtained sequences were quality checked and filtered using Trimmomatic (Illumina) (Bolger et al., 2014). The trimmed sequence was mapped to the *B. cereus* P5 strain genome (GenBank assembly accession: GCA_012395425.1) sequenced in the previous section 3. 9. 5 using Bowtie2 (version 2.3.4.3) (Langmead et al., 2009). FeatureCounts was used to quantify the number of reads mapped to each gene (Liao et al., 2014). The alignment, trimming sequence and read counting was conducted by Custom Science (New Zealand). Differential gene transcription between two types of cells (biofilm *vs* planktonic) was evaluated using an "RNA-seq: Interactive Differential Expression Analysis" platform (https://viewlytix.shinyapps.io/idea-new/). The thresholds for significant changes in gene transcription were folded changes greater than 2 and a P-value value of 0.05. DAVID (Database for Annotation, Visualization and Integrated Discovery) (Jiao et al., 2012) was used to identify and analyse differentially expressed genes between the two cell populations – biofilm *vs* planktonic. Differentially expressed genes ($P < 0.05$) were aligned against the DAVID database. The Gene Set Enrichment Analysis (GSEA) was also used for KEGG (Kyoto Encyclopaedia of Genes and Genomes) pathway analysis. The GSEA analysis was performed by Custom Science using the Fgsea package in Rstudio® (Korotkevich et al., 2019). P values and FDR values (False discovery rate; also called q value or adjusted P-value) were used to account for multiple hypothesis testing.

3.10 Statistical analysis

All of the experiments used biological triplicates, and cereulide quantification was performed with six biological replicates. All graphs interpreting results were created and analysed by GraphPad Prism 7. The data expressed in the figures/tables in this thesis were generated from the average value of at least 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.

Chapter 4 Phenotypic properties and genotyping analysis of *Bacillus cereus* group isolates from dairy and potato products

4.1 Introduction

The *Bacillus cereus* group, also called *B. cereus sensu lato* (*B. cereus s.l.*) comprise diversus species including *B. cereus* and *Bacillus thuringiensis* (Jensen et al., 2003; Lechner, 2009; Guinebretière et al., 2010). *B. toyonensis* and *B. paranthracis* were recently identified as belonging to *B. cereus s.l.* (Jiménez et al., 2013; Liu et al., 2017). *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 milk pasteurization (72 °C for 15 sec) (Wijnands et al., 2006b; Bartoszewicz et al., 2008; Watterson et al., 2014; Gopal et al., 2015). *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 and cooked mashed potato (Thomas et al., 2002; Rajkovic et al., 2006; Turner et al., 2006; Heini et al., 2018). *B. cereus* can survive the processing of potato products (e.g., heating and dehydration), therefore it is regarded as a significant foodborne pathogen related to processed potato-based foods (Doan and Davidson, 2000; Heini et al., 2018). This study used isolates from milk and potato products.

Biofilm and spore formation are used by *B. cereus* to adapt and survive in the presence of environmental stresses (Flemming et al., 2016), making *B. cereus* difficult to control in food production environments. *B. cereus* is a potential foodborne pathogen that can cause diarrhoea and/or emesis, mainly due to the production of toxins such as haemolytic enterotoxin (Hbl), non-haemolytic enterotoxin (Nhe), cytotoxin K (CytK) and emetic toxin cereulide (Beecher et al., 1995; Granum et al., 1999; Lund et al., 2000; Rajkovic et al., 2008). This study investigated the phenotypic properties (biofilm and sporulation) and genetic information (biofilm-related and toxin genes) within the *B. cereus* group isolates from dairy and potato products.

4.2 Experimental procedures

4.2.1 Bacterial isolates, selection, and culture

Eight food isolates, comprising potato isolates (P2, P4 and P5) and dairy isolates (M1, M2, M3, M4 and M5) were confirmed as *B. cereus* group species based on 16S ribosomal sequencing (Chapter 3, section 3. 9. 3) from 20 presumptive *B. cereus* isolated on MYP plates (Chapter 3, section 3. 1. 1). Two reference strains (ATCC 14579 and F4810/72) were included in this study. The overnight inoculum used was described previously (Chapter 3, section 3. 1. 2) and TSB was used throughout this section.

4.2.2 Genotype analysis of isolates

The DNA of eight *B. cereus* food isolates (P2, P4, P5, M1, M2, M3, M4 and M5) was extracted as described in Chapter 3, section 3. 9. 1. The genetic information of these isolates was analysed as described below:

- PCR-based toxin gene profiling (Chapter 3, section 3. 9. 3) to identify the presence toxin genes in *B. cereus* isolates.
- Multi-locus sequencing (MLST; Chapter 3, section 3. 9. 4) to identify the sequencing type (ST) of isolates.
- Whole-genome sequencing (WGS; Chapter 3, section 3. 9. 5) to confirm *B. cereus* species and compare the differences between isolates at the genome level by using Average Nucleotide Identity analysis (ANI). The presence of biofilm, sporulation, toxin genes and MLST genes were also extracted from WGS data and compared with the PCR-based method above. The WGS and analysis was done by Prof. Ding and Shubo from Guangdong Institute of Microbiology in China.

4.2.3 Biofilm formation on stainless-steel coupons and pellicle formation

The biofilm formation of all ten *B. cereus s.l.* isolates was investigated on stainless-steel (SS) coupons as described in Chapter 3, section 3. 2. 3. Both the crystal violet (CV) assay and biofilm cell enumeration (Chapter 3, section 3. 2. 3) were used to evaluate the biofilm-forming ability of these isolates. Pellicle formation was observed in the well-plate without SS coupon insertion (Chapter 3, section 3. 2. 2). Biofilm formation was grown in either static or shaken at 120 rpm for one, two and three days at 30°C.

4.2.4 Spore formation in biofilm and planktonic populations

The planktonic cultures and biofilms grown on SS coupons were described previously in Chapter 3, sections 3. 2. 1 and 3. 2. 3, respectively. The sporulation percentages in both biofilm and the planktonic populations was assessed and calculated (refer to Chapter 3, section 3. 3).

4.2.5 Statistical analysis

The data expressed in the figures were generated from the average value of three independent biological repetitions and statistical analysis was done using GraphPad Prism 7 (refer to Chapter 3, section 3. 10).

4.3 Results

4.3.1 Bacterial species identification using WGS

The genomes of all eight food isolates were compared with all reference 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 the type strain of *B. paranthracis* and only 91.8% identity with 76% coverage to the

type strains of *B. cereus*, therefore, M2 was assigned as *B. paranthracis.* The results are summarised in Table 4. 1.

Table 4. 1 Species identification of eight *B. cereus* food isolates.

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*

4.3.2 Genetic diversity between isolates based on WGS

4.3.2.1 ANI analysis

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 was calculated (Supplementary data, section 4. 9. 1, Table S4. 1). Based on the coverage, the pairwise genome comparisons of percent identities (ANI metrics) between isolates were analysed as shown in Fig. 4. 1. An ANI above 95% between the two genomes is an indication that the two strains have highly similar genetic backgrounds (Goris et al., 2007). The genome of M2 was very similar to the emetic reference strain *B. cereus* F4810/72 (ANI > 99%), although it was identified as *B. paranthracis*. Isolates P4, M1 and M3 were clustered with ATCC 14579 (ANI > 98%), while P2 and P5 were genetically different from ATCC 14579 (ANI < 97%). The genome of M4 presented 95% similarity (ANI) with M2 and F4810/72 but was shown an ANI below 91% from other tested isolates (M5 was different from all the other isolates (ANI < 91%) and was identified as *B. toyonensis.*

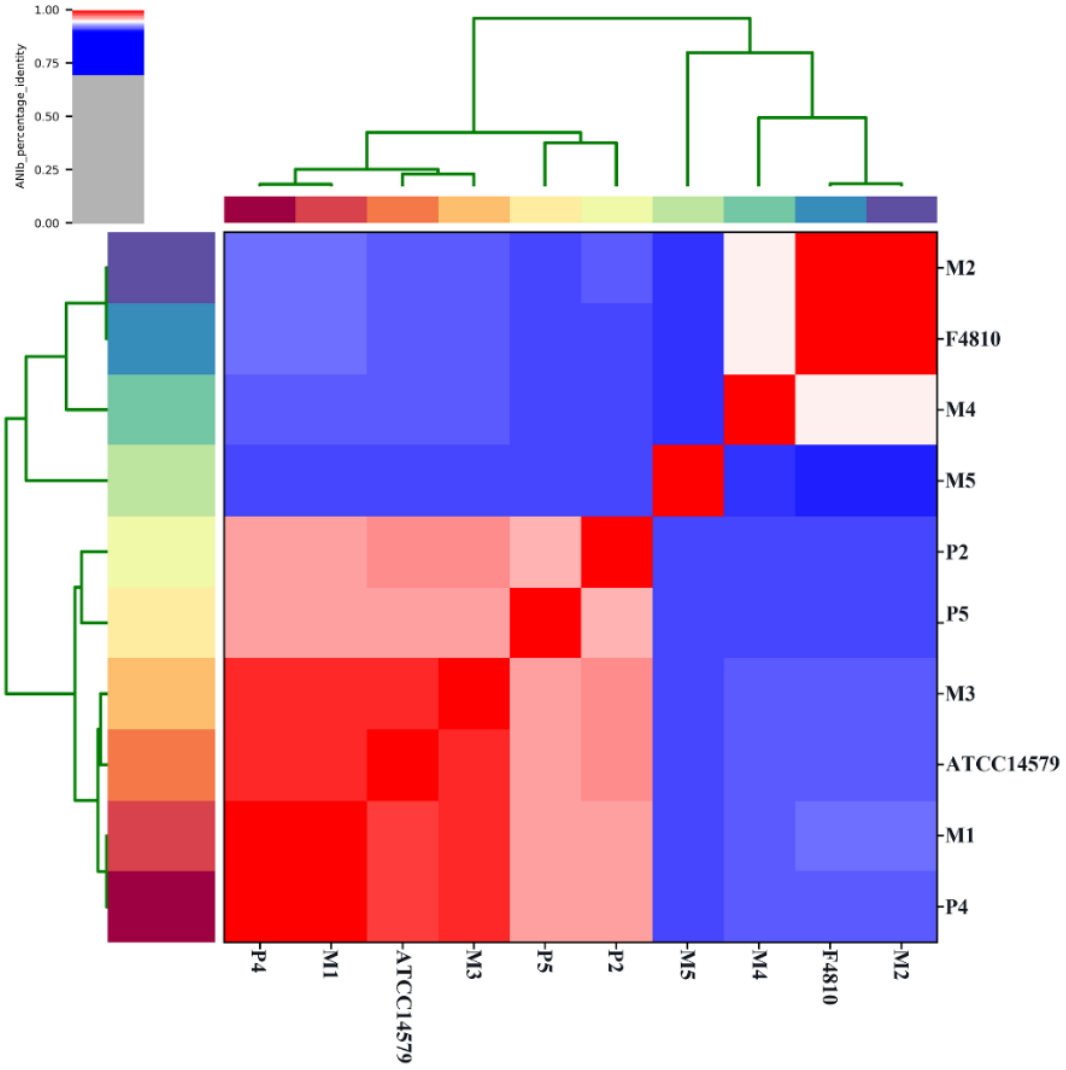


Figure 4. 1 Average nucleotide identity (ANI) among eight *B. cereus s.l.* food isolates and two reference strains. The dendrogram directly reflects the degree of identity between genomes.

4.3.2.2 MLST scheme

To further identify the genetic diversity and sequence type (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 analysed as shown in Table 4. 2. Not all the loci were identified in the isolates based on the MLST database, expressed with "b" in Table 4. 2. For example, there was no exact allelic profile match for the *glp* loci for P2. 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 STs. 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.

WGS data were used to extract sequence data for seven house-keeping genes that are used in the *B. cereus* MLST typing scheme. The loci of the house-keeping genes based on WGS are shown in Table 4. 2. The unmatched results found in WGS compared with MLST are indicated (marked with "c" in Table 4. 2) while other allelic profiles of genes were comparable with the MLST results, therefore, no extra indication is included in the table. The incompletely matched sequences via MLST such as for the loci of *pycA* of P5 and *gmk* for M4 could be identified using WGS. Interestingly, the loci of *pur* of P2 and *ilv* of M2 matched using WGS but showed differences in MLST. From the data extracted from WGS, *B. paranthracis* M2 was assigned to ST-26, which is the same as *B. cereus* F4810/72.

Table 4. 2 The allelic profiles of seven house-keeping genes in the MLST scheme of these isolates are based on MLST and WGS.

Locus	*B. cereus* P2	*B. cereus* P4	*B. cereus* P5	*B. cereus* M1	*B. cereus* M3	*B. cereus* M4	*B. paranthracis* M2	*B. toyonensis* M5	ATCC1579	F4810/72
glp	37[b]	122	93	122	12	65	3	43	13	3
gmk	9	8	6	8	8	1[b]/1[c]	2	26	8	2
ilv	76	8	170[b]	8	9	93	326[b]/31[c]	35	8	31
pta	18	11	9	11	14	1	5	42	11	5
pur	16[b]/12[c]	9	4	9	11	51	16	39	11	16
pycA	14	12	7[b]/7[c]	12	12[b]	52	3	41	12	3
tpi	7	10	21	10	10	24	4	63[b]	7	4
ST[a]	ND	427	ND	427	ND	ND	ND/26[c]	ND	4	26

[a] ST Sequence Types.
[b] allele not found in the *B. cereus* MLST database, shown as the closeted matched alleles.

[c] allele found by extracted data from WGS and show the difference with MLST.
ND Not determined.

4.3.2.3 Biofilm- and toxin-related gene profiling

The existence of biofilm- and toxin-related genes were identified by extracting the data from WGS. Summary information is shown in Table 4. 3. The genes in ATCC 14579 and F4810/72 were identified by extracting from GenBank. Most of the tested food isolates contained a variety of biofilm-regulating genes, while M4, F4810/72, *B. paranthracis* M2 and *B. toyonensis* M5 all lack the *plcR* which is a well-known pleiotropic transcriptional regulator influencing biofilm formation and toxin production for *B. cereus* (Hsueh et al., 2006). Isolates showed various combinations of toxin genes (Table 4. 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 P2 and M4 contained only the *nhe* and *cytK* genes. *B. toyonensis* M5 contained both the *hbl* and *nhe* genes, while *B. paranthracis* M2 contained only the *nhe* gene. The results from the PCR assay using specific toxin gene primers that related to corresponding toxins (Chapter 3, section 3. 9. 3) showed the same results as the data extracted from WGS.

Table 4. 3 The existence of biofilm- and toxin-related genes selected based on current databases.

	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 et al., (2014)
	SipW	+	+	+	+	+	+	+	+	+	+	Matrix protein encoding gene; Signal peptidase I	
Toxin-related gene	*Hbl*	+	-	-	+	+	+	-	+	-	+	Haemolytic toxin	Ehling-Schulz et al., (2006b)
	Nhe	+	+	+	+	+	+	+	+	+	+	Non-haemolytic toxin	
	CytK	+	-	+	+	+	+	-	+	+	-	Cytotoxin	
	Ces	-	+	-	-	-	-	-	-	-	-	Emetic toxin	

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

4.3.3 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 or shaken at 120 rpm, shown in Fig. 4. 2A and Fig. 4. 2B, respectively. The numbers of biofilm cells grown on SS coupons are expressed in log CFU/cm^2 (Fig. 4. 2). In static incubation (Fig. 4. 2A), M1 showed significantly ($P < 0.05$) higher biofilm-forming capacity (>7 log CFU/cm^2) after one-day incubation, however, it showed lower numbers of biofilm cells (less than 5 log CFU/cm^2, $P < 0.05$) after two days compared with all the other isolates. M3 showed significantly ($P < 0.05$) lower biofilm formation at day one (around 3 log CFU/cm^2) but this increased at day two and three (6 log CFU/cm^2). P2 and *B. toyonensis* M5 were strong biofilm-formers, producing approximately 7 log CFU/cm^2 after two days incubation (Fig. 4. 2A). Most of the isolates tested formed stable or increasing of numbers of biofilm cells after three days in static incubation, except for M1 and M2, which showed decreased numbers of biofilm cells after two and three days respectively compared to day one (Fig. 4. 2A). The two reference isolates showed either comparable or slightly lower biofilm numbers than the food isolates in a static condition (Fig. 4. 2A).

In shaking (120 rpm) conditions (Fig. 4. 2B), P5 was the strongest biofilm-former ($P < 0.05$) at days one, two and three, producing approximately 7.5 log CFU/cm^2 of biofilm cells on the SS coupons after one day incubation. P2 and *B. toyonensis* M5 also produced significantly ($P < 0.05$) higher biofilm cells (7 log CFU/cm^2) compared to other isolates tested after two days in the shaking incubation. The statistical comparison of biofilm formation in static and shaking conditions was expressed as "H" in Fig. 4. 2B indicating significantly higher ($P < 0.05$) cell numbers in shaking compared to static incubation. It showed that either comparable or significantly higher ($P < 0.05$) numbers of biofilm cells formed in shaking condition at the early time points (one- or two-day incubation). However, there was a difference between the shaken and static biofilms after three days incubation, in that shaken coupons tended to show some release of biofilm while the static coupons contained stable amounts of biofilm cells over the three days of incubation. Isolates P2 and M4 differed from this trend (Fig. 4. 2B).

The biofilm biomass on SS coupon was stained by CV and quantified by measuring OD_{595nm} (Fig. 4. 3) in either static or shaken at 120 rpm. The P2 isolate showed significantly ($P < 0.05$) higher biofilm biomass than other isolates at day one and three in static conditions (Fig. 4. 3A) and P5 was a significantly ($P < 0.05$) higher biofilm former ($OD_{595nm} > 1.5$) after two days of shaken incubation based on the CV assay (Fig. 4. 3B). These results are comparable with the cell counts in Figs 4. 2A and 4. 2B. While the M5 that formed significantly ($P < 0.05$) higher numbers of biofilm cells among all tested isolates (Fig. 4. 2A), did not show higher biomass compared to other isolates based on CV assay (Fig. 4. 3A) in static incubation, suggesting the numbers of biofilm cells based on cell counting and biomass based on CV assay are not always correlated.

Similarly, ATCC 14579, P5 and M1 which showed significantly higher ($P < 0.05$) biofilm cells with shaken, also showed significantly ($P < 0.05$) higher biomass in the CV assay (Fig. 4. 2B and 4. 3B), compared to static incubation. However, for isolates P2, P4 and M2, the number of cells within the biofilms did not correlate with the results from CV staining when comparing static and shaken incubation. For example, P2 showed significantly higher (day two, $P < 0.05$) or lower (day one and three, $P < 0.05$) biomass based on the CV assay (Fig. 4. 3) in shaken compared to static incubation, however, the numbers of biofilm cells were comparable between two the incubations (Fig. 4. 2). Furthermore, the stability of the biofilms was observed differently using the two methods. For example, F4810/72 showed stable numbers of biofilm cells over the three days of incubation (Fig. 4. 2A) while the biomass decreased over time (Fig. 4. 3A). However, most of the isolates showed reduced amounts of total biomass overtime under shaken incubation (Fig. 4. 3B) which is in line with the results for the numbers of culturable biofilm cells (Fig. 4. 2B).

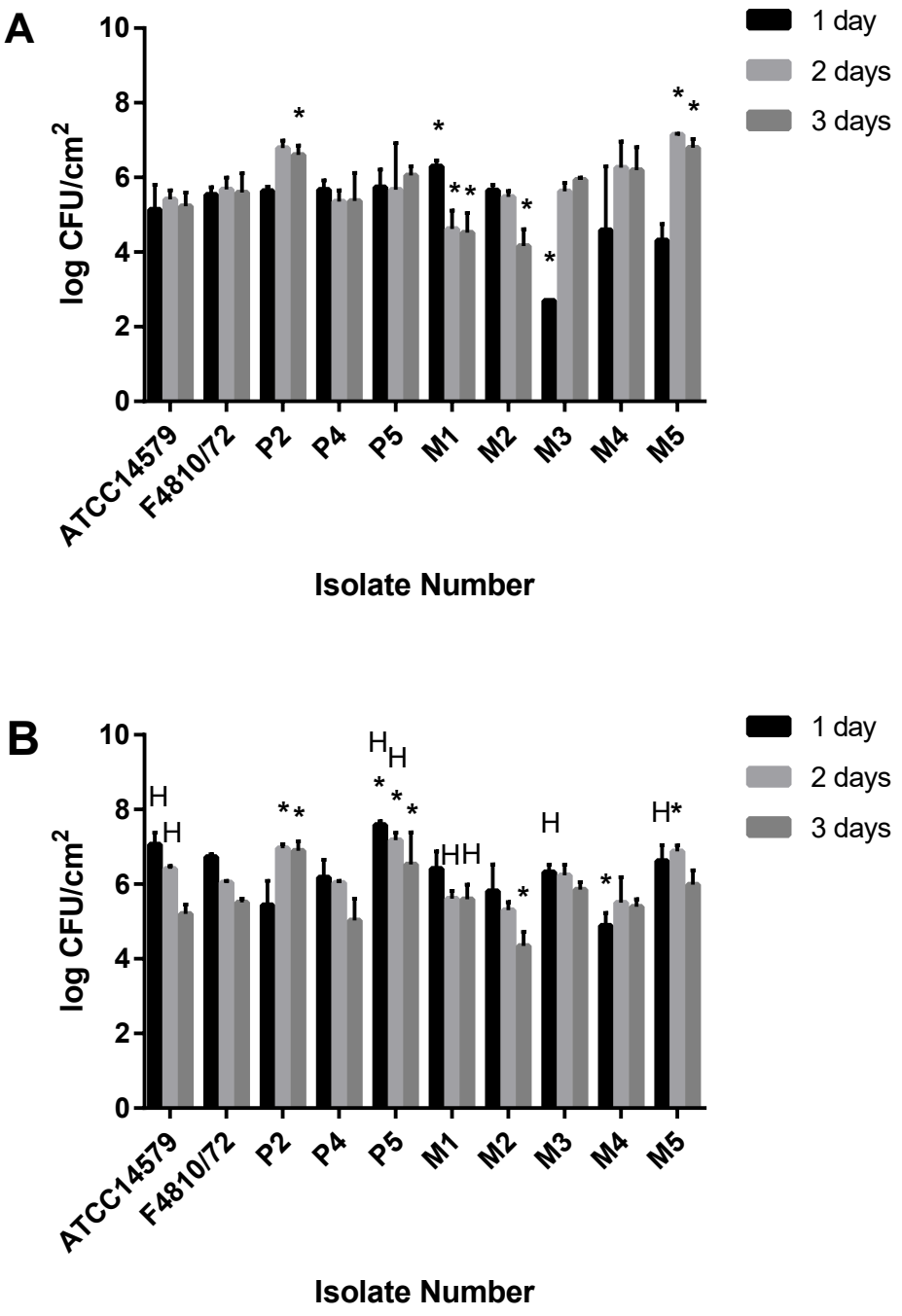


Figure 4. 2 Biofilm cells of ten *B. cereus s.l.* isolates in static (A) or 120 rpm shaking (B) incubation at 30°C. The numbers of culturable cells based on plating method. The growth of biofilms was counted after one-, two- and three-days incubation. Biofilms were grown on SS coupons vertically inserted into a 48-well plate filled with TSB. Each column indicates the average number within three 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 is expressed with "H" in Fig. B, meaning significantly higher cell counts in shaken compared with static incubation.

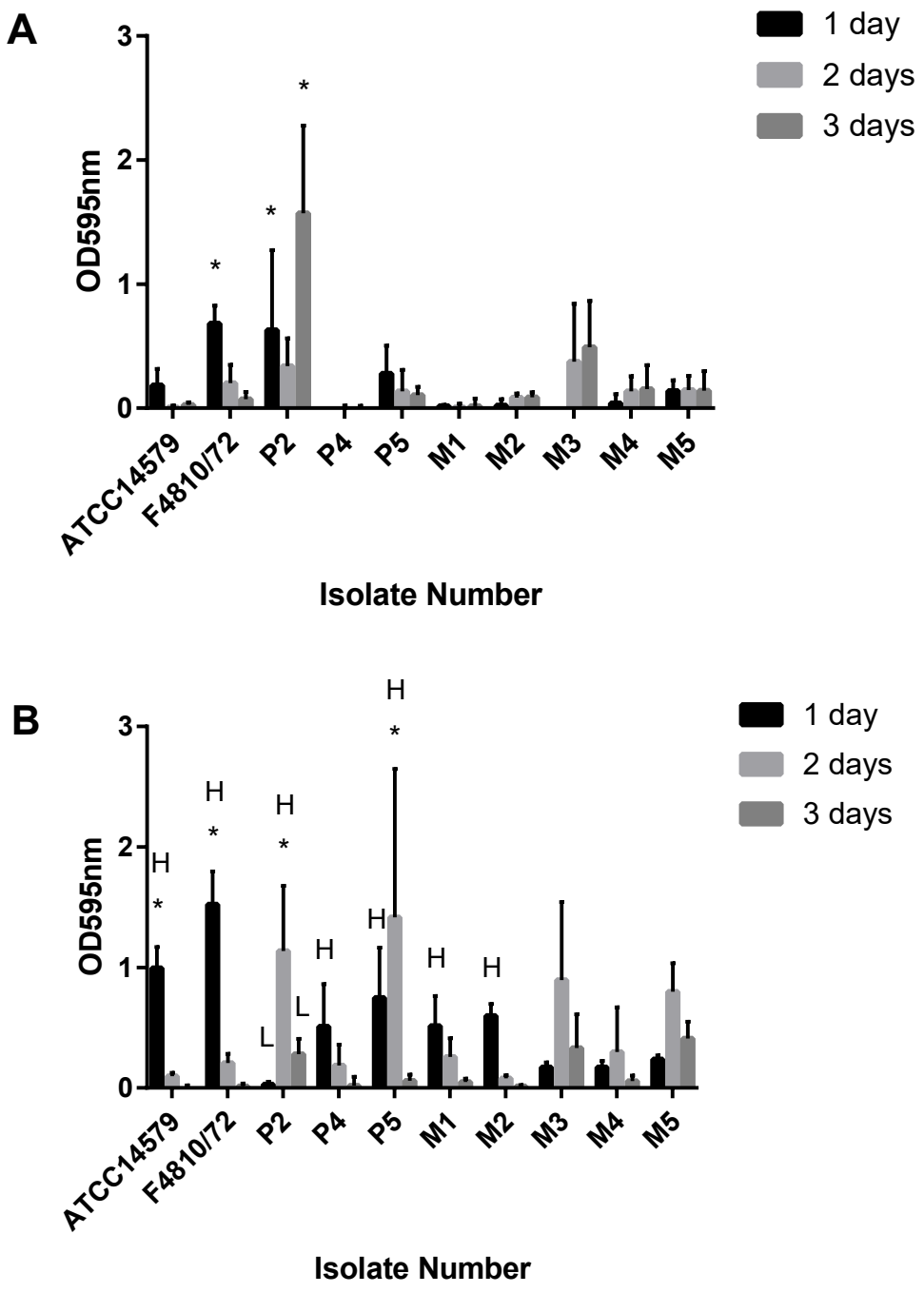


Figure 4. 3 Biofilm biomass quantification of ten *B. cereus s.l.* isolates in static (A) or 120 rpm shaking (B) incubation at 30°C using the CV assay. The biomass was measured after one-, two- and three-days incubation. Biofilms were grown on SS coupons vertically inserted into a 48-well plate filled with TSB. Each column indicates the average number within three biological replicates and error bars represent standard deviation. The symbol "*" 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 is expressed with "H" in Fig. B, meaning significantly higher biomass in shaking compared with static incubation.

4.3.4 Pellicle formation in static and shaking incubation

Pellicles formed by *B. cereus s.l.* isolates were formed in 48-well plates and pictures of two representative wells were taken (Fig. 4. 4). In static incubation (Fig. 4. 4 left), most of the isolates started to produce visible pellicle after two days incubation, except for *B. paranthracis* M2 which showed visible pellicle at day one. Interestingly, P2 did not show strong pellicle formation in static incubation even after three days incubation (Fig. 4. 4 left), which is contradictory to the results obtained from biofilm formed on SS coupons in that P2 was one of the strongest biofilm formers under the same conditions (Fig. 4. 2A). This shows that the ability to form pellicle is not necessarily related to biofilm formation on a solid surface. When incubating plates under 120 rpm shaking, all isolates showed visibly higher and/or earlier pellicle formation compared with static incubation, which was comparable with biofilms formed on SS coupons described previously. Most of the isolates started to produce pellicles from day one in shaking incubation, especially for P5 and *B. paranthracis* M2. P5 was a significantly ($P < 0.05$) higher biofilm former on SS coupons in the same condition (Fig. 4. 2B). The location and status of pellicle formation varied between isolates. For example, P5 formed a complete piece of pellicle in the air-liquid interface under shaking incubation (Fig. 4. 4 right), while other strains such as ATCC 14579 and F4810/72 formed fragmented pellicles floating within the medium. Like the SS coupon where biofilm was partially released, the pellicles also disintegrated after three days incubation.

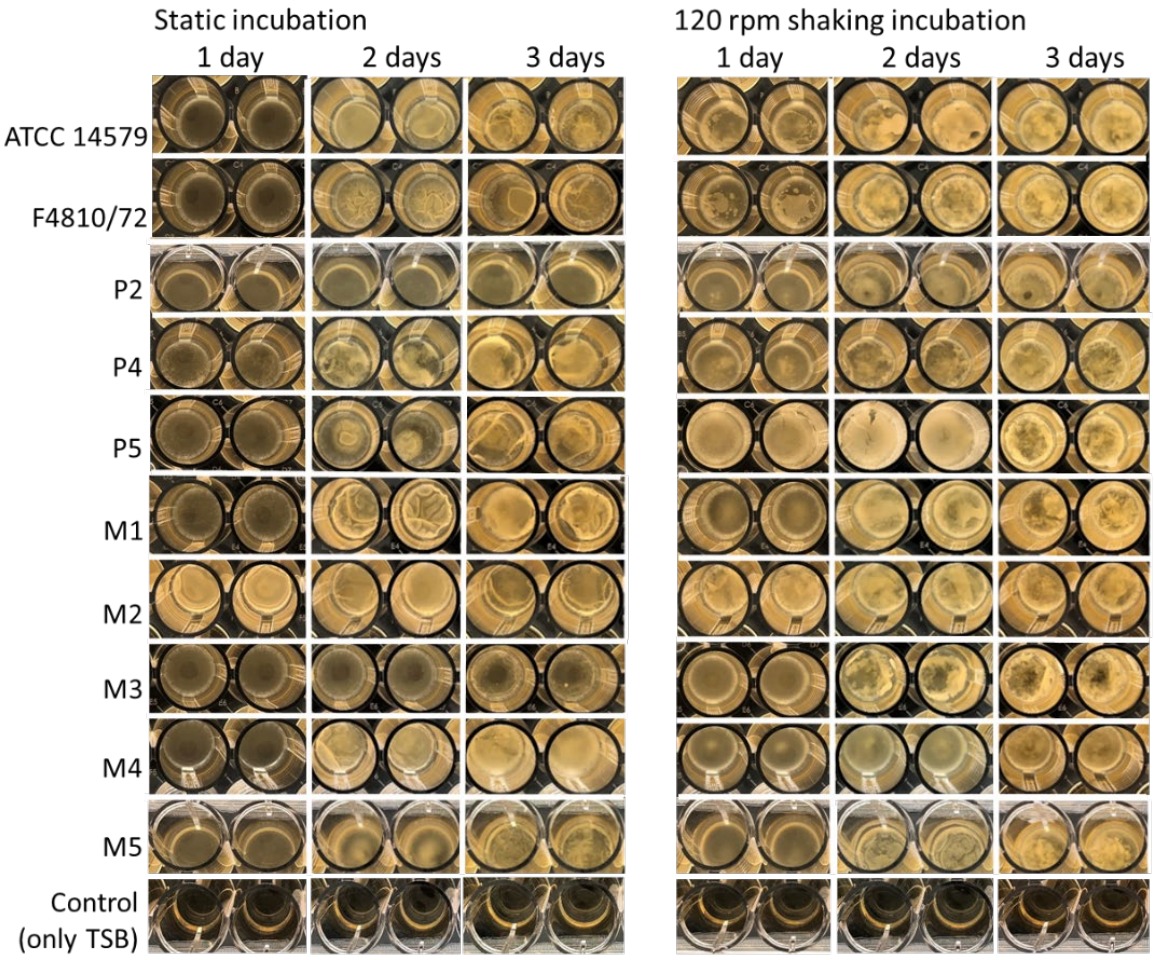


Figure 4. 4 The floating pellicle of ten *B. cereus s.l.* isolates in TSB. Pellicle was grown in a 48-well plate filled with TSB and incubated at 30°C for one, two and three days in static (left) and shaken at 120 rpm (right). Pellicle formation of each isolate in each condition was performed with three biological repetitions, although only two representative wells are shown here. Negative controlled wells with only TSB are also shown in the last row.

4.3.5 The sporulation within planktonic or biofilm populations

The sporulation percentage in planktonic and biofilm communities was calculated based on the spore counts divided by the total cell counts. This is because the numbers of total viable cells of planktonic and biofilm cultures are different therefore it is hard to compare the spore-forming ability of these two types of cells with only spore 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. The original plate counting results of planktonic total cells, spores isolated from planktonic

culture, biofilm total cells and spores isolated from biofilms over three days incubation are included in the Supplementary file (section 4. 9. 2, Fig S4. 1 - 3).

The sporulation percentages of planktonic cultures were below 1% for all isolates at day 3 (Fig. 4. 5). 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, an overall trend of higher sporulation percentage was observed within biofilms grown in both static and 120 rpm shaking incubation, although the differences were not statistically significant ($P < 0.05$) for all isolates. The sporulation of the *B. toyonensis* M5 was low compared with other isolates after three days of incubation, for both planktonic and biofilm cultures. *B. paranthracis* M2 showed significantly higher ($P < 0.05$) sporulation within biofilms compared to planktonic populations, representing over 50% of spores within the total biofilm cells at days 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 ($P < 0.05$) higher sporulation percentages in biofilms compared to planktonic cultures. Most of the isolates reached significantly ($P < 0.05$) higher or comparable amounts of spores when placed in shaking compared with the static incubation, including F4810/72 (both day two and three), M2 and M3 at day two, and P4, M1 and M4 at day three (Fig. 4. 5).

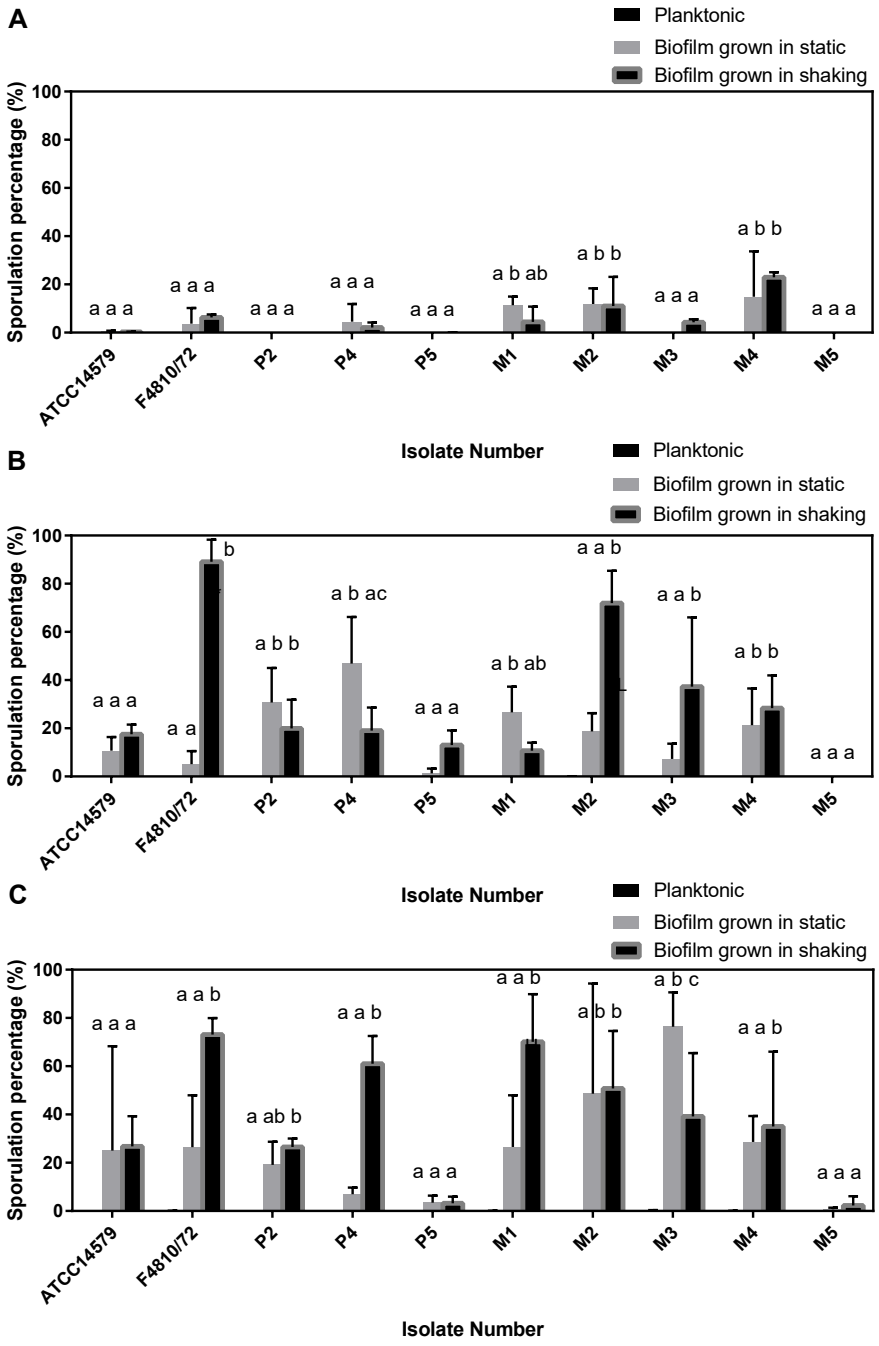


Figure 4. 5 The sporulation percentages of ten *B. cereus s.l.* isolates in planktonic culture, and biofilm grown in static and in 120 rpm incubation at 30°C after one day (A), two days (B) and three days (C) incubation. Biofilms were grown on SS coupons vertically inserted into a 48-well plate filled with TSB, while planktonic culture was grown in a shaken flask filled with 50 mL TSB. The sporulation percentages were calculated by (spore numbers / total cell numbers × 100) %. Each column represents the average number within three biological replicates and error bars represent standard deviation. To compare the sporulation percentages of the planktonic population, biofilm grown in static and shaking condition for each isolate, Turkey's multiple comparison test was performed. Different letters within each condition indicate a significant difference ($p < 0.05$).

4.4 Discussion

There is an extensive nucleotide diversity among *B. cereus* group strains therefore there is potential to find previously unidentified sequence types (STs) (Carter et al., 2018). The genetic diversity was shown within the isolates tested in this study based on MLST and WGS, and many of them (including P2, P5, M3, M4 and M5) have not previously been 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 as dairy isolates (M2 and M5, respectively). 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 existed in New Zealand but under the name of *B. cereus*, as these two species have been described recently (Jiménez et al., 2013; Liu et al., 2017). 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). Carter et al., (2018) concluded that the seven-loci MLST is inadequate and sometimes incorrect to reveal the diversity of *B. cereus*. It should be noted that the Platinum™ Green Hot Start PCR Master Mix used to conduct the PCR assay for MLST contained *Taq* DNA polymerase, which is not a high-fidelity polymerase and may cause errors in the PCR (McInerney et al., 2014). This may be the reason why different results were observed for MLST from the PCR-based assay and WGS. WGS is still the preferred and most comprehensive method to determine the genetic sequence and diversity of bacteria (Carter et al., 2018). However, isolates that share highly similar genomes, could have different biofilm-forming and sporulation ability.

In this study, the results of toxin gene profiling were the same from the end-point PCR assay and WGS, suggesting that the PCR assay with specific toxin primers is still an effective method to determine the presence of toxin genes in *B. cereus* strains. Most of tested *B. cereus s.l.* isolates showed the presence of biofilm-related genes (Table 4. 3), however, with exceptions. *PlcR* is a pleiotropic transcriptional regulator involved in biofilm formation and a virulence factor (Hsueh et al., 2006; Ramarao and Lereclus, 2006). F4810/72, M4, *B. paranthracis* M2 and *B. toyonensis* M5 all

lack the *plcR* gene, however, the biofilm formation (both cell number and CV staining) of these isolates was not affected. For example, M5 showed significantly ($P < 0.05$) higher biofilm cells compared to other isolates (Fig. 4. 2). Although the biofilm-related genes existed in the genomes of isolates, the actual expression level of these genes is unknown.

The *B. cereus s.l.* isolates used in this study showed a variety of combinations of toxin genes, fitting the expectation that toxin gene prevalence and distribution among the *B. cereus* group strains varies greatly (Carter et al., 2018). Wijnands et al., (2006a) and Saleh-Lakha et al., (2017) showed that the dairy isolates contained various combinations of enterotoxin genes. All the food isolates in this study contained the *nhe* genes while only some isolates contained *hbl* and *cytK* genes (Table 4. 3), which agrees with previous studies done by Wijnands et al., (2006a) and Park et al., (2009) indicating the strain-dependence of *hbl* and *cytK*. None of the food isolates in this study contained the emetic toxin-related *ces* gene, as the presence of emetic *B. cereus* strains is rare (Agata et al., 2002; Altayar and Sutherland, 2006). This result could be due to the limited numbers of isolates tested in this study. The potato *B. cereus* isolates, P4 and P5, carried enterotoxin-related genes including *hbl*, *nhe* and *cytK*, suggesting a possible safety risk related to *B. cereus* in potato-based food. *B. paranthracis* and *B. toyonensis* isolates found in this study also showed the presence of *hbl* and *nhe* toxin genes. *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 Abdulmawjood et al., (2019) showed that the enterotoxin levels in *B. toyonensis* were much lower than *B. cereus* strains and in some cases, even absent. The presence of toxin genes only means the potential for toxin production, as Kovac et al., (2016) indicated that the presence of *hblACD* and *nheABC* is not enough to produce detectable levels of 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 the SS-liquid-air interface and floating pellicles varies between isolates, which is in line with Hussain and Oh (2017) indicating the biofilm formation

is influenced by the source of *B. cereus* strains. In this study, P2, P5 and *B. toyonensis* M5 isolates produced significantly ($P < 0.05$) higher amounts of biofilm on SS coupons than other isolates, and P5 and *B. paranthracis* M2 isolates were strong pellicle formers in TSB. This is the first study showing the biofilm formation of *B. toyonensis* and *B. paranthracis*, indicating the potential biofilm-forming ability of these two species. Interestingly, P2 that can produce significantly ($P < 0.05$) higher amounts biofilm than other isolates on SS coupons, was not necessarily a strong pellicle former in the same condition (Fig. 4. 2 – 4. 4), which suggests that the mechanism behind pellicle formation of *B. cereus* strains could be different from other types of biofilms. Okshevsky et al., (2018) concluded that the genes required for submerged biofilm and pellicle formation are different. Pellicles can also be regarded as one type of "air-liquid biofilm", although it is a floating structure and lacks a solid surface for attachment (Branda et al., 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 pellicle studies focus on visualization (Bridier et al., 2011; Chabane et al., 2014; Gao et al., 2015). Ostrov et al., (2019) showed that dairy-associated *Bacillus* strains produced higher amounts of submerged biofilm and pellicles than non-dairy isolates based on visualization under confocal laser scanning microscopy. This was also observed in this study where dairy-related *B. cereus s.l.* isolates could not only form strong biofilm on SS but formed strong pellicle, suggesting that food safety issues caused by *B. cereus* in the dairy industry may not only focus on the contamination on the SS processing line but pellicle formation in tanks and other similar environments may also be important. The isolation of *B. cereus* cells from tank milk has been reported (Svensson et al., 2004; Vissers et al., 2007). Unlike well-studied dairy-related *B. cereus*, the biofilm formation of *B. cereus* potato isolates has not been reported. *B. cereus* isolates such as P2 and P5 from potato products produced significantly ($P < 0.05$) higher amounts of biofilms than other isolates both in biofilm cell and biomass. This was especially true for the P5 isolate, that not only produced a significantly ($P < 0.05$) higher biofilm on SS coupons (Fig. 4. 2 and 4. 3), but also produced higher amounts of floating pellicles (Fig. 4. 4) in shaking incubation.

Hussain and Oh (2017) indicated that the amount of biomass quantified by CV assay was highly correlated with number of biofilm cells at 24 h but the correlation decreased after 72 h incubation and may be caused by the formation of extracellular polymeric substance (EPS) after a longer period time of incubation, as CV can bind to both biofilm cells and EPS. In this study, a lack of correlation between biomass and cell numbers was observed for F4810/72 showing a stable number of cells while the biomass decreased over three days incubation (Fig. 4. 2A and 4. 3A). The possible composition and function of EPS has been discussed and reviewed by Flemming (2016), suggesting a possible enzyme production by *B. cereus* F4810/72 and therefore breakdown of the EPS. This may be interesting to investigate in the future.

The sporulation of *B. cereus s.l.* isolates in this study varied from below 0.001% to 1% in planktonic populations after three days incubation, which agrees with Wijman et al., (2007) and Faille et al., (2014) who reported that sporulation is a strain-dependent characteristic for *B. cereus*. In this study, the overall trend of higher sporulation within biofilms than planktonic cells, fits the hypothesis that biofilms could be a better reservoir for spores compared with planktonic cultures (Burgess et al., 2014; Hayrapetyan et al., 2015a). This may be explained by the high cell density reached in biofilm resulting in nutrient or oxygen limitation favouring sporulation for survival (van Gestel et al., 2012; Hayrapetyan et al., 2016). Spores within biofilms of *B. cereus* can be released and form new biofilms, causing recontamination in the food processing line (Wijman et al., 2007), therefore increasing the risk caused of *B. cereus* spore contamination of food products. This study found that isolates considered as strong spore formers in a biofilm may not necessarily be good spore formers in the planktonic state, suggesting that different mechanisms may exist for the regulation of sporulation within the biofilm and planktonic states. Intertwined regulatory pathways of sporulation and biofilm formation have been proposed for *B. subtilis* (Vlamakis et al., 2013), however, this remains to be determined for *B. cereus*.

In this study, shaking seems to be a better condition for biofilm growth and biofilm reaches peak yield earlier compared to static incubation, both at the SS-liquid-air interface or as pellicle. Hayrapetyan et

al., (2015a) and Wijman et al., (2007) indicated that *B. cereus* produced the strongest biofilm around the air-liquid interface which could be due to the aerotaxis (migration towards oxygen) of *B. cereus* (Daniel et al., 1984). It is hypothesised that shaking could increase the availability of oxygen therefore help *B. cereus* to form more biofilm in the air-liquid interface and more intensive/efficient nutrient exchange during shaking supporting biofilm formation (Oosthuizen et al., 2002; Moreira et al., 2013). Motility has been identified as an important factor to form strong biofilms especially in the air-liquid interface biofilms for *B. cereus* (Houry et al., 2010), while shaking could provide additional movement of *B. cereus* cells enhancing the interaction with a surface and accelerating nutrient transfer and distribution. 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 et al., 2014). However, to our knowledge, this is the first report comparing the biofilm formation in static and shaking incubation for *B. cereus*.

4.5 Conclusions

Dairy and potato products are sources of *B. cereus s.l.*. *B. toyonensis* and *B. paranthracis* were reported from dairy products in the New Zealand market for the first time, showing biofilm and spore forming ability and the presence of enterotoxin genes. Potato isolates were strong biofilm formers in this study, 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 the first study to compare the biofilm formation of *B. cereus* including pellicle formation in static and shaken incubation suggesting that food processing conditions with shaking 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 diversus at the genetic level. Genetically closely related *B. cereus s.l.* isolates may display significant differences in biofilm and sporulation ability. This section provides basic phenotypic information of

selected *B. cereus* isolates and those isolates confirmed as *B. cereus* species (P2, P4, P5, M1, M3 and M4) were used in the following sections.

4.6 Accession numbers

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).

4.7 Acknowledgement

We would like to express our special thanks to Prof. Ding and Shubo from Guangdong Institute of Microbiology, China, for helping us with the whole genome sequencing.

4.8 Copyright information

Parts of this study have been published in LWT- Food Science and Technology, and the Online Statement of Contribution form is attached in Appendix II.

4.9 Supplementary file

4.9.1 The alignment coverage between isolates

Table S4. 1 The alignment coverage between isolates. The data indicates how long the sequences are relative to each other. "1.00" means 100% coverage.

	F4810/72	ATCC14579	P5	P4	P2	M5	M4	M3	M2	M1
F4810/72	1.00	0.74	0.74	0.75	0.73	0.71	0.77	0.73	0.94	0.77
ATCC14579	0.76	1.00	0.85	0.89	0.85	0.77	0.75	0.90	0.75	0.89
P5	0.70	0.79	1.00	0.80	0.78	0.74	0.69	0.79	0.70	0.79
P4	0.74	0.85	0.84	1.00	0.81	0.74	0.74	0.86	0.73	0.94
P2	0.71	0.80	0.79	0.79	1.00	0.73	0.70	0.80	0.69	0.80
M5	0.66	0.70	0.72	0.70	0.70	1.00	0.66	0.70	0.66	0.70
M4	0.82	0.78	0.78	0.79	0.77	0.76	1.00	0.79	0.82	0.80
M3	0.76	0.90	0.85	0.90	0.85	0.78	0.76	1.00	0.76	0.90
M2	0.92	0.72	0.73	0.73	0.71	0.69	0.76	0.72	1.00	0.75
M1	0.75	0.85	0.81	0.94	0.81	0.74	0.74	0.86	0.74	1.00

4.9.2 Total cells and spores counts in planktonic and biofilm population for ten isolates

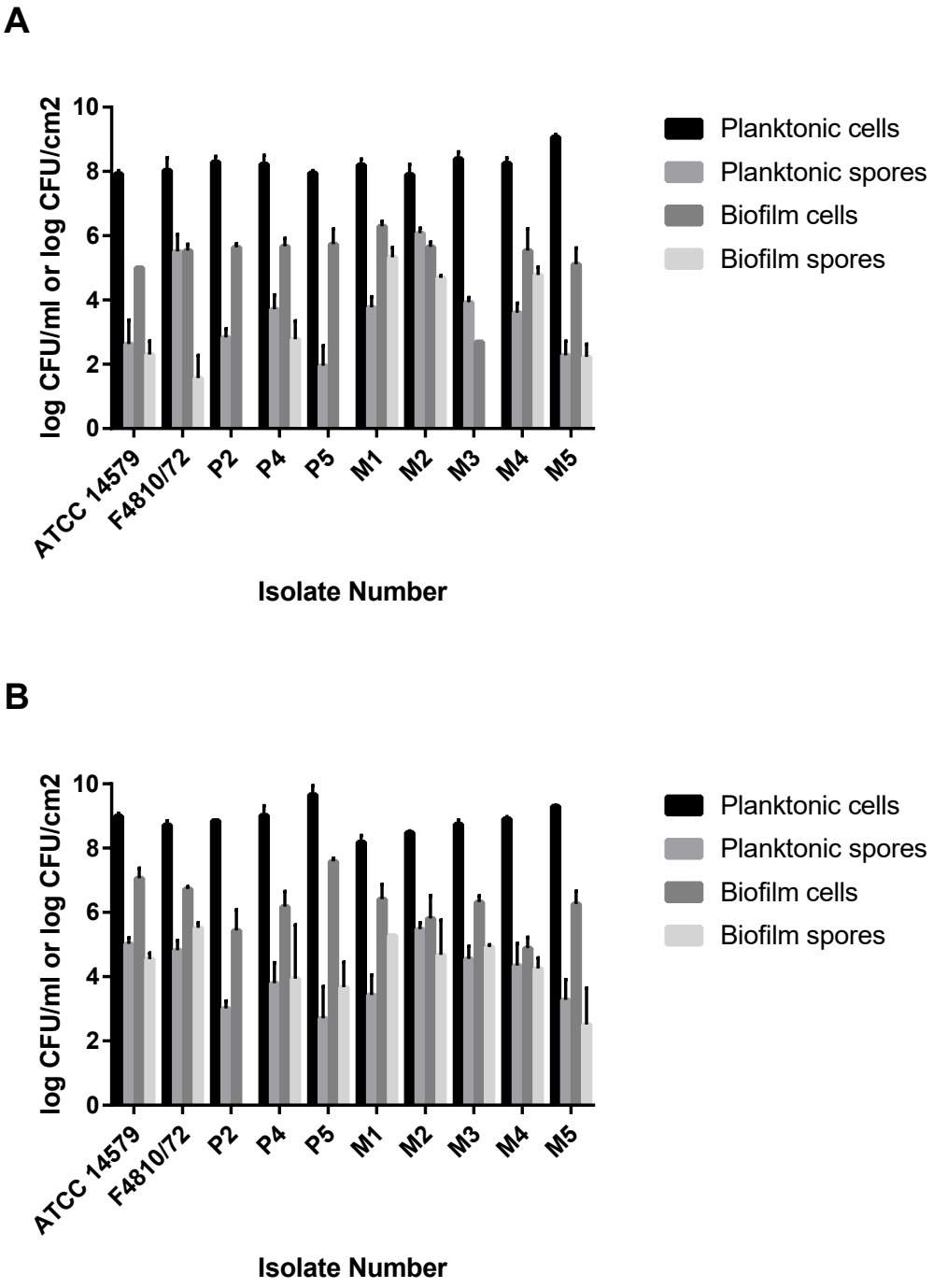


Figure S4. 1 The number of planktonic total cells, planktonic spores, biofilm total cells and biofilm spores after one day in static (A) or 120 rpm shaking (B) incubation at 30°C. Each column indicates the average number within replicates and error bars represent standard deviation.

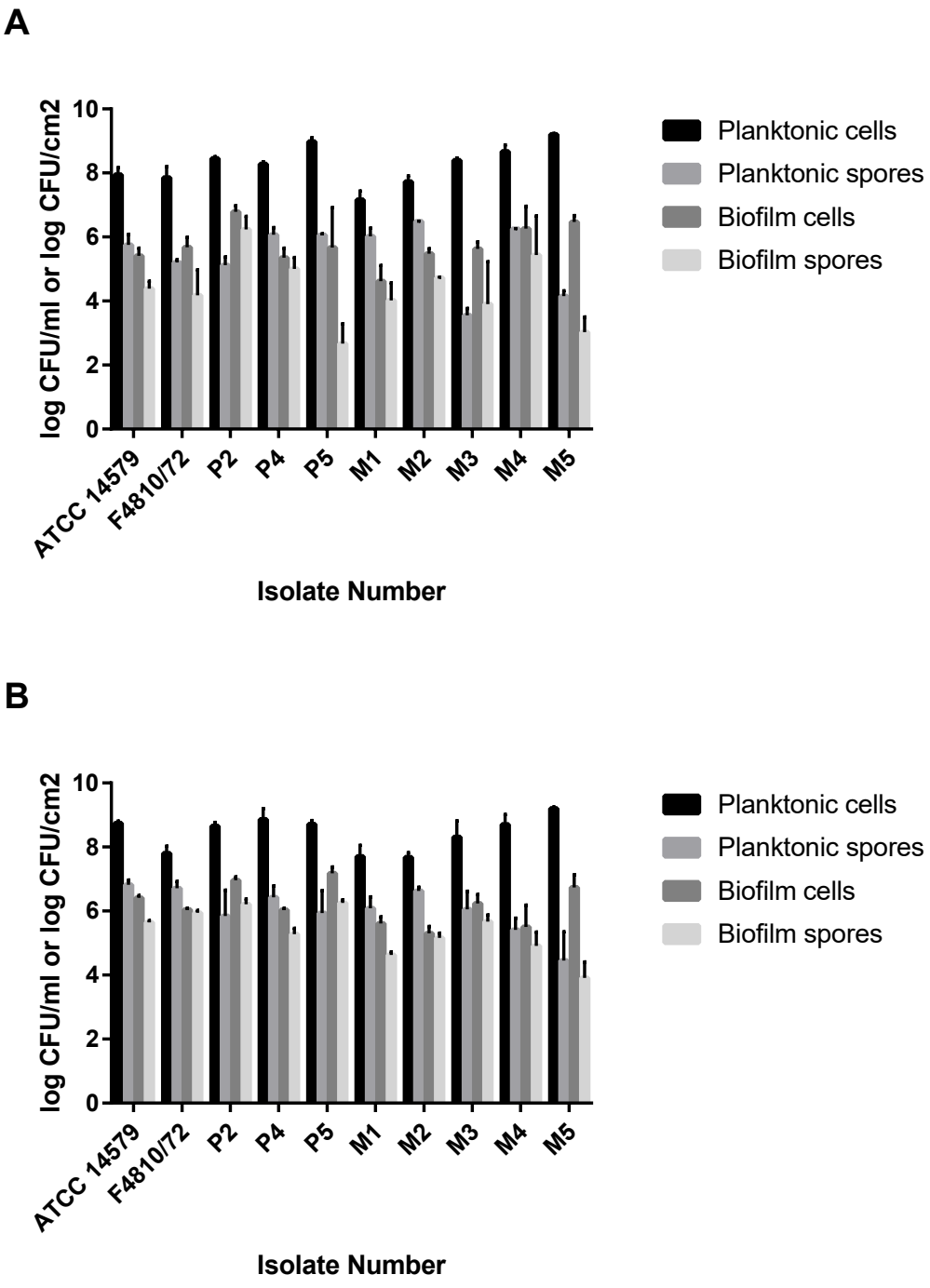


Figure S4. 2 The number of planktonic total cells, planktonic spores, biofilm total cells and biofilm spores after two days in static (A) or 120 rpm shaking (B) incubation at 30°C. Each column indicates the average number within replicates and error bars represent standard deviation.

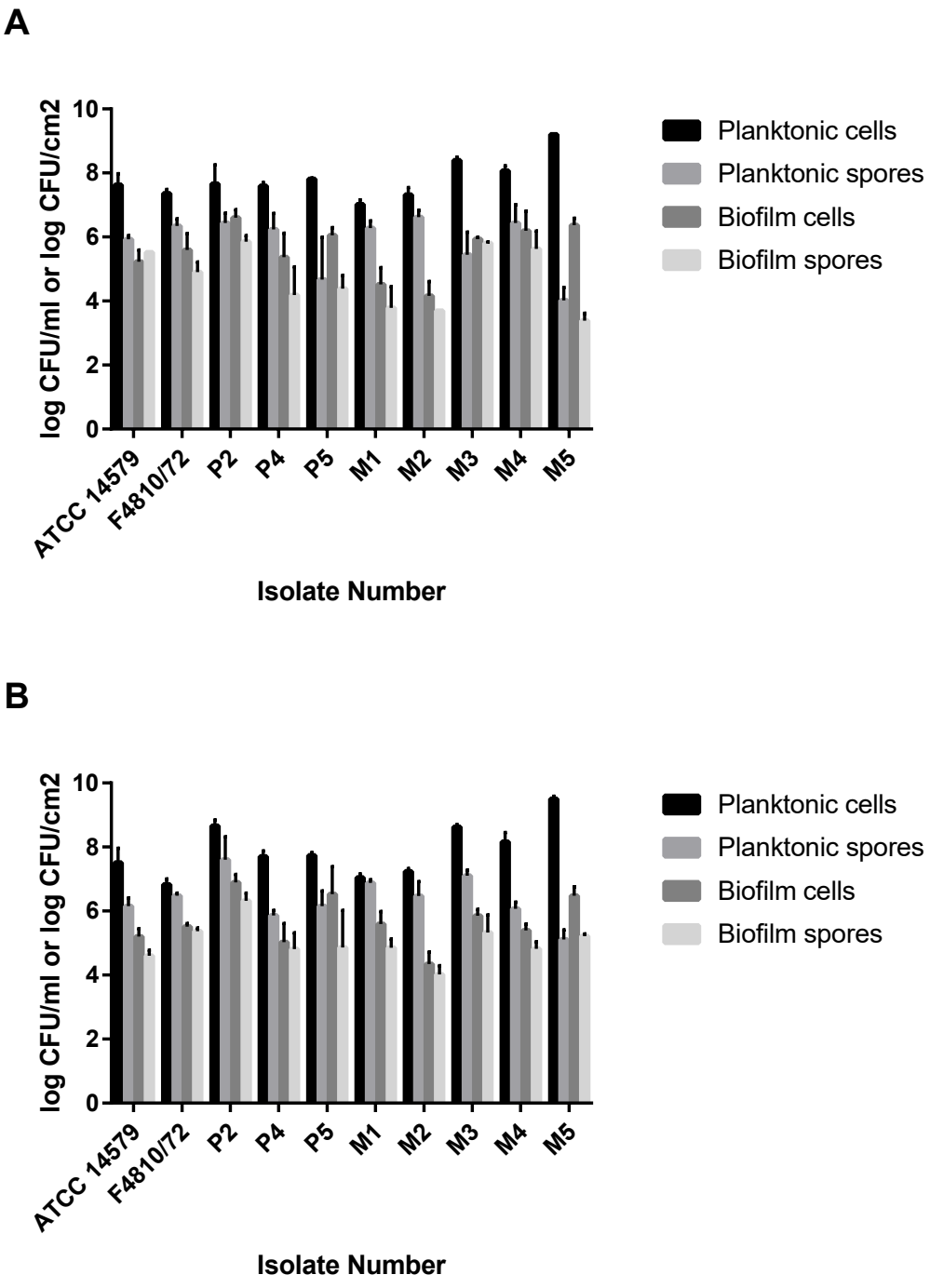


Figure S4. 3 The number of planktonic total cells, planktonic spores, biofilm total cells and biofilm spores after three days in static (A) or 120 rpm shaking (B) incubation at 30°C. Each column indicates the average number within replicates and error bars represent standard deviation

Chapter 5 Transcriptome comparison of biofilm and planktonic cells of *Bacillus cereus* P5

5.1 Introduction

B. cereus is a biofilm-forming bacterium and produces an extracellular polymeric substance (EPS) matrix which contains proteins, carbohydrates and DNA. The EPS provides a protective environment for *B. cereus* and therefore increases the cell's ability to adapt and survive sudden environmental changes compared with planktonic cells (Vilain et al., 2009; Karunakaran and Biggs, 2011; Flemming et al., 2016). Biofilm cells show different physiology compared to planktonic cells and display a special gene-expression pattern which is not observed in free-living cells (Davies, 2003). The physiological changes of *B. cereus* type strain ATCC 14579 to switch the lifestyle from planktonic to biofilm were reported recently, showing 23.5% of the total gene content was expressed differently in the two subpopulations of cells (Joaquín et al., 2019). Joaquín et al., (2019) also showed that biofilm cells of *B. cereus* formed on a polystyrene well-plate utilize strategies such as synthesis of EPS and rearrange metabolism of amino acid to defend itself from competitors. Charlebois et al., (2016) showed that 25.7% of genes were expressed differently with 12.8% genes upregulated in the biofilms of *Clostridium perfringens* (*C. perfringens*) developed on a polystyrene well-plate compared to its counterpart planktonic cells, with upregulation in inorganic ion metabolism and the stress response system. Yao et al., (2005) indicated that 12% of genes were differently expressed with the same amount of genes downregulated and upregulated (~6%) in biofilms of *Staphylococcus epidermidis* (S. *epidermidis*) grown on a polystyrene well-plate and showed less-aggressive, low metabolic activity and longer survival of biofilms compared to its planktonic populations.

Bacterial cells firmly adhere and grow on inert materials, including stainless-steel (SS) commonly used in food processing (Wiencek et al., 1991). SS is a preferred material to use in the food manufacturing equipment due to its resistance to chemicals and its ability to resist corrosion. Wang et al., (2015) showed 1515 genes (618 genes upregulated and 897 genes downregulated) were expressed differently in biofilms of *Salmonella* Typhimurium grown on

a stainless-steel surface compared to its planktonic cells. Wang et al., (2015) indicated the importance of genes related to membrane proteins, transcriptional regulation and stress response in biofilm development of *Salmonella*. However, the transcriptomic comparison between biofilm grown on stainless-steel and planktonic cells of *B. cereus* is unknown. Here, the transcriptomic changes between biofilm developed on SS and planktonic cells of a strong biofilm former, *B. cereus* food isolate P5 (Chapter 4), were determined using RNA sequencing (seq).

5.2 Experimental procedures

5.2.1 Bacterial strain and culture condition

The bacterium used in this study was *B. cereus* potato isolate P5 (Genome sequence accession number: JAAVIR000000000). TSB was used in this study using the culturing methods described in Chapter 3, section 3. 1.

5.2.2 Planktonic and biofilm sampling

Planktonic culture and biofilm grown on SS coupons were described previously (Chapter 3, sections 3. 2. 1 and 3. 2. 3). Both planktonic and biofilm cultures were grown at 30°C for 24 h with 120 rpm shaking. Swabbing was used to collect biofilm cells from 120 SS coupons for each replicate sample (Chapter 3, section 3. 2. 3. 2).

5.2.3 Total RNA isolation

Total RNA was extracted using the TRI-reagent, which is described in Chapter 3, section 3. 9. 2, method 1.

5.2.4 RNA sequencing and transcriptomic data analysis

The RNA sequencing and the analysis was performed as described in Chapter 3, section 3. 9. 7. The sequencing was done by the Novogene sequencing facility (Beijing, China) and the

analysis were conducted with the assistance of the bioinformatician in Custom Science (New Zealand). Gene set enrichment analysis (GSEA) was used to determine if a gene set is correlated with a phenotypic functional group (Subramanian et al., 2005). Briefly, the analysis starts with a defined gene set which encodes a functional group, such as a pathway or Gene Ontology (GO) category. The experimental gene expression data were ranked (L) based on differential expression between two biological samples. The goal of GESA is to determine if the gene set tends to occur in the top, bottom or is randomly distributed in the ranked list (L). The enrichment score (ES) was calculated to reflect the degree of a gene set overrepresented in the top or bottom of the ranked list (L). For example, a positive ES indicates that genes in the defined gene set are mostly at the top of the ranked list. In this study, the ranked list was set from the most highly expressed genes to the lower expressed genes in biofilm cells compared to planktonic cells, therefore, a positive ES means enrichment in biofilm cells. FDR values (False discovery rate; also called q value or adjusted P-value) were used to account for multiple hypothesis testing to determine the statistically significant difference of expression of a gene in two types of cells.

5.3 Results and Discussion

5.3.1 Biofilm formation of *B. cereus* P5 strain

P5 was grown on a SS coupon to obtain approximately 7.5 log CFU/cm^2 biofilm cells (Chapter 4, Fig. 4. 2). Biofilm cells from 120 SS coupons were harvested to achieve cell counts similar to those obtained with planktonic growth (9 log CFU/mL, Chapter 4, Fig. 4. 2). Biofilms were visible on SS coupons after 24 h incubation (Fig. 5. 1), and this time point was chosen for the RNA isolation and subsequent sequencing.

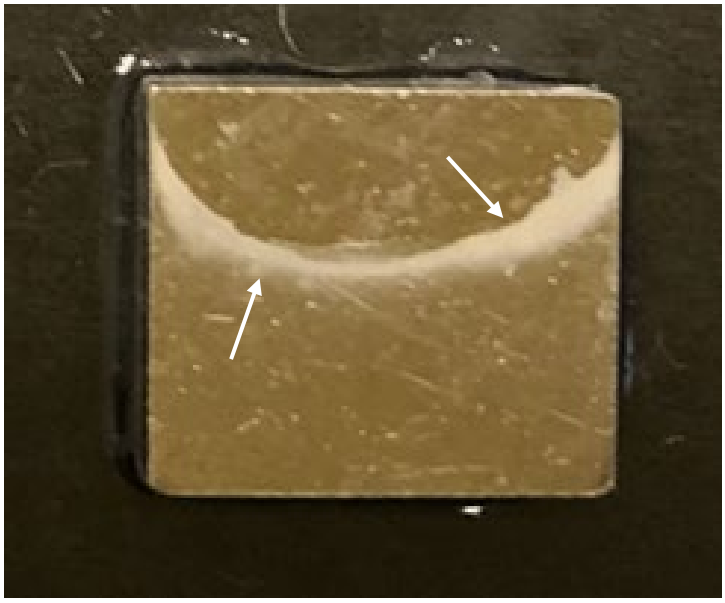


Figure 5. 1 Biomass on a SS coupon formed by P5 after 24 h of incubation. The white arrow indicates the visible biofilm.

5.3.2 Differences in gene transcription levels of biofilm and planktonic cells

A total of 5836 genes were identified after mapping to the P5 genome (RNA-seq mapping statistic is shown in supplementary files, section 5. 7. 1). RNA-seq analysis showed that 2833 genes (48.5% of total gene content) were significantly ($P < 0.05$) differentially expressed in biofilm on SS compared to planktonic cells. The DeSeq2 analysis generated a volcano plot (Fig. 5. 2) that showed the up-regulated (1067 genes) and down-regulated genes (1766 genes) in the biofilm population in comparison to the planktonic population. The larger proportion of the transcriptome was downregulated in the biofilm population, potentially indicating that the biofilm population is metabolically more inert than the planktonic cells. The log2 fold change (Lfc) was used to indicate the difference between two populations throughout the study, and

Lfc < 0" denotes up-regulated genes in the planktonic cells while "Lfc > 0" denotes up-regulated genes in the biofilms.

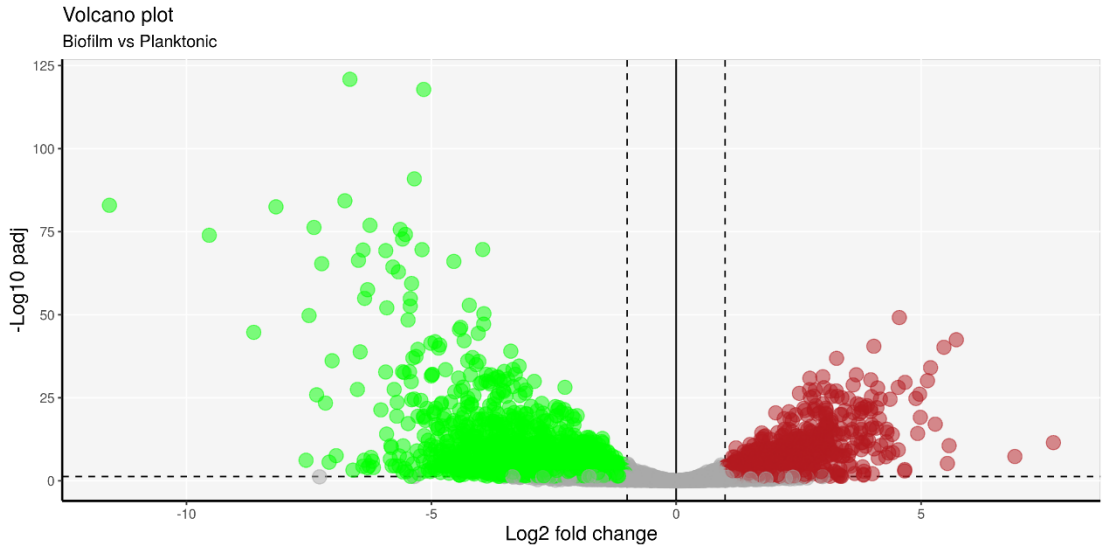


Figure 5. 2 A volcano plot representation of the differential expressed genes in biofilm and planktonic populations. The down-regulated genes (P < 0.05) in the biofilm population are shown as green dots; the up-regulated genes (P < 0.05) in biofilms are shown as red dots; Genes that were not significantly differentially expressed (P > 0.05) genes shown as grey dots.

5.3.3 Genes related to biofilm formation and sporulation

5.3.3.1 Genes related to biofilm formation

A list of biofilm-related genes presented in P5 was extracted from whole-genome sequencing data shown in Chapter 4, Table 4. 3, and the expression levels of those genes from the RNA seq dataset is displayed in Table 5. 1. *Spo0A* is a transcriptional factor required for sporulation and the *spo0A* deletion mutant shows a defect in biofilm formation (Hamon and Lazazzera, 2001). *ComER* also plays a positive role in biofilm formation and sporulation for *B. cereus* (Yan et al., 2016). However, in the present study, the expression of these two genes (*spo0A* (BC_4170) and *comER* (BC_4325)) were significantly downregulated (Lfc = -1.23 and -1.12, respectively; FDR < 0.05) in biofilms compared to planktonic cells (Table 5. 1). This may be explained by the fact that the strain P5 used in this study is not a model spore former with relatively low sporulation efficacy in either planktonic or biofilm populations, and no

significant difference (P > 0.05) was observed in sporulation percentages between the planktonic cells and biofilms of P5 (Chapter 4, Fig. 4. 5). *AbrB* (BC_2444) is another regulator known to play a critical role in the early stage of biofilm development of *B. cereus* and is related to cell mobility enabling cells to search a suitable substratum for expansion (Ma et al., 2017; Park et al., 2019), which was upregulated (Lfc = 4.91) in biofilms (Table 5. 1). This agrees with Ma et al., (2017) who observed the upregulation of *abrB* in 24 h biofilm cells of *B. subtilis*. *SinR* is a master biofilm repressor that directly represses genes related to building biofilm matrix for *B. subtilis* (Yan et al., 2017) and the central role of the *spo0A-sinI-sinR* regulatory circuit in biofilm formation for one *B. cereus* strain (*B. cereus* AR156) was proposed by Xu et al., (2017). A comparable expression of *sinR* (BC_1282, Lfc = 0.54, FDR > 0.05) between biofilm and planktonic cells was observed for the P5 isolate (Table 5. 1), while the anti-repressor *sinI* (BC_1283), which counteracts the effect of *sinR*, was significantly upregulated (Lfc = 0.51; FDR < 0.05) in biofilm cells (Bai et al., 1993). A similar observation was shown by Ikram et al., (2019) who reported a significant increase in the expression of *sinI* in biofilm cells of *B. cereus* while the expression of *sinR* was comparable between planktonic and biofilm cells using quantitative PCR. Gao et al., (2015) showed that a mutation in the *sinI* gene was defective in pellicle formation for *B. cereus*. Notably, P5 was a strong pellicle former (Chapter 4, Fig 4. 4), suggesting the dominant role of *sinI* in biofilm matrix development for P5 and may be involved in pellicle formation. Contradictorily, the upregulation of both *sinR* and *sinI* was observed in biofilm grown on a polystyrene surface by *B. cereus* ATCC 14579 (Joaquín Caro-Astorga et al., 2019), suggesting the role of *sinR-sinI* in biofilm regulation may depend on strain variability and biofilm substrates. This needs to be confirmed in the future.

Table 5. 1 A list of genes related to biofilm formation, which were differentially expressed in biofilm *versus* planktonic cells of P5.

Gene_ id	Protein	Biofilm counts	Planktonic counts	Lfc[a]	FDR[b]
BC_4170	Biofilm regulator; Sporulation transcription factor (*spo0A*)	34	85	-1.23	1.13E-11
BC_4325	Biofilm and sporulation regulator (*comER*)	63	137	-1.12	2.83E-07
BC_5350	Pleiotropic transcriptional regulator (*plcR*)	12	47	-1.88	3.12E-19
BC_3826	GTP-sensing pleiotropic transcriptional regulator (*codY*)	4556	3717	0.29	0.0045
BC_2444	DNA-binding domain-containing protein (*abrB*)	1193	41	4.91	2.19E-32
BC_1282	Helix-turn-helix domain-containing protein (*sinR*)	26	18	0.54	0.15
BC_1283	DNA-binding anti-repressor SinI (*sinI*)	93	65	0.51	0.0007

[a] Log 2-fold change, biofilm *vs* planktonic.
[b] False discovery rate, adjusted P-value.

5.3.3.2 Genes related to spore germination

The planktonic environment favours spore germination, as genes related to spore germination proteins (e.g., BC_3574 – 3576; BC_4731 – 4733) were significantly (FDR < 0.05) upregulated in planktonic populations (Table 5. 2). This may be explained by the availability of nutrients for vegetative cell growth in planktonic culture, as nutrients are one key factor triggering the germination of spores (Setlow, 2003). Germinant receptors consist of three components, GerA, GerB and GerC (Moir et al., 2002; Setlow, 2003; Hornstra et al., 2006; Abee et al., 2011). In this study, genes related to the receptor family (BC_0782, BC_0783 and BC_0784) were significantly (FDR < 0.05) upregulated in planktonic cells. Genes related to spore germination protein I (BC_4731 - 4733) and S (BC_3574 - 3576) family were also significantly (FDR < 0.05) upregulated in planktonic cells, however, the specific function of these two types of protein is unknown in the germination process. Spore germination is an irreversible process to reactivate spores into vegetative cells (Abee et al., 2011; Setlow, 2003), and the more active germination in planktonic cells may result in vegetative cells dominating, while in the biofilm, the predominance of spores may contribute to its higher

tolerance. The cell wall hydrolase (BC_2753; *cwlJ*) is a cortex-lytic enzyme contributing peptidoglycan cortex degradation for spores and was suggested as a candidate lytic enzyme playing a role in the early stage of germination (Ishikawa et al., 1998; Setlow, 2003), which was also significantly upregulated (Lfc = -3.01, FDR < 0.05) in planktonic cells.

Table 5. 2 A list of genes related to spore germination, which were differentially expressed in biofilm *versus* planktonic cells.

Gene_id	Protein	Biofilm counts	Planktonic counts	Lfc[a]	FDR[b]
BC_0782	Spore germination protein, GerB family	2	19	-3.02	1.05E-25
BC_0783	Spore germination protein, GerC family	8	86	-3.41	1.53E-26
BC_0784	Spore germination protein, GerA family	0	19	-4.55	2.95E-26
BC_2753	Cell wall hydrolase cwlJ	5	48	-3.01	1.61E-39
BC_3111	Spore germination protein BB	122	637	-2.38	3.46E-11
BC_3574	Spore germination protein SA	4	30	-2.61	1.08E-24
BC_3575	Spore germination protein SB	2	76	-5.12	2.91E-95
BC_3576	Spore germination protein SC	14	585	-5.29	2.58E-132
BC_4319	Spore protease	5	34	-2.55	3.89E-19
BC_4731	Spore germination protein IA	13	40	-1.55	4.24E-17
BC_4732	Spore germination protein IB	5	19	-1.69	2.09E-10
BC_4733	Spore germination protein IC	8	37	-2.13	7.71E-25

[a] Log 2-fold change, biofilm *vs* planktonic.
[b] False discovery rate, adjusted P-value.

5.3.3.3 Ribosome/translation process in biofilms

Biofilm formation is a survival strategy for *B. cereus* in facing environmental changes and stress (Flemming et al., 2016). In this study, biofilm cells were more active (enriched using GSEA, P < 0.05) in the ribosome/translation process (Supplementary files, section 5. 7. 2) where the synthesis of proteins or polypeptides takes place. The function of ribosomal proteins (r-proteins) as a sensor in heat and cold shock response was postulated for *Escherichia coli* (Vanbogelen and Neidhardt, 1990) and *Bacillus subtilis* (Weber and Marahiel, 2002). Fiedoruk et al., (2017) suggested that ribosomal proteins may link to the thermotypes (e.g., thermotolerant, mesophilic, psychrotolerant) of *Bacillus*, and the

divergence in r-proteins may be related to the adaption of the *B. cereus* group to different thermal conditions. However, the role of r-proteins in *B. cereus* species is still unclear. Highly expressed r-proteins (such as BC_0135 – 0148) may be involved supporting biofilm cells to survive temperature stress. The resistance of biofilm cells is generally regarded as being associated with extracellular substances (EPS) in biofilms (Costerton, 1995; Karunakaran and Biggs, 2011), speculating that r-proteins may be involved in the EPS composition of *B. cereus*. Tetracycline resistance protein (BC_3026, *tetP*) was significantly (FDR < 0.05) more highly expressed in biofilms than planktonic cells (Supplementary files section 5. 7. 2), suggesting the better antibiotic resistance in biofilm cells.

5.3.4 Virulence factors and secretion

Comparative analysis of differentially expressed toxin-related genes in biofilm and planktonic cells showed upregulation of enterotoxin (Hbl, Nhe and CytK) related genes (BC_3101, BC_3103 – 3104, BC_1809 – 1810, BC_1110) in biofilm compared to planktonic cells, indicating the potential for increased enterotoxin-producing ability in biofilms compared to planktonic cells (Table 5. 3). This is contradictory to the results shown by Joaquín Caro-Astorga et al., (2019) who indicated lower *hbl* and *nhe* expression in biofilms swabbed from the wells of a polystyrene well-plate compared to planktonic cells. However, biofilm cells in this study were grown on SS, suggesting that the substates for biofilm growth may influence the enterotoxin production for *B. cereus*. PlcR (BC_5350) and CodY (BC_3826) are two important transcriptional regulators positively correlated in both biofilm and enterotoxin production for *B. cereus* (Gohar et al., 2008; Lindbäck et al., 2012; Huang et al., 2020). The expression of *plcR* was significantly (FDR < 0.05) upregulated in the planktonic population while the CodY regulator was upregulated in biofilm cells. This suggests that the expression of CodY may solely or with other regulators, apart from PlcR, regulate enterotoxin gene expression by biofilm cells.

The expression of pore-forming haemolysins differs between planktonic and biofilm cells (Table 5. 3). *B. cereus* expresses various types of haemolysins causing the lysis of erythrocytes, leukocytes, and platelets by producing pores in the cytoplasmic membrane promoting the damage of intestinal cells (Ramarao and Sanchis, 2013), however, the mechanism of secretion of different haemolysins is unknown. In this study, haemolysin I (thiol-activated cytolysin or cholesterol-dependent cytolysin; BC_5101) and haemolysin II (BC_3523) were significantly (FDR < 0.05) downregulated in biofilm cells, while haemolysin III (BC_2196) was upregulated, suggesting that the invasion strategies differ between biofilm and planktonic cells (Ramarao and Sanchis, 2013). Haemolysin III was also upregulated in the biofilms swabbed from a polystyrene well-plate compared to planktonic cells shown by Joaquín Caro-Astorga et al., (2019), however, the role of haemolysin III in biofilms for *B. cereus* is still unknown.

β-lysine acetyltransferase (BC_2249) providing resistance to β-lysine, an antibacterial compound produced by platelets inducing bacterial cell lysis (Hamzeh-Cognasse et al., 2015), was significantly (FDR < 0.05) upregulated in biofilm cells. β-lysine acetyltransferase could be synthesized by *B. cereus* to adapt to an osmotic environment (Triadó-Margarit et al., 2011), and may also contribute to the survival of bacterial cells facing the host immune system response to infection (Hamzeh-Cognasse et al., 2015), which may be used by biofilm cells to survive the immune system causing human disease. InhA metalloproteases of *B. cereus* are another important virulence factor thought to protect the bacteria against the host immune system (Ramarao and Lereclus, 2005; Guillemet et al., 2010; Enosi Tuipulotu et al., 2020).

One of the homologs of InhA1 (BC_1284) was significantly upregulated (Lfc = 7.69, FDR < 0.05) in biofilm cells, while the other two, InhA2 and InhA3 (BC_0666 and BC_2984) were downregulated. InhA1 is a key factor involved in the spore exosporium (Charlton et al., 1999) and biofilm is a preferred environment for *B. cereus* to sporulate (Chapter 4), which may explain the higher expression of InhA1 in biofilms. Besides, InhA in *Bacillus anthracis* plays

a role in degrading the epithelial barrier proteins (Chung et al., 2006), and presumably has a similar function in *B. cereus*. It is assumed that only cells that reach the mucus layer on arrival in the small intestine can survive and adhere to the host cells followed by outgrowth and enterotoxin production, causing diarrhoea (Ceuppens et al., 2012a). Therefore, forming biofilms may support the adhesion on host cells and increase the possibility of illness.

Table 5. 3 A list of genes related to toxin production and virulence factors, which were differentially expressed in biofilm *versus* planktonic cells.

Gene_id	Protein	Biofilm counts	Planktonic counts	Lfc[a]	FDR[b]
BC_1110	Cytotoxin K	6	12	-1.09	0.00082
BC_5101	Thiol-activated cytolysin	0	7	-5	2.70E-10
BC_3523	Hemolysin II	14	37	-1.31	2.42E-12
BC_0666	Immune inhibitor A precursor (InhA2)	119	306	-1.35	2.85E-56
BC_2984	Immune inhibitor A precursor (InhA3)	789	2134	-1.43	2.40E-09
BC_5350	Transcriptional activator PlcR	12	47	-1.89	3.12E-19
BC_3101	Hemolytic enterotoxin Hbl binding subunit B	39	4	2.74	3.91E-14
BC_3104	Hemolytic enterotoxin Hbl lytic component L2	143	14	3.14	9.57E-36
BC_3103	Hemolytic enterotoxin Hbl lytic component L1	96	15	2.63	2.69E-22
BC_1809	Non-hemolytic enterotoxin Nhe subunit A	194	41	2.26	1.12E-39
BC_1810	Non-hemolytic enterotoxin Nhe subunit B	2069	252	3.06	9.04E-62
BC_2196	Hemolysin III	201	55	1.97	5.65E-51
BC_2249	Beta-lysine acetyltransferase	247	71	1.86	1.18E-23
BC_1284	Immune inhibitor A precursor (InhA1)	408	1	7.69	5.19E-37
BC_3826	Transcription pleiotropic repressor CodY	4556	3717	0.29	0.0045

[a] Log 2-fold change, biofilm *vs* planktonic.
[b] False discovery rate, adjusted P-value.

Pathogens use their secretion system to secrete virulence factors from the cytosol of the bacteria into host cells, causing disease, and possibly competing with other bacteria in the environment (Mecsas and Green, 2016). The transcriptomic analysis showed that biofilm cells not only express more enterotoxin-related genes (*hbl* and *nhe*, shown in Table 5. 3), but also, genes related to the general secretion pathway (Sec) pathway and the two-arginine (Tat) system were also upregulated in biofilm cells (Table 5. 4 and supplementary file, section 5. 7. 3). Secreted proteins are commonly translocated across the single membrane by the general secretion pathway (Sec) or the two-arginine (Tat) pathway in Gram-positive bacteria (Anné et al., 2016), and the secretion of Hbl and Nhe proteins has been suggested via the Sec system based on its amino acid sequences (Senesi and Ghelardi, 2010). This would also explain why the biofilm cells showed less cytotoxicity than planktonic cells by Joaquín Caro-Astorga et al., (2019), as the toxins were not bound to the cells and biofilms tend to secrete Hbl toxin into the environment.

Table 5. 4 A list of protein export and bacterial secretion system genes that are differentially expressed in biofilm *versus* planktonic cells.

Gene_id	Protein	Biofilm counts	Planktonic counts	Lfc[a]	FDR[b]
BC_3070	Signal peptidase I	190	8	4.17	9.94E-31
BC_2197	Sec-independent protein translocase protein TatA	271	76	1.93	6.45E-51
BC_4405	Protein translocase subunit SecD / Protein translocase subunit SecF	29	8	1.49	0.0097
BC_4410	Protein translocase subunit YajC	28	11	1.48	1.81E-05
BC_3843	Signal recognition particle, subunit Ffh/SRP54	1062	391	1.43	1.31E-28
BC_3845	Cell division protein FtsY	467	299	0.67	1.56E-06
BC_2740	Protein translocase subunit SecY	26	15	0.55	0.016
BC_0115	Protein translocase subunit SecE	7068	5210	0.44	0.028

[a] Log 2-fold change, biofilm *vs* planktonic.
[b] False discovery rate, adjusted P-value.

5.3.5 Biosynthesis in biofilms

Biofilms expressed higher levels of genes related to biotin and siderophore biosynthesis in this study (Table 5. 5). Biotin (vitamin H or vitamin B7) is an essential enzyme cofactor involved in carboxyl transfer for all living organisms (Attwood and Wallace, 2001). Genes involved in biotin synthesis (BC_2897, BC_4114 - 4118) were significantly (FDR < 0.05) more highly expressed in the biofilm population (Table 5. 5), suggesting that cells within a biofilm produce more biotin than planktonic cells of *B. cereus*, however, the link between biotin synthesis and biofilm formation is unknown. *B. cereus* species are biotin-producers and biotin has been suggested as a regulator in glutamate production and contributes to the lipid composition of the cell membrane in *B. cereus* (Sasaki, 1965; Hubbard and Hall, 1968). Hubbard and Hall (1968) showed that biotin may affect cell permeability by altering the composition of fatty acids in the cell membrane, more specifically, the biotin-deficient cells contained lower amounts of straight-chain and higher amounts of branched-chain fatty acids. Two genes, *bioC* (BC_4115) and *bioH* (BC_4118), were involved in biotin synthesis for *Escherichia coli* and *B. cereus* at the beginning of fatty acid synthetic pathway (Lin et al., 2010; Lin and Cronan, 2011 & 2012), which were upregulated (Lfc = 3.3 and 3.39, respectively) in biofilms (Table 5. 5). This suggests that a diverse fatty acid composition may exist in biofilm and planktonic cells of *B. cereus*, and the regulatory pathway may differ for two types of cells. Biotin supports the growth of *Bacillus*, especially in minimal nutritional conditions (Proom and Knight, 1955) which may be relevant to biofilm growth, however, the role of biotin/vitamin biosynthesis in biofilm development is unknown and the actual production of biotin by biofilm cells still needs to be elucidated.

Table 5. 5 A list of genes related to biotin synthesis and biosynthesis of siderophore group non-ribosomal peptides, which were differentially expressed in biofilm *versus* planktonic cells.

	Gene_id	Protein	Biofilm counts	Planktonic counts	Lfc[a]	FDR[b]
Biotin synthesis	BC_2897	(3R)-hydroxymyristoyl-[acyl carrier	899	114	2.98	5.9E-254

		protein] dehydratase				
	BC_4114	Biotin synthase	114	10	3.41	9.72E-26
	BC_4115	Biotin synthesis protein bioC	136	13	3.33	4.53E-24
	BC_4116	BioH protein	263	15	4.16	2.01E-30
	BC_4117	8-amino-7-oxononanoate synthase	174	12	3.78	7.85E-30
	BC_4118	ATP-dependent dethiobiotin synthetase BioD	372	34	3.39	1.71E-48
Biosynthesis of siderophore group nonribosome peptides	BC_1978	Siderophore biosynthesis protein	69	17	1.90	2.37E-16
	BC_1979	Siderophore biosynthesis protein	42	9	2.24	3.53E-14
	BC_1980	AMP-(fatty)acid ligases	859	344	1.32	7.78E-32
	BC_1982	Petrobactin biosynthesis protein AsbE	34	6	2.21	8.14E-12
	BC_2302	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase	70	16	1.99	1.84E-15
	BC_2303	Isochorismate synthase	133	51	1.35	1.06E-14
	BC_2304	2,3-dihydroxybenzoate-AMP ligase	82	12	2.75	2.48E-27
	BC_2305	Isochorismatase	956	186	2.37	5.53E-55

[a] Log 2-fold change, biofilm *vs* planktonic.
[b] False discovery rate, adjusted P-value.

Siderophores are iron acquisition cofactors used by pathogens to uptake sufficient ion for proliferation and are designed to form a stable complex with ferric ion (Bidlack, 1999; Miethke and Marahiel, 2007). In this study, the genes related to siderophore biosynthesis were upregulated in biofilms (Table 5. 5). Biosynthesis of siderophores is a common strategy by microorganisms facing a low iron environment (Fischer et al., 1990), however, the biosynthesis-related genes were upregulated in biofilm cells grown on stainless-steel where sufficient iron availability is expected. Stainless-steel may stimulate the formation of those iron complexes supporting biofilm growth. Similarly, genes involved in iron acquisition were

also upregulated in biofilms of other bacteria, such as *Clostridium perfringens* (*C. perfringens*) and *Staphylococcus aureus* (Lin et al., 2012; Charlebois et al., 2016), suggesting the importance of iron in biofilm development. *B. cereus* strains can excrete two types of siderophores, petrobactin and bacillibactin (Wilson et al., 2006). The genes involved in synthesis (BC_1979, BC_1982 and BC_1978) were upregulated in the biofilm population alongside its enzymes (BC 2302 - 2305). Petrobactin belongs to the group of iron transport cofactors that have been suggested to enhance microbial virulence (Challis, 2005; Wilson et al., 2006). The biosynthesis of siderophores may enhance the virulence of biofilm cells for *B. cereus*. However, the role and actual production of siderophores in biofilms is unknown.

5.3.6. Different sigma factors expressed in biofilm and planktonic cells

Sigma factors are regulators/proteins needed in the initiation of transcription that control promotor selection (Gruber and Gross, 2003). These transcriptional regulons controlled by sigma factors may play a critical role in the phenotypic diversity of the *B. cereus* group (Schmidt et al., 2011). Different sigma factors were expressed in biofilm and planktonic cells in this study (Table 5. 6), speculating that the divergence of phenotypic characteristics of two types of cells may be as a result of the different sigma factors expressed. Oosthuizen et al., (2002) observed an increased level of sigma proteins (sigma B and H) in the attached biofilm and planktonic cells in the presence of biofilm, suggesting the important role of sigma proteins in regulating the biofilm phenotype.

ECF (extracytoplasmic function) sigma factors belong to the sigma 70 family and the expression is related to extracellular stimulation, which is associated with functions including response to stress and resistance to antimicrobial compounds, ion transportation and virulence (Missiakas and Raina, 1998; Heimann, 2002; Kazmierczak et al., 2005; Butcher and Helmann, 2006; Ross et al., 2009). However, most of the roles and mechanisms of ECF are unknown. In the present study, genes related to ECF sigma factors, including BC_2469, BC_1698, BC_2386, BC_0647 and BC_5363, were upregulated in planktonic cells, while

BC_2794 and BC_1731 were significantly (FDR < 0.05) more highly expressed in biofilm cells (Table 5. 6). Sigma E, F, G, H and K are involved in transcribing sporulation-related genes for *B. subtilis* (Gruber and Gross, 2003). In this study, sigma E (BC_3904), G (BC_3903), and K (BC_4336) were upregulated in planktonic cells, while sigma F (BC_4072) and H (BC_0114) were upregulated in biofilm cells. This may explain the different sporulation efficacy in the two types of cells, as biofilm is regarded as the preferred condition for *B. cereus* to sporulate (Chapter 4). However, the role of sigma factors in sporulation needs to be investigated in the future.

Sigma B is a primary alternative (PA) sigma factor, playing an important role in the adaptive stress response of *B. cereus* (Price, 2001; van Schaik et al., 2004), and the gene-related sigma B (BC_1004) and its regulators including RsbV (BC_1002) and RsbW (BC_1003), were upregulated in biofilm cells (Table 5. 6). The active expression of sigma B factor in biofilm cells may be caused by the environmental changes from floating to sessile cells, and biofilm is regarded as a high cell density and nutrient-limited condition (van Gestel et al., 2012) which may trigger the expression of sigma B factor. However, the role of nutrient stress in sigma B activation needs to be determined. Sigma B was also shown to be involved in protecting cells from heat stress (van Schaik et al., 2004), suggesting the possible role of sigma B in the heat resistance of biofilm cells. The role of sigma B in biofilm formation was proposed in *B. subtilis*, showing that *sigB*-deficient biofilm expressed accelerated cell death and greater sensitivity to stresses although the biofilm formation of the *sigB*-deficient mutant was higher than the wild type (Bartolini et al., 2018). However, the role in *B. cereus* is unknown. In *B. subtilis*, the deletion of *sigB* results in deficient sporulation and the expression of *sigB* happens when the cell faces stress (Price, 2001; Méndez et al., 2004). This has been suggested to be similar in *B. cereus* by De Vries et al., (1994) with the peak of *sigB* expression of *B. cereus* observed in the early stationary phase where sporulation happens. It is possible that *sigB* contributes to the resistance of biofilms of *B. cereus* by regulating sporulation. The function of the sigma B-dependent protein is largely unknown for *B. cereus*,

but further investigation may reveal a novel mechanism for stress adaption (De Vries et al., 2004). The induced stress-related and resistance-related genes were also found in the biofilm transcriptome studies of *E. coli*, *C. perfringens* and *S. epidermidis* (Yao et al., 2005; Wood, 2009; Charlebois et al., 2016), however, how these bacteria utilize the stress-response mechanism in biofilms is not fully understood.

Sigma 54 (*RpoN*, BC_5143) is a pleiotropic transcriptional regulator supporting motility, biofilm formation and virulence for *B. cereus* (Hayrapetyan et al., 2015b), and was upregulated in the planktonic population in this study. Interestingly, *RpoN* is involved in biofilm formation through controlling motility (Hayrapetyan et al., 2015b; Huang et al., 2020), however, it was not the case when biofilm grew in the shaking condition used in this study. This leads us to hypothesize that the regulation of gene expression for biofilm formation may be altered when environmental conditions change.

Table 5. 6 A list of genes related to sigma factor activity, which were differentially expressed in biofilm versus planktonic cells.

Gene_id	Protein	Biofilm counts	Planktonic counts	Lfc[a]	FDR[b]
BC_1819	Deoxyribonucleoside regulator	16	46	-1.56	2.53E-12
BC_0019	Hypothetical Cytosolic Protein	2	19	-2.63	6.87E-13
BC_2469	RNA polymerase ECF-type sigma factor	18	60	-1.67	4.68E-21
BC_1698	RNA polymerase ECF-type sigma factor	8	540	-5.92	7.2E-26
BC_2386	RNA polymerase ECF-type sigma factor	0	3	-3.51	1.97E-05
BC_0647	RNA polymerase ECF-type sigma factor	3	27	-2.92	2.24E-33
BC_5363	RNA polymerase ECF-type sigma factor	9	23	-1.25	6.81E-07
BC_5251	RNA polymerase sigma factor	0	17	-4.15	1.29E-27
BC_1114	RNA polymerase sigma factor sigM	0	8	-4.8	8.68E-14
BC_5143	RNA polymerase sigma-54 factor rpoN	11	52	-2.22	7.02E-41
BC_3904	RNA polymerase sigma-E factor	17	127	-2.86	2.48E-17

BC_3903	RNA polymerase sigma-G factor	39	139	-1.87	8.42E-75
BC_4336	RNA polymerase sigma-K factor	37	195	-2.37	1.09E-16
BC_1002	Anti-sigma B factor antagonist RsbV	69	25	1.47	1.62E-10
BC_1003	anti-sigma B factor RsbW	113	24	2.27	6.36E-26
BC_2794	RNA polymerase ECF-type sigma factor	22	6	1.78	4.59E-06
BC_1731	RNA polymerase ECF-type sigma factor	80	49	0.71	7.95E-06
BC_4289	RNA polymerase sigma factor rpoD	112	73	0.65	1.03E-07
BC_1004	RNA polymerase sigma factor SigB	99	27	1.86	9.96E-23
BC_3426	RNA polymerase sigma factor SigI	146	43	1.75	1.02E-36
BC_4072	RNA polymerase sporulation sigma factor SigF	217	100	1.18	4.45E-07
BC_0114	RNA polymerase sporulation sigma factor SigH	2550	1284	0.98	2.79E-14
BC_2766	sigma-54-dependent Fis family transcriptional regulator	3435	1673	1.04	5.29E-06

[a] Log 2-fold change, biofilm *vs* planktonic.
[b] False discovery rate, adjusted P-value.

5.3.7 Survival strategy used by planktonic cells

The phosphotransferase system (PTS) is the major mechanism used by bacteria for the uptake and transport of carbohydrates. Planktonic cells actively use this system, demonstrated by the upregulation of glucose (BC_0414), sucrose (BC_0842) and trehalose (BC_0631) genes (Supplementary file, section 5. 7. 4). The pentose phosphate pathway (PPP) which belongs to the central carbohydrate metabolism, was also enriched in the planktonic population (Supplementary file, section 5. 7. 5). Energy from the environment can support cell growth and enhance the transmission and pathogenicity of *B. cereus* (Ceuppens et al., 2013). Planktonic cells thrive through continually absorbing available nutrients from the environment. On the other hand, biofilm cells have limited exposure to the environment, relying on limited resources. The slow cell growth of biofilm cells was shown in the

transcriptomic analysis of other bacteria, such as *Staphylococcus epidermidis*, *Porphyromonas gingivalis* and *C. perfringens* (Yao et al., 2005; Lo et al., 2009; Charlebois et al., 2016).

Better motility and active flagellar activity were indicated in the planktonic population, as genes involved in flagellar assembly and motility were upregulated (Supplementary file, section 5. 7. 6). Ghelardi et al., (2007) observed higher Hbl toxin detection in the swarming than non-swarming *B. cereus* strains using an immunoblot assay, and Mazzantini et al., (2016) showed that a depletion mutant of *flhF*, which is a flagellar protein, had a significant decrease in secreting virulence factors (e.g., Hbl toxin, sphingomyelinase and cytotoxin K) using an electrophoresis method for *B. cereus*. Furthermore, Ramarao and Lereclus (2006) indicated that a strain lacking the *flhA* gene, which encodes for a flagellar protein, showed impairment in cell adhesion onto epithelial cells suggesting flagella are important virulence factors. These results suggest that the swarming ability/flagella of *B. cereus* may increase toxin secretion and enhance the pathogenicity of *B. cereus*, although there was no significant difference in the expression of *flhF* and *flhA* in this study (FDR > 0.05, data not shown). The link between swarming ability and toxin production was also suggested by Senesi and Ghelardi (2010) who reviewed the essential role of flagellar proteins in secretion from bacteria. However, how these flagellar proteins provide advantages to planktonic cells in producing and secretion of virulence factors is still unknown.

The presence of flagella and the motility of cells is known to affect biofilm formation, however, this may not necessarily be important in flow cells (Houry et al., 2010). This may explain why we did not observe the high expression of motility genes in biofilm cells grown in a shaking condition, as this would allow interaction with the surface in a similar way to flow cells, without the requirement for motility. Chemotaxis is used for sensing chemical gradients and followed by movement to more favourable conditions and this belongs to a stress response mechanism (Den Besten et al., 2009; Ganesh Babu et al., 2011). Chemotaxis response genes *cheY* (BC_1627) and *cheA* (BC_1628) (Celandroni et al., 2000) were

upregulated in planktonic cells suggesting that motility and chemotaxis may be used by planktonic cells as a survival strategy when facing stress.

5.4 Conclusions

Biofilm cells display a different lifestyle than planktonic cells, supported by the 48.5% of the total gene content being significantly ($P < 0.05$) differently expressed in this study. Factors, including lower germination ability, more highly expressed ribosome protein and active biosynthesis may contribute to the resistance mechanism (e.g., chemical or antibiotics) of biofilm cells. Biofilms grown on stainless-steel could be more pathogenic than planktonic cells, which is not only because of the higher expression of enterotoxin genes (*hbl*, *nhe* and *cytK*), but also the up-regulated secretion pathways. There was variability in the expression of sigma factors in biofilm and planktonic cells, which may contribute to the phenotypic diversity of the two types of cells. The expression of *SigB* was significantly increased in biofilm cells which may contribute to the ability of biofilms to adapt to changing conditions. Planktonic cells were actively taking up energy from surrounding environments and expressed different virulence factors than biofilms, indicating that different mechanisms may exist in planktonic and biofilm cells in producing and releasing virulence factors.

5.5 Acknowledgement

We would like to express our special thanks to Ali Saei and Simon Zhang from Custom Science, New Zealand, for helping me with RNA sequencing and bioinformatics analysis.

5.6 Copyright information

Parts of this study is intended to be submitted to a journal for publication and the Online Statement of Contribution form is attached in Appendix III.

5.7 Supplementary files

5.7.1 RNA sequencing mapping statistic

Table S5. 1 RNA sequencing general statistics.

Sample	Raw reads[a]	Q20(%)[b]	Q30(%)[b]	GC(%)[c]	Duplicate reads	Unique reads
Planktonic_1_1	6641430	98.75	95.94	46.8	3122589	198126
Planktonic_1_2					3092340	228375
Planktonic_2_1	6549082	98.84	96.13	47.59	3086268	188273
Planktonic_2_2					3069331	205210
Planktonic_3_1	8248108	98.51	95.35	46.38	3850325	273729
Planktonic_3_2					3818799	305255
Biofilm_1_1	16429018	98.35	94.83	40.7	7089909	1124600
Biofilm_1_2					6967907	1246602
Biofilm_2_1	6732372	98.55	95.35	38.5	2651181	715005
Biofilm_2_2					2533983	832203
Biofilm_3_1	10281340	98.2	94.58	38.14	4188995	951675
Biofilm_3_2					4041879	1098791

[a] Raw read: the total amount of reads of raw data. For paired-end sequencing, it equals the amount of read1 and read2. All the samples have sequences of 150 bp.
[b] Q20, Q30: (Base count of Phred value > 20 or 30) / (Total base count).
[c] GC: GC content. (G & C base count) / (Total base count).

5.7.2 Ribosome/translation

Figure S5. 1 Gene set enrichment analysis of ribosome pathway ($P < 0.05$). The enrichment score reflects the degree which the genes in a gene set are overrepresented at the top or bottom of the entire ranked list of genes. Rank was based on the expression levels of genes.

Table S5. 2 A list of genes related to ribosome/translation, which were differential expressed in biofilm *versus* planktonic cells.

Gene_id	Protein	Biofilm counts	Planktonic counts	Lfc[a]	FDR[b]
BC_0119	LSU ribosomal protein L10P	1515	2143	-0.50	0.02
BC_0120	LSU ribosomal protein L12P (L7/L12)	1093	1979	-0.86	0.0001
BC_0155	LSU ribosomal protein L36P	1	13	-3.32	3.31E-19
BC_0156	SSU ribosomal protein S13P	4	21	-2.07	2.29E-10
BC_0157	SSU ribosomal protein S11P	760	1015	-0.42	4.33E-07
BC_3806	SSU ribosomal protein S15P	1096	1639	-0.58	0.002
BC_3923	LSU ribosomal protein L32P	46	62	-0.44	0.002
BC_4339	LSU ribosomal protein L33P	0	10	-7.06	1.03E-14
BC_4436	LSU ribosomal protein L27P	684	1473	-1.11	1.64E-12
BC_4438	LSU ribosomal protein L21P	1758	2416	-0.46	0.002
BC_4573	LSU ribosomal protein L20P	273	464	-0.77	3.69E-05
BC_4655	SSU ribosomal protein S4P	1391	2274	-0.71	9.49E-05
BC_5331	LSU ribosomal protein L31P	487	694	-0.51	0.004
BC_0134	LSU ribosomal protein L2P	936	580	0.68	0.0001
BC_0135	SSU ribosomal protein S19P	1261	608	1.04	2.14E-07
BC_0136	LSU ribosomal protein L22P	890	463	0.93	6.53E-06
BC_0137	SSU ribosomal protein S3P	1249	657	0.91	3.07E-08
BC_0138	LSU ribosomal protein L16P	228	122	0.86	4.41E-07
BC_0139	LSU ribosomal protein L29P	874	397	1.12	1.7E-11
BC_0140	SSU ribosomal protein S17P	2883	866	1.72	1.1E-32
BC_0141	LSU ribosomal protein L14P	982	369	1.39	9.69E-18

BC_0142	LSU ribosomal protein L24P	1363	460	1.55	1.74E-56
BC_0143	LSU ribosomal protein L5P	446	126	1.79	2.45E-65
BC_0144	SSU ribosomal protein S14P	56	22	1.48	1.74E-10
BC_0145	SSU ribosomal protein S8P	875	276	1.62	6.23E-74
BC_0146	LSU ribosomal protein L6P	883	321	1.42	3.5E-61
BC_0147	LSU ribosomal protein L18P	2483	721	1.76	1.1E-110
BC_0148	SSU ribosomal protein S5P	319	172	0.92	2.4E-19
BC_0149	LSU ribosomal protein L30P	55	35	0.67	0.0007
BC_0150	LSU ribosomal protein L15P	36	25	0.54	0.005
BC_0159	LSU ribosomal protein L17P	219	83	1.47	3.61E-30
BC_0164	LSU ribosomal protein L13P	144	28	2.28	2.15E-43
BC_0165	SSU ribosomal protein S9P	468	56	3.03	3E-114
BC_1498	SSU ribosomal protein S1P	149	24	2.69	1E-40
BC_3825	SSU ribosomal protein S2P	110	42	1.36	4.7E-21
BC_3838	LSU ribosomal protein L19P	81	67	0.28	0.04
BC_3842	SSU ribosomal protein S16P	47	24	1.05	2.47E-06
BC_3856	LSU ribosomal protein L28P	238	198	0.31	0.004
BC_4263	LSU ribosomal protein L33P	51	13	1.83	2.13E-12
BC_4307	SSU ribosomal protein S21P	7512	5370	0.48	3.83E-05
BC_4320	SSU ribosomal protein S20P	260	147	0.82	0.0006
BC_5471	LSU ribosomal protein L9P	329	254	0.42	0.0001
BC_5476	SSU ribosomal protein S6P	352	119	1.62	6.38E-78
BC_5490	LSU ribosomal protein L34P	461	200	1.24	9.6E-14

[a] Log 2-fold change, biofilm *vs* planktonic.
[b] False discovery rate, adjusted P-value.

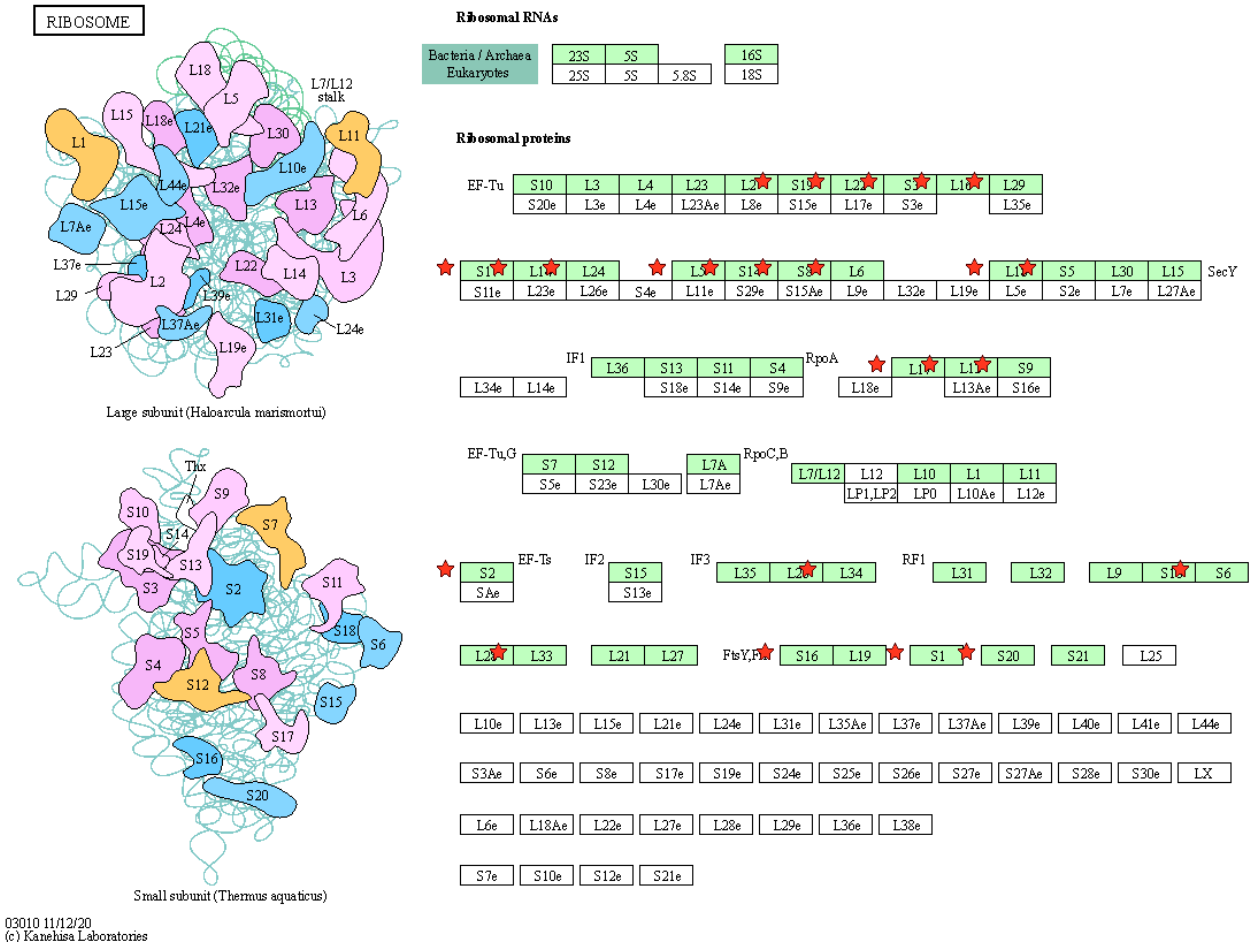


Figure S5. 2 KEGG pathway enrichment of ribosome is found in differentially expressed genes of biofilm *versus* planktonic. The red stars are genes in the dataset that were significantly differentially expressed.

5.7.3 Protein export & Bacterial secretion system

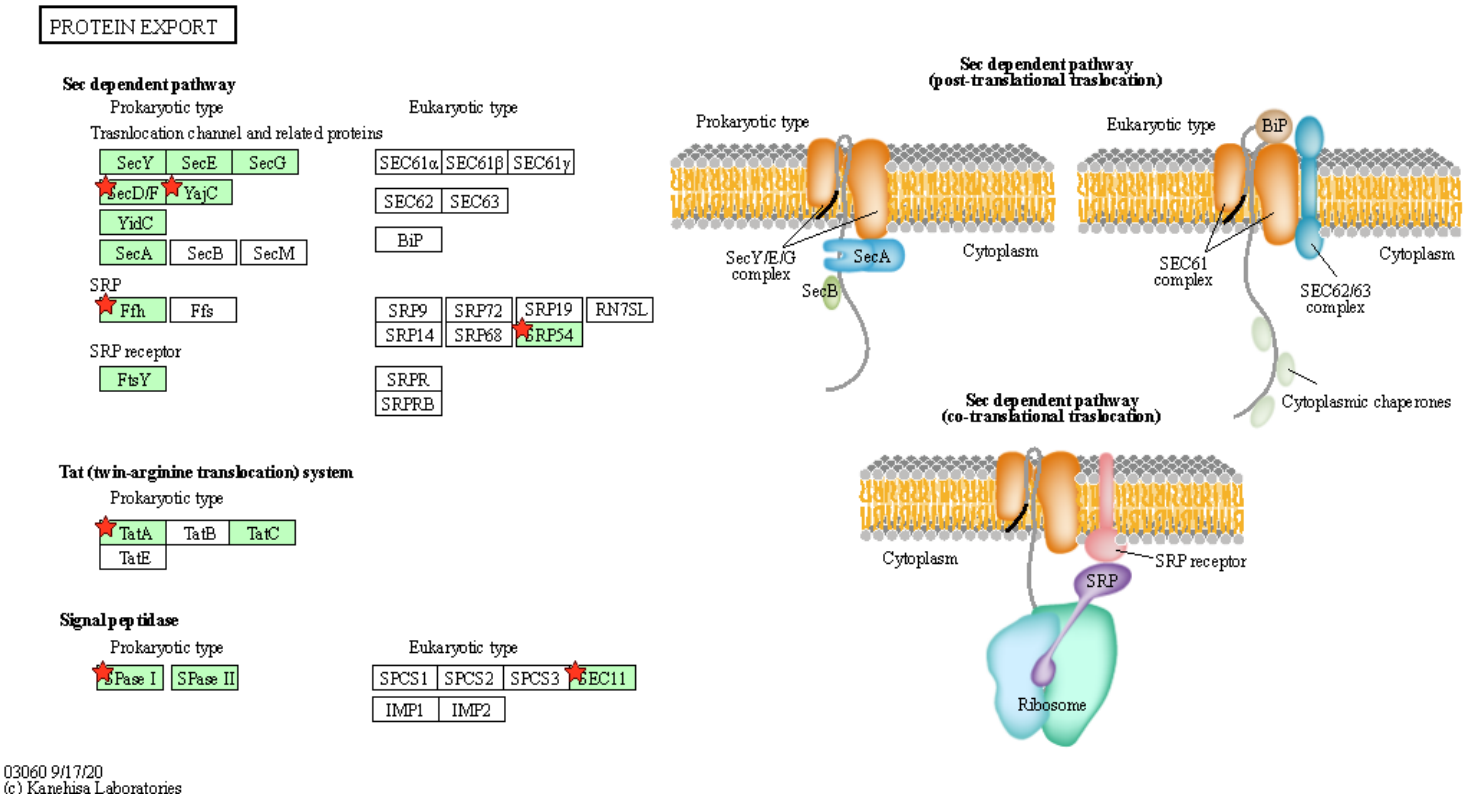


Figure S5. 3 KEGG pathway enrichment of protein export is found in differentially expressed genes of biofilm *versus* planktonic. The red stars are genes in the dataset that were significantly differentially expressed in biofilm population.

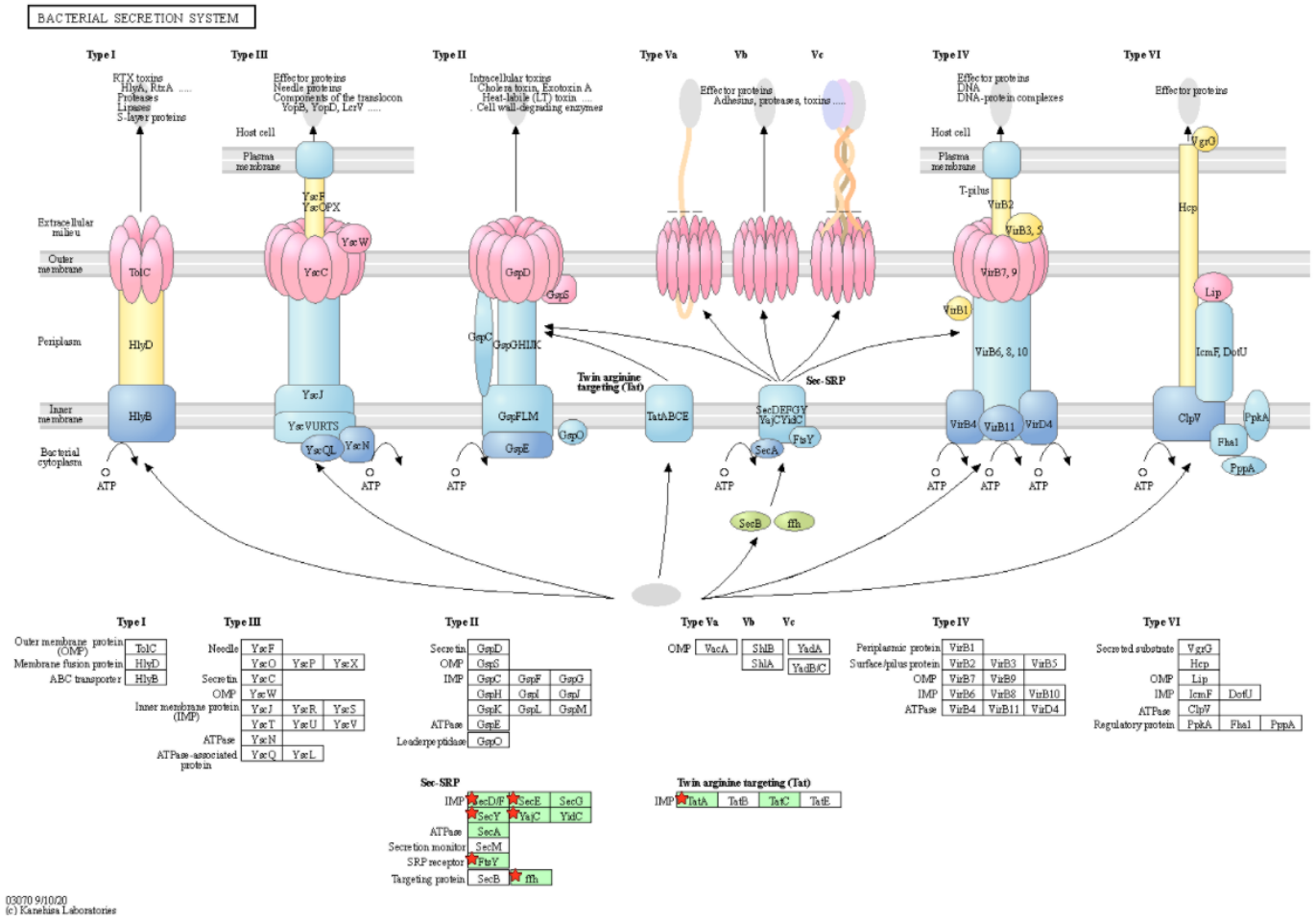


Figure S5. 4 KEGG pathway enrichment of bacterial secretion system is found in differentially expressed genes of biofilm *versus* planktonic. The red stars are genes in the dataset that were significantly differentially expressed in biofilm populations.

5.7.4 Phosphotransferase system (PTS)

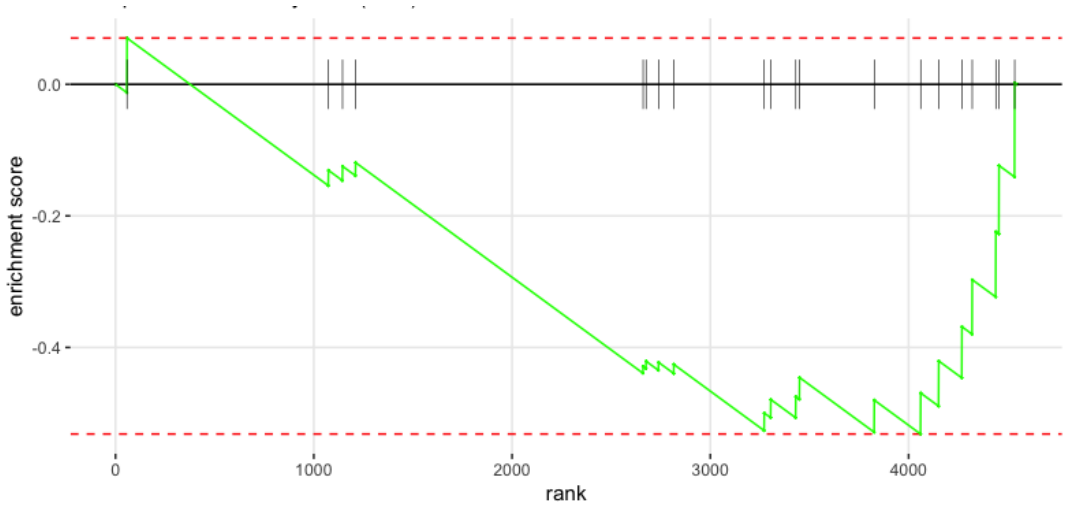


Figure S5. 5 Gene set enrichment analysis of PTS system ($P < 0.05$). The enrichment score reflects the degree which the genes in a gene set are overrepresented at the top or bottom of the entire ranked list of genes. Rank was based on the expression levels of genes.

Table S5. 3 A list of genes related to PTS system, which were differential expressed in biofilm *versus* planktonic cells.

Gene_id	Protein	Biofilm counts	Planktonic counts	Lfc[a]	FDR[b]
BC_0414	PTS system, glucose specific IIBC component	6	577	-6.49	4.52E-250
BC_0631	PTS system, trehalose specific IIBC component	1	11	-3.53	3.1E-19
BC_0842	PTS system, sucrose specific IIBC component	2	21	-3.11	1.85E-28
BC_5210	PTS system, lichenan oligosaccharide specific IIA component	9	264	-4.71	1.48E-130
BC_5211	PTS system, lichenan oligosaccharide specific IIC component	2	50	-4.50	1.6E-54
BC_5215	PTS system, lichenan oligosaccharide specific IIA component	2	29	-3.75	2.96E-38
BC_5216	PTS system, lichenan oligosaccharide specific IIC component	1	12	-2.82	0.000008

[a] Log 2-fold change, biofilm *vs* planktonic.
[b] False discovery rate, adjusted P-value.

5.7.5 Pentose phosphate pathway

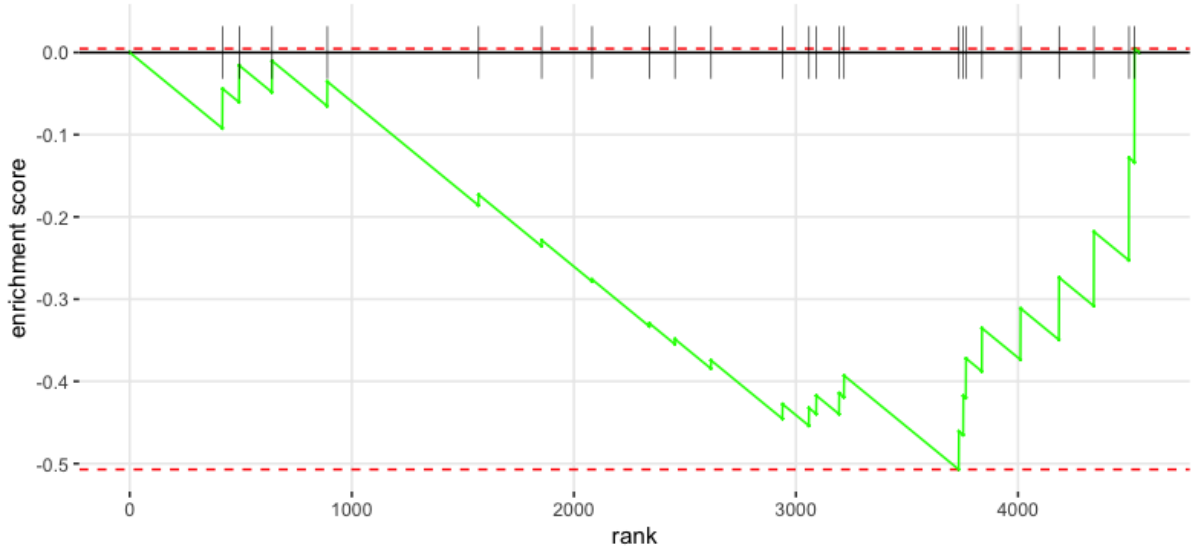


Figure S5. 6 Gene set enrichment analysis of pentose phosphate pathway ($P < 0.05$). The enrichment score reflects the degree which the genes in a gene set are overrepresented at the top or bottom of the entire ranked list of genes. Rank was based on the expression levels of genes.

Table S5. 4 A list of genes related to pentose phosphate pathway, which were differential expressed in biofilm *versus* planktonic cells.

Gene_id	Protein	Biofilm counts	Planktonic counts	Lfc[a]	FDR[b]
BC_1820	Deoxyribose-phosphate aldolase	1	8	-2.66	4.17E-09
BC_2223	Gluconokinase	3	10	-1.97	1.54E-06
BC_3368	6-phosphogluconolactonase	7	32	-2.05	1.16E-22
BC_3371	Transaldolase	4	66	-3.85	9.25E-33
BC_3372	6-phosphogluconate dehydrogenase	4	199	-5.31	8.49E-152
BC_4600	6-phosphofructokinase	194	922	-2.24	2.97E-159
BC_4843	2-dehydro-3-deoxygluconokinase	11	45	-2.02	7.64E-30
BC_4898	Glucose-6-phosphate isomerase	1	13	-3.23	5.73E-14
BC_5318	Ribose 5-phosphate isomerase	0	47	-5.90	1.92E-50

[a] Log 2-fold change, biofilm *vs* planktonic.
[b] False discovery rate, adjusted P-value.

5.7.6 Cell motility/flagellar/chemotaxis

Table S5. 5 A list of genes related to cell motility/flagellar/chemotaxis, which were differential expressed in biofilm *versus* planktonic cells.

Gene_id	Protein	Biofilm counts	Planktonic counts	Lfc[a]	FDR[b]
BC_0404	Methyl-accepting chemotaxis protein	20	102	-2.25	1.48E-68
BC_0678	Methyl-accepting chemotaxis protein	3	21	-2.69	3.23E-22
BC_1627	Chemotaxis protein chey	40	489	-3.61	1.3E-140
BC_1628	Chemotaxis protein chea	19	66	-1.75	4.81E-14
BC_1632	Chemotaxis protein methyltransferase	25	61	-1.22	1.39E-18
BC_1636	Flagellar hook-associated protein flgk	10	162	-3.97	6E-174
BC_1637	Flagellar hook-associated protein flgl	14	173	-3.55	1.24E-37
BC_1638	Flagellar capping protein	4	23	-2.29	1.6E-16
BC_1639	Flagellar protein flis	0	14	-3.77	4.89E-19
BC_1641	Flagellar basal-body rod protein flgb	0	7	-3.12	7.49E-09
BC_1642	Flagellar basal body rod protein flgc	1	16	-3.69	5.38E-20
BC_1643	Flagellar hook-basal body protein flie	14	264	-4.21	2.1E-144
BC_1644	Flagellar ms-ring protein	14	54	-1.97	1.88E-20
BC_1645	Flagellar motor switch protein g	5	24	-2.05	8.75E-17
BC_1646	Flagellar assembly protein flih	9	65	-2.75	8.82E-27
BC_1647	Flagellum-specific atp synthase	38	150	-1.93	2.14E-15
BC_1650	Flagellar basal body rod modification protein	30	309	-3.35	8.7E-174
BC_1651	Flagellar hook protein flge	12	61	-2.33	3.45E-10
BC_1656	Flagellin	46	4172	-6.47	1.9E-102
BC_1659	Flagellin	2	22	-3.23	1.4E-10
BC_1661	Flagellar motor switch protein	4	68	-4.02	1.7E-99
BC_1662	Flagellar motor switch protein flim	2	15	-2.85	6.35E-20
BC_1663	Flagellar motor switch protein	1	6	-2.58	5.69E-08
BC_1664	Flagellar motor switch protein flin	2	34	-3.48	3.85E-38
BC_1665	Flagellar biosynthetic protein flip	1	6	-1.94	0.000392
BC_1666	Flagellar biosynthesis protein fliq	3	24	-2.78	8.95E-27

BC_1667	Flagellar biosynthesis protein flir	4	23	-2.25	1.36E-19
BC_1668	Flagellar biosynthesis protein flhb	8	49	-2.46	9.02E-37
BC_4512	Flagellar motor protein motb	6	113	-4.15	1.2E-100
BC_4513	Flagellar motor stator protein mota	3	103	-4.95	7.68E-74
BC_5009	Methyl-accepting chemotaxis protein	9	24	-1.31	2.25E-08
BC_5034	Methyl-accepting chemotaxis protein	11	30	-1.39	1.71E-11

[a] Log 2-fold change, biofilm *vs* planktonic.
[b] False discovery rate, adjusted P-value.

Chapter 6 The heat resistance of spores from biofilms of *Bacillus cereus* grown in tryptic soy broth and milk

6.1 Introduction

Bacillus cereus (*B. cereus*) is a common foodborne pathogenic and spoilage microorganism of concern in the dairy industry where spores survive the pasteurization of milk and grow well in nutrient rich milk (Flint et al., 1997; García-Armesto and Sutherland, 1997; Larsen and Jørgensen, 1997). *B. cereus* vegetative cells and spores can adhere to materials such as stainless-steel and polymers used in food processing to form biofilms (Wiencek et al., 1991; Bremer et al., 2009). Biofilms of *B. cereus* are sources of spores contaminating the food processing line and resulting in the presence of the bacteria in final products. However, the percentage of spores within biofilms varies between studies and may be influenced by factors including strain difference, growth temperature, and the substratum for biofilm growth (Lindsay et al., 2005; Faille et al., 2014; Hussain and Oh, 2018).

In Chapter 4, the sporulation percentages in biofilm and planktonic populations were calculated, indicating biofilm was a preferred niche for spores. The transcriptomic study (Chapter 5) showed that genes related to germination factors were upregulated in planktonic cells compared to biofilms, supporting the observation that biofilms contain higher levels of spores comparted to planktonic populations. These findings warrant further investigation into the characteristics of spores isolated from biofilms, one being heat resistance. Heat stable spores of *B. cereus* are an important source of contamination for milk-derived products, such as milk powder and infant food formulas (Becker et al., 1994; Svensson et al., 2006; Watterson et al., 2014). However, the heat resistance of spores grown in milk or dairy environments from biofilms is unknown, as most of the studies use lab media or sporulation medium to cultivate spores (Simmonds et al., 2003; Hayrapetyan et al., 2016). Six *B. cereus* food isolates confirmed as *B. cereus* species (Chapter 4) were included in this study, with lab medium (TSB, tryptic soy broth) and milk used to assess the sporulation ability and heat resistance of spores in biofilms grown on stainless-steel.

6.2 Experimental procedures

6.2.1 Bacterial isolates and culture conditions

Six food isolates (P2, P4, P5, M1, M3 and M4) identified as *B. cereus* were used in this study. Overnight cultures of these isolates were prepared as described in Chapter 3, section 3. 1. 2.

6.2.2 Planktonic and biofilm growth in TSB and milk

The planktonic and biofilm cells of six *B. cereus* isolates were grown in TSB and UHT milk (Anchor, New Zealand). Planktonic cultures were grown as described in Chapter 3, section 3. 2. 1. Biofilms were developed on stainless-steel (SS) coupons as described in Chapter 3, section 3. 2. 3 and incubated statically to obtain comparable amounts of spores to those grown in planktonic conditions.

The number of planktonic and biofilm cells grown on SS coupons was assessed using spread plating after one-, two- and three-day incubation (refer to Chapter 3, section 3. 1. 3). The detachment and enumeration of biofilm cells was conducted using glass beads (refer to Chapter 3, section 3. 2. 3. 2). The spore formation and the sporulation percentages in either planktonic or biofilm populations were counted and calculated as described in Chapter 3, section 3. 3.

6.2.3 Preparation of spore suspension and heat treatment

Spores were harvested after three days incubation at 30°C in either TSB or milk. The preparation of crude suspensions and the heat treatment (90°C) of spores are described in Chapter 3, section 3. 4. Spore suspensions were heated at 80°C for 10 min to kill vegetative cells leaving spore counts.

6.2.4 Estimation of dipicolinic acid content of spores

The dipicolinic acid (DPA) content of spores isolated from either planktonic or biofilm populations was measured (refer to Chapter 3, section 3. 5) to evaluate the correlation between DPA content and heat resistance of spores.

6.2.5 Transmission electron microscopy (TEM)

The structure of spores harvested from planktonic culture and biofilms was visualized under TEM as described in Chapter 3, section 3. 6.

6.2.6 Statistical analysis

The data expressed in the figures were generated from the average value of three independent biological replicates and the statistical analysis was performed refer to Chapter 3, section 3. 10.

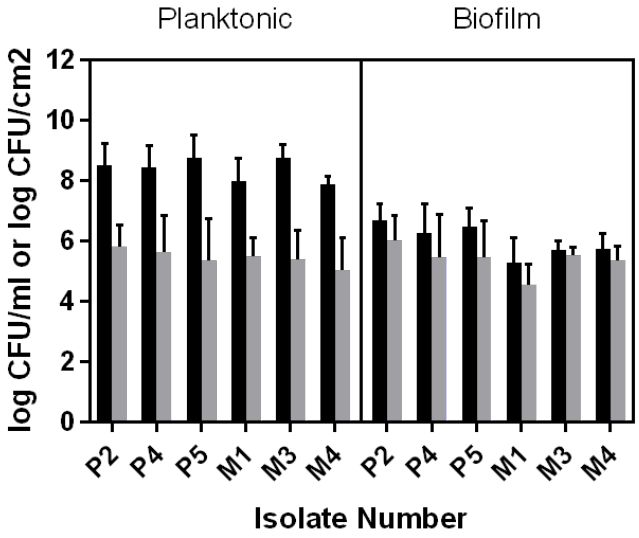
6.3 Results

6.3.1 Sporulation percentages in planktonic and biofilm populations

The total cells and spores for six *B. cereus* isolates grown in TSB and milk after three days of incubation are in Fig. 6. 1 and Fig. 6. 2, respectively, showing the different sporulation percentages between isolates. The corresponding sporulation percentages are in Fig. 6. 1B and Fig. 6. 2B. The cell and spore count for one- and two-days incubation are shown in supplementary files (section 6. 8. 1 and 6. 8. 2).

In TSB after three days incubation at 30°C, all the isolates reached 8 to 9 log CFU/mL in planktonic growth. Biofilm numbers varied between isolates with 6 to 7 log CFU/cm^2 (Fig. 6. 1A). The spore percentage in the planktonic population was all less than 5% for all isolates except M1 which had 6.43% spores (Fig. 6. 1B). Within the biofilm populations, the spore percentage varied between 23.85% for P5 and up to 69.36% for M3, with all of the isolates containing higher percentages of spores in biofilm than planktonic population (Fig. 6. 1B).

A



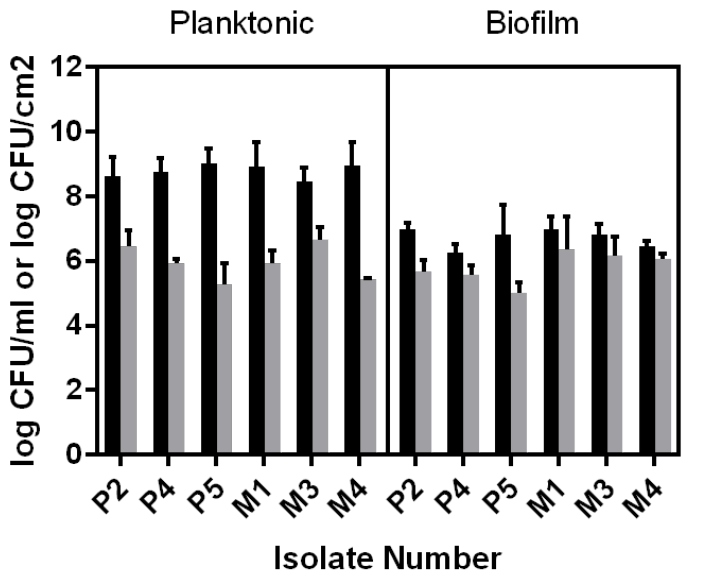
B

	Planktonic (%)	Biofilm (%)
P2	2.59	27.52*
P4	2.07	29.60*
P5	0.43	23.85
M1	6.43	28.02
M3	0.18	69.36*
M4	1.14	45.51*

Figure 6. 1 The sporulation percentages of six *B. cereus* isolates in planktonic culture and biofilms grown on SS coupons in TSB after three days of incubation at 30°C. The corresponding sporulation percentages were calculated and recorded in table B. Each column/data in the table indicates the average number within three independent biological replicates and error bars in the graph represent the standard deviation. Statistical analysis was conducted to compare the significant difference in sporulation between the planktonic and biofilm populations, expressed with "*" ($P < 0.05$).

In milk, after 3 days incubation at 30°C, the total number of planktonic and biofilm cells was like TSB with approximately 9 log CFU/mL of total planktonic cells and 6 – 7 log CFU/cm^2 total biofilm cells for all six isolates (Fig. 6. 2). The spore percentage in the planktonic culture, was less than 2% for all isolates except M3 which had 4.1% of spores (Fig. 6. 2B). In the biofilm population, the percentage of spores was higher the planktonic population for all isolates, especially for M1 and M4, showing significantly ($P < 0.05$) higher percentages in biofilm compared to planktonic populations (Fig. 6. 2B).

A



B

	Planktonic (%)	Biofilm (%)
P2	1.21	5.21
P4	0.18	24.08
P5	0.03	13.27
M1	0.14	44.80*
M3	4.10	26.87
M4	0.08	44.92*

Figure 6. 2 The sporulation percentages of six *B. cereus* isolates in planktonic culture and biofilms grown on SS coupons in milk after three days of incubation at 30°C. The corresponding sporulation percentages were calculated and recorded in table B. Each column/data in the table indicates the average number within three independent biological replicates and error bars in the graph represent standard deviation. Statistical analysis was conducted to compare the significant difference in sporulation between the planktonic and biofilm populations, expressed with "*" ($P < 0.05$).

6.3.2 Heat resistance of spores

The survival curves of spores during heat treatment are included in supplementary files (section 6. 8. 3 and 6. 8. 4). The D value is the time it takes to reduce a microbial population by one log at a defined temperature, therefore, the higher D value at a defined temperature the greater the heat resistance. Fig. 6. 3 and Fig. 6. 4 show the $D_{90°C}$ values of spores grown in TSB and milk, respectively. The initial spore counts for both planktonic and biofilm spores for all isolates were 10^6-10^7 CFU/mL.

The heat resistance of spores grown in biofilm was higher than those grown in planktonic culture, especially for P2, P4, M3 and M4 where the $D_{90°C}$ values of spores isolated from biofilms ($D_{90°C}$ = 7.22 ± 1.36 min, 12.82 ± 1.18 min, 12.09 ± 0.55 min and 6.89 ± 0.25 min, respectively) were significantly ($P < 0.05$) higher than spores isolated from planktonic cultures ($D_{90°C}$ = 3.08 ± 0.52 min, 6.47 ± 0.11 min, 5.67 ± 1.1 min and 4.16 ± 0.76 min, respectively). Although there was no

statistically significant difference in the $D_{90°C}$ values between planktonic and biofilm spores for P5 and M1, the overall trend was the same with biofilm spores more resistant than planktonic spores.

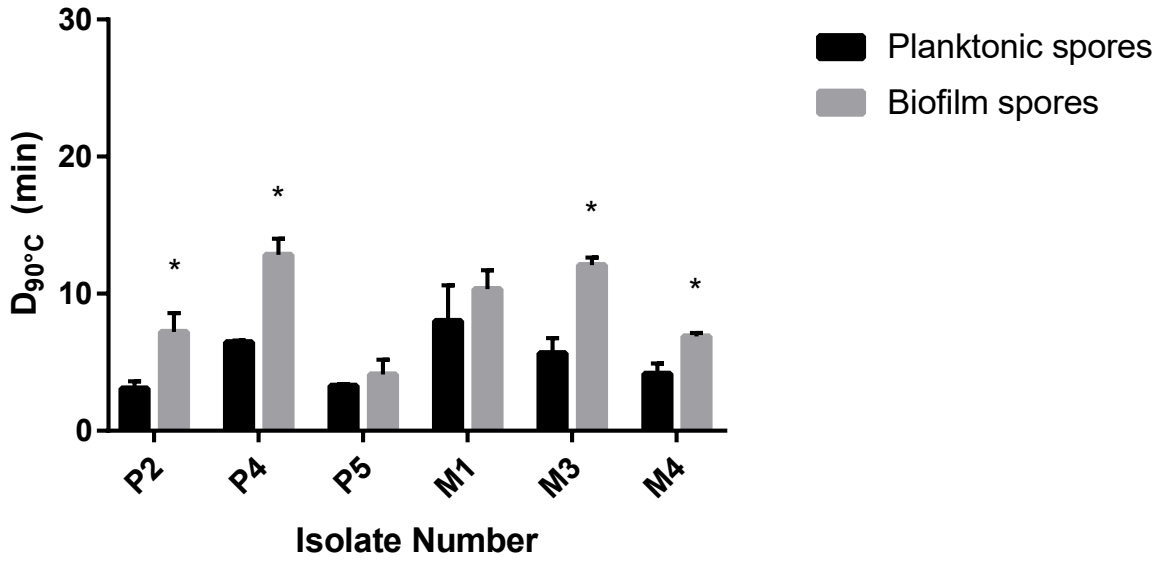


Figure 6. 3 The $D_{90°C}$ values of spores from either planktonic or biofilm populations of six isolates grown in TSB. The $D_{90°C}$ values were the average values derived from three independent biological replicates. Significant differences are indicated by showing "*" indicating $P < 0.05$.

The spores of six isolates were harvested from milk and the heat resistance of the planktonic and biofilm spores was compared, with results shown in Fig. 6. 4. The heat resistance of spores grown in milk were similar to the spores grown in TSB, with an overall trend of higher heat resistance of spores from biofilm compared to the corresponding spores from planktonic culture. This was especially true for P4 and M1 that were significantly ($P < 0.05$) higher with $D_{90°C}$ values of 21.52 ± 3.4 min and 17.65 ± 5.02 min, respectively, for spores from biofilms, compared to $D_{90°C}$ values of 12.48 ± 4.49 min and 8.76 ± 1.39 min, respectively, for spores from planktonic culture. Notably, the spores harvested from milk were more heat resistant than those harvested from TSB for both planktonic and biofilm spores, although this was not statistically significant ($P > 0.05$, statistical data is not shown).

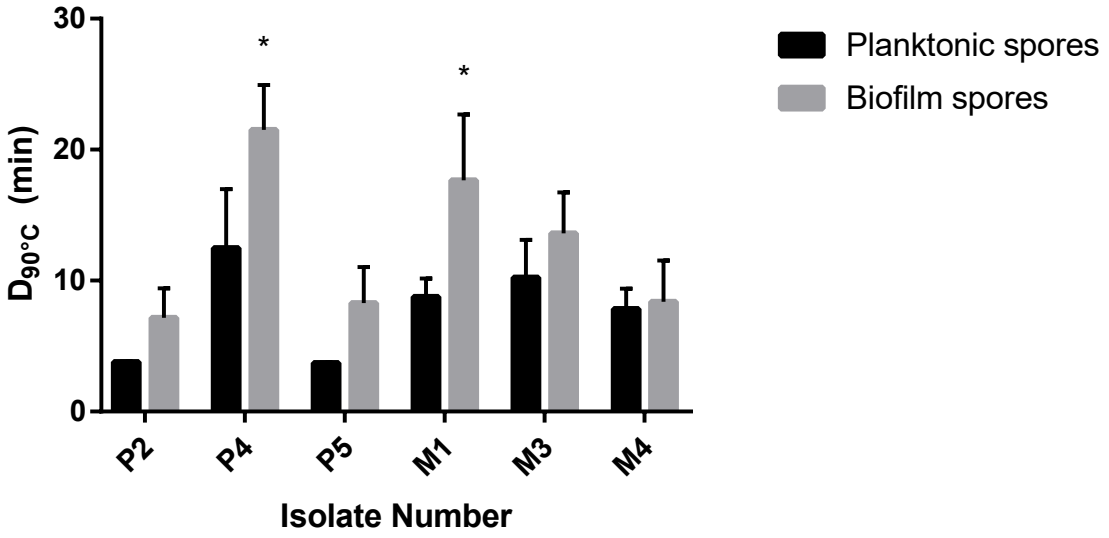


Figure 6. 4 The $D_{90°C}$ values of spores from either planktonic or biofilm populations of six isolates grown in milk. The $D_{90°C}$ values were the average value derived from three independent biological replicates. Significant differences are indicated by showing "*" indicating $P < 0.05$.

6.3.3 DPA content

The DPA content released from spores harvested from TSB and milk was measured using a Spectro fluorimeter after binding with terbium ions (Tb^{3+}), shown in Figs 6. 5 and 6. 6, respectively. The DPA content in spores of M4 from biofilms cultivated in TSB was significantly higher ($P < 0.05$) than spores from planktonic culture (Fig. 6. 5), suggesting the DPA content may play a role in heat resistance for M4 biofilm spores. The DPA content in the biofilm and planktonic spores of P2 and P4 were comparable ($P > 0.05$; Fig. 6. 5). Interestingly, significantly higher ($P < 0.05$) DPA was measured in spores from planktonic culture of M1 than from biofilms (Fig. 6. 5) while there was no significant difference in the heat resistance between the spores isolated from the two populations when grown in TSB (Fig. 6. 3). On the other hand, the heat resistance of spores harvested from biofilms was significantly ($P < 0.05$) higher than from the planktonic population for M3 when it was grown in TSB (Fig. 6. 3), but the DPA content between these two types of spores for this isolate was comparable (Fig. 6. 5).

M3 showed a significantly higher ($P < 0.05$) amount of DPA in its spores from planktonic culture compared with biofilms when grown in milk (Fig. 6. 6), while the heat resistance of spores from the

two populations was comparable (Fig. 6. 4) although spores from biofilm showed more heat resistance than those from planktonic cells (the reverses of what we would expect based on the DPA content). No significant difference in DPA content in biofilm and planktonic spores was seen for isolates P2, P4, P5, M1 and M4 when cultivated in milk, except for M3 (Fig. 6. 6).

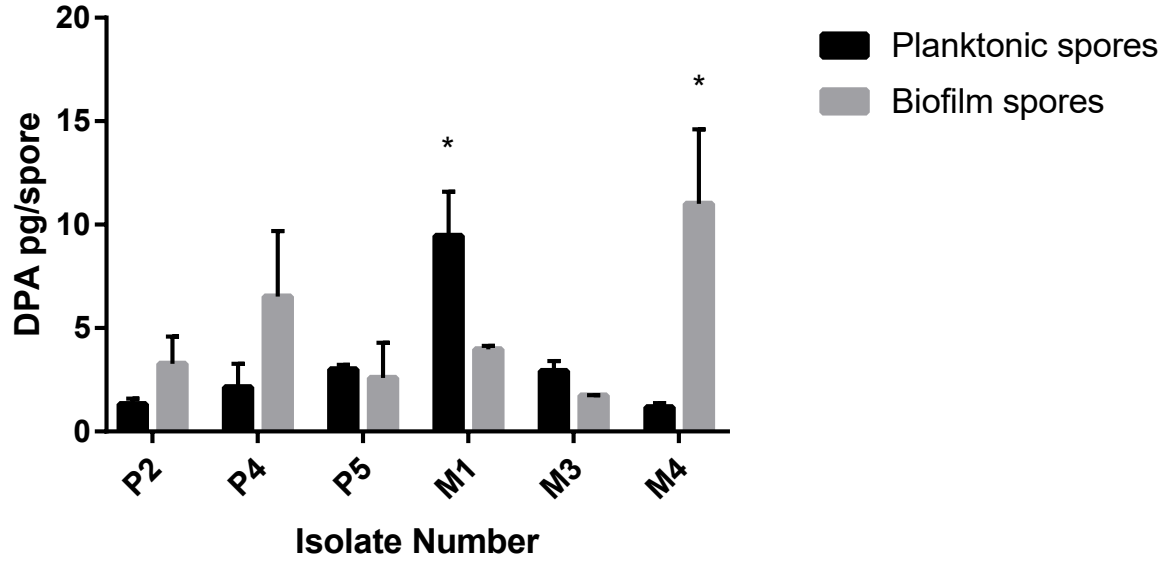


Figure 6. 5 DPA content of spores harvested from TSB. The DPA pg/spore was the average value derived from three independent biological replicates for each isolate. Significant differences between planktonic and biofilm spores are indicated by "*" indicating $P < 0.05$.

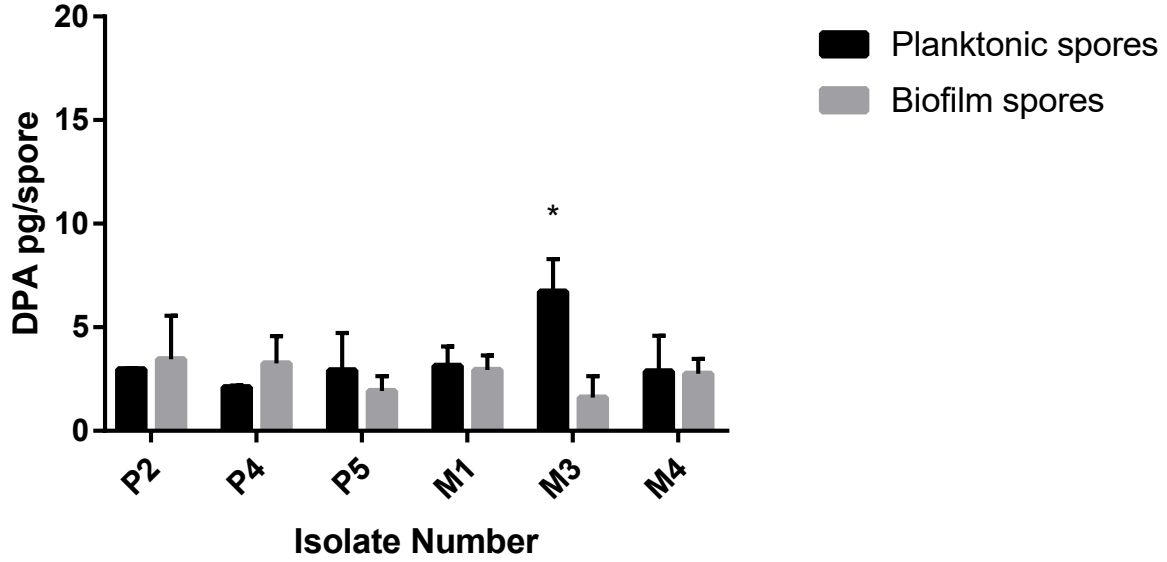


Figure 6. 6 DPA content of spores harvested from milk. The DPA pg/spore was the average value derived from three independent biological replicates for each isolate. Significant differences between planktonic and biofilm spores are indicated by "*", indicating $P < 0.05$, respectively.

6.3.4 TEM of spores

TEM was used to compare the intracellular structural differences between spores harvested from planktonic and biofilm populations. Three representative TEM pictures of spores isolated from planktonic and biofilm cultures of P4, M1 and M3 are shown in Figs. 6. 7 – 6. 12 cultured in either TSB or milk. The named structures within the spore were referenced to Lv et al., (2019).

When grown in TSB, the biofilm spores of P4 were highly structured with exosporium, coat, cortex and core observed under TEM (Fig. 6. 7, B1 – B3). However, the one layer labelled the coat, appeared to be fragmented in spores of P4 from the planktonic culture compared to spores from biofilms (Fig. 6. 7, A1 - A3). Irregular shaped coats were observed for M1 and M3 from planktonic culture grown in TSB (Figs. 6. 8 and 6. 9, A1 – A3), while the coat for biofilm spores was clearly defined with solid integrity (Figs. 6. 8 and 6. 9, B1 – B3).

When grown in milk, the spores from biofilm and planktonic cultures of the P4 showed a similar structure (Fig. 6. 10), although parts of the coat of the spores from planktonic culture tended to be broken (Fig. 6. 10, A1 – A3). The coats of spores from planktonic cultures of M1 and M3 cultivated

in milk were irregular, while the coat for spores from biofilm were clearly defined (Fig. 6. 11 and 6. 12), like the spores from TSB.

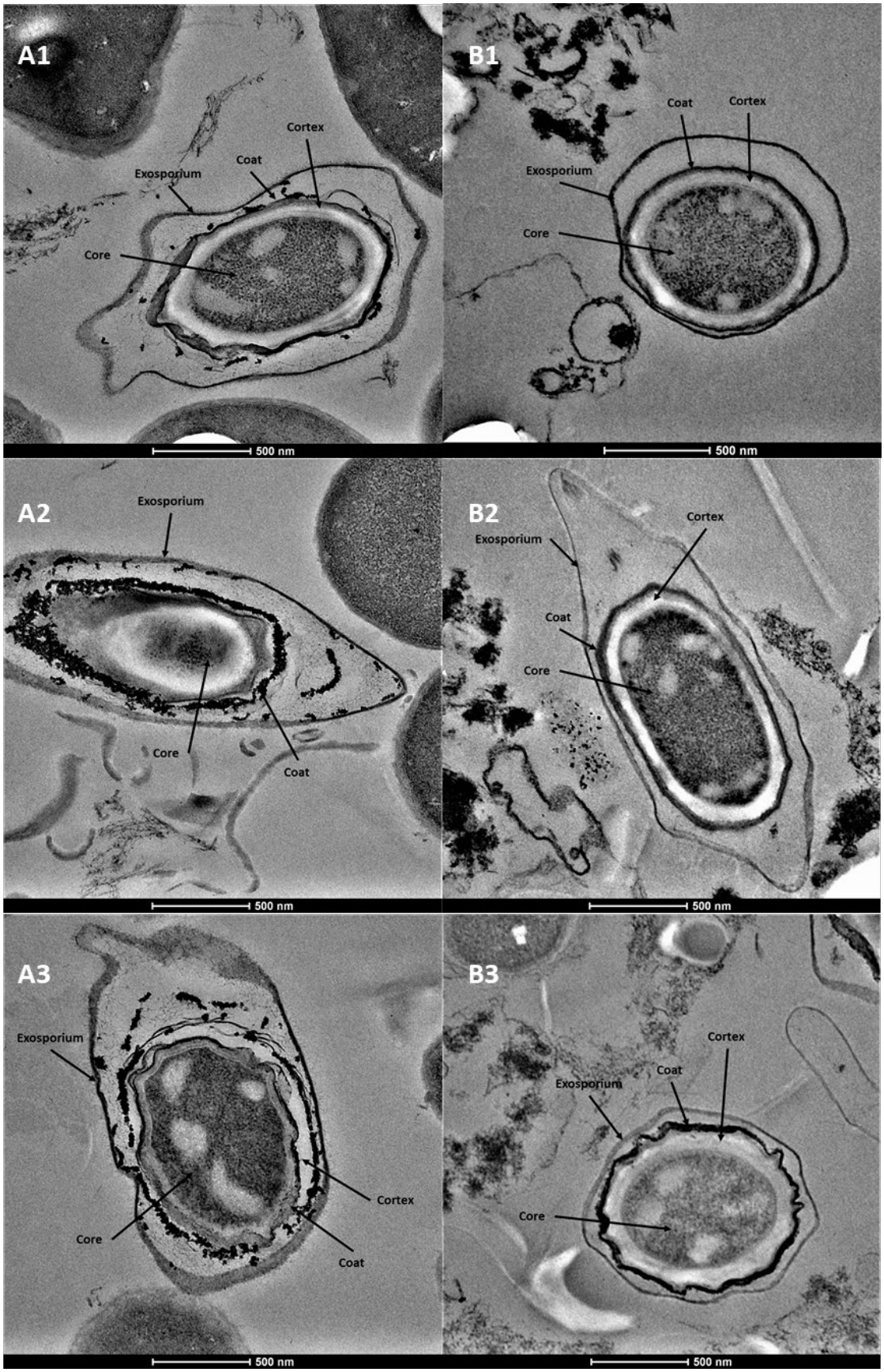


Figure 6. 7 Three representative TEM pictures of spores for P4 grown in TSB. A1, A2 and A3 are spores from planktonic culture, while B1, B2 and B3 are spores from biofilm.

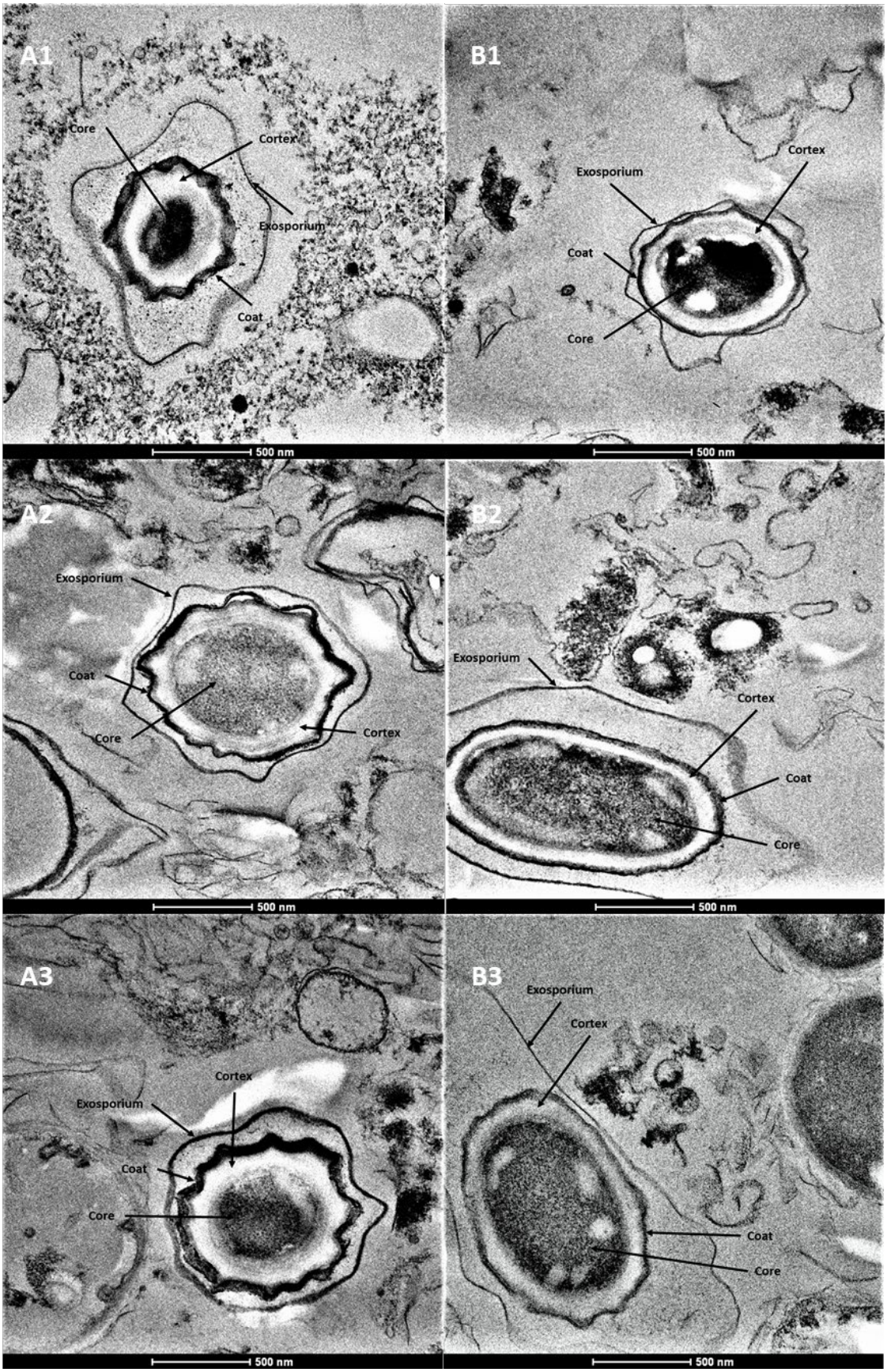


Figure 6. 8 Three representative TEM pictures of spores for M1 grown in TSB. A1, A2 and A3 are spores from planktonic culture, while B1, B2 and B3 are spores from biofilm.

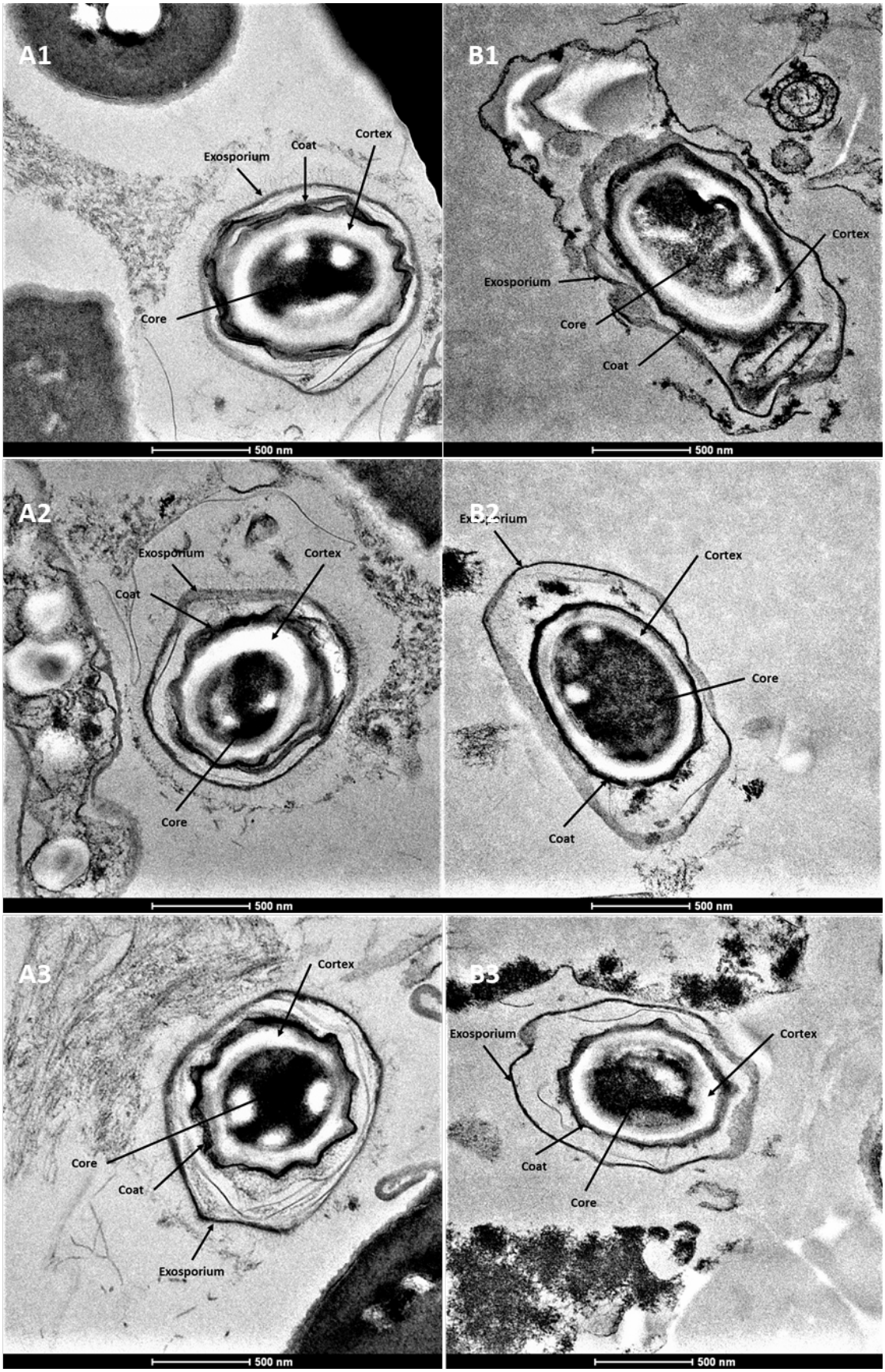


Figure 6. 9 Three representative TEM pictures of spores for M3 grown in TSB. A1, A2 and A3 are spores from planktonic culture, while B1, B2 and B3 are spores from biofilm.

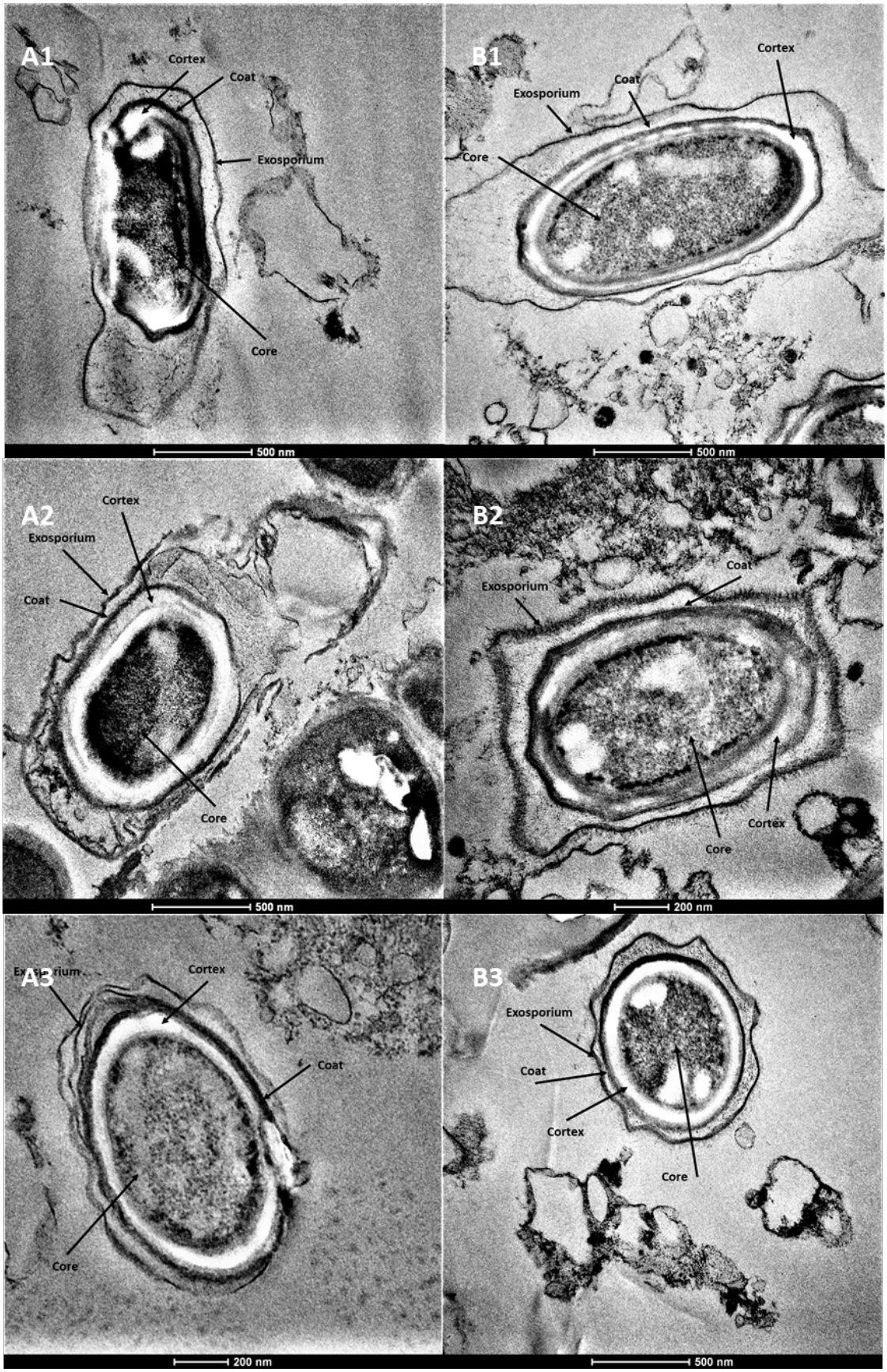


Figure 6. 10 Three representative TEM pictures of spores for P4 grown in milk. A1, A2 and A3 are spores from planktonic culture, while B1, B2 and B3 are spores from biofilm.

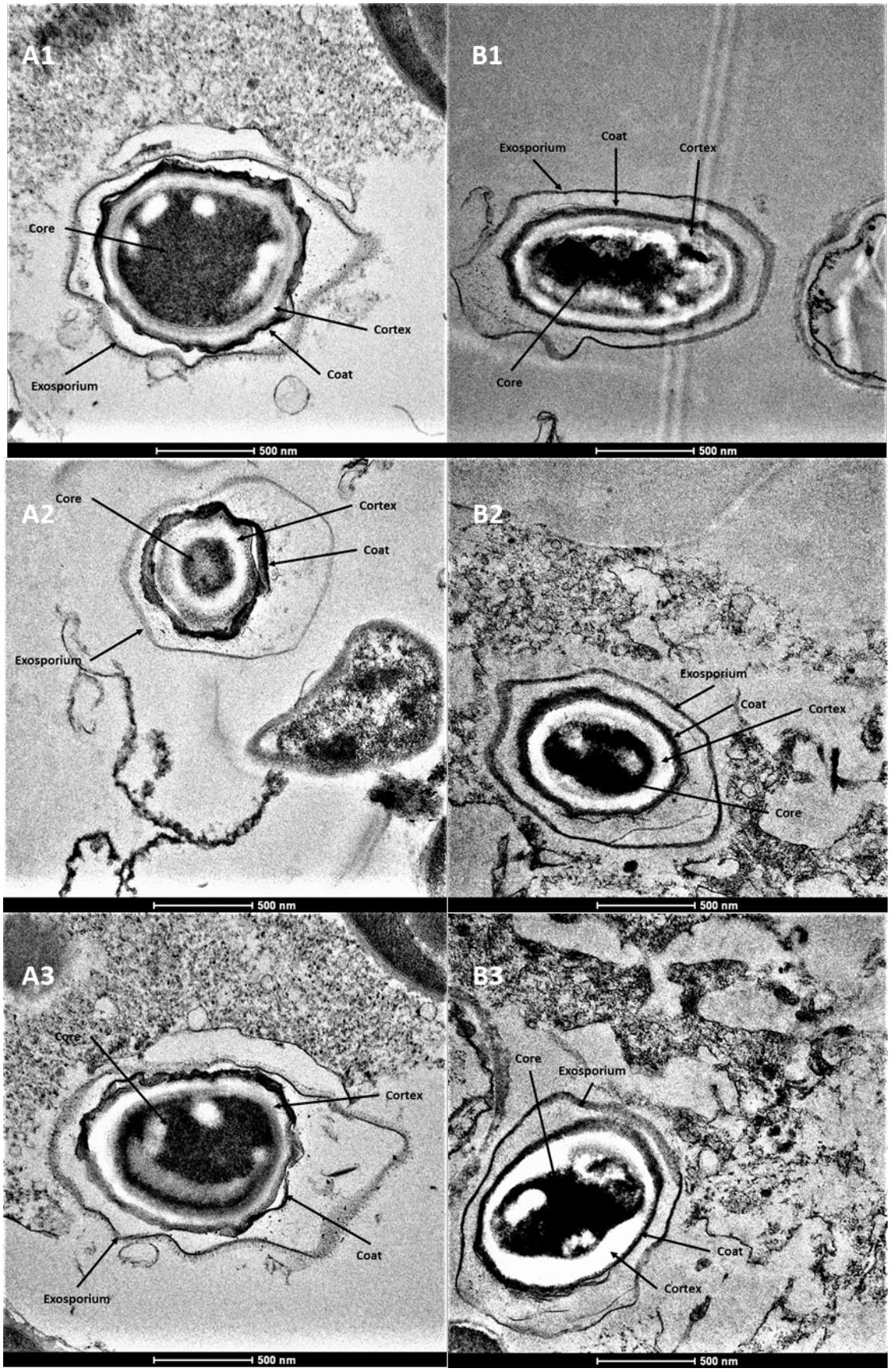


Figure 6. 11 Three representative TEM pictures of spores for M1 grown in milk. A1, A2 and A3 are spores from planktonic culture, while B1, B2 and B3 are spores from biofilm.

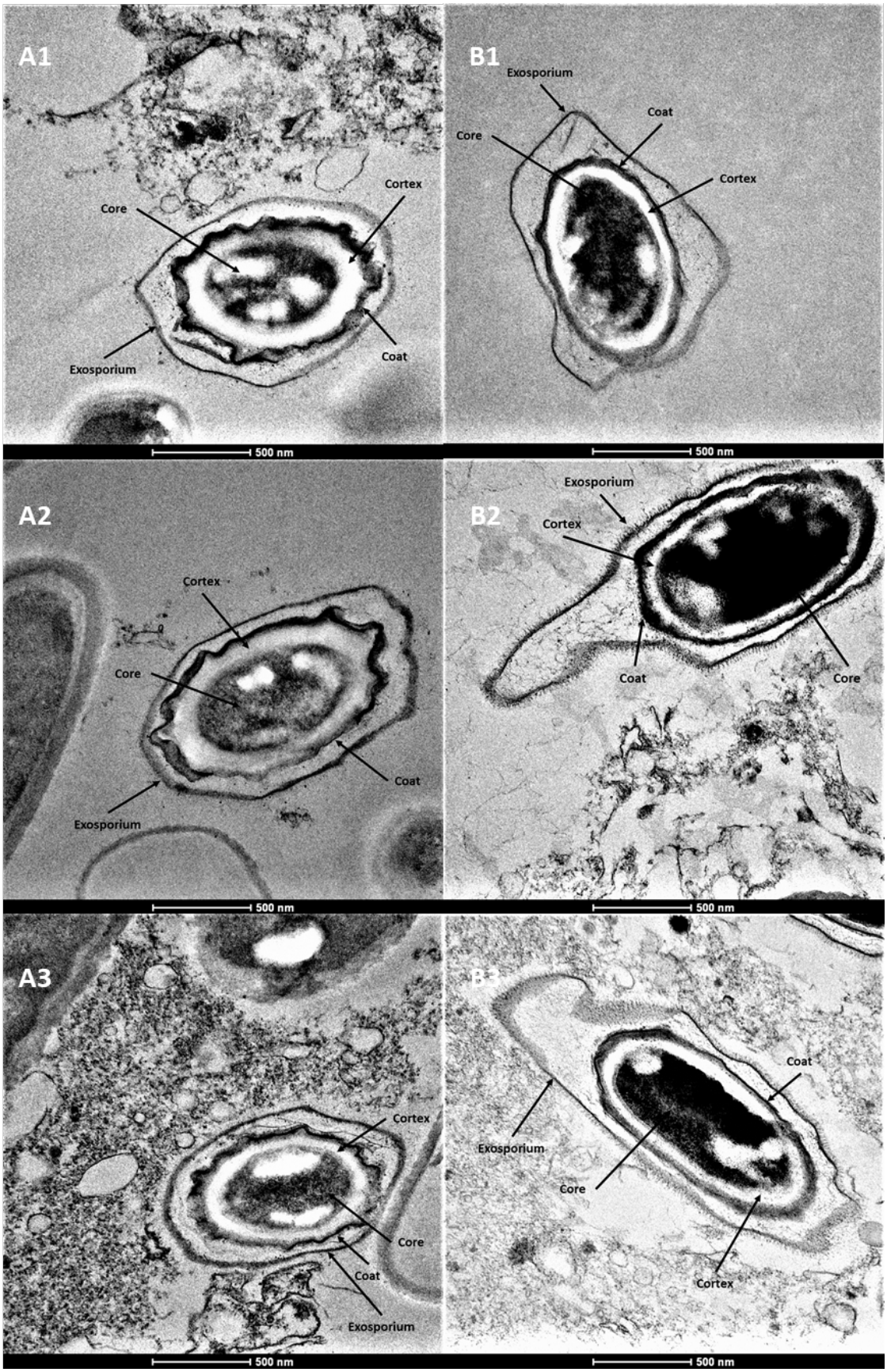


Figure 6. 12 Three representative TEM pictures of spores for M3 grown in milk. A1, A2 and A3 are spores from planktonic culture, while B1, B2 and B3 are spores from biofilm.

6.4 Discussion

Biofilms of *B. cereus* in food manufacturing plants can be a source of contamination for food products. The spores of *B. cereus* are of particular concern as they are resistant to heat and chemical treatment. To characterize the spores in the biofilms, six *B. cereus* food isolates were grown in both TSB and milk, on stainless-steel (SS) coupons and compared with planktonic cultures. The sporulation percentages were investigated for all six isolates in both TSB and milk. Higher sporulation percentages in the biofilm population were observed compared to the planktonic culture from days 1 – 3 of growth. This is consistent with the study done by Wijman et al., (2007) who reported that most cells (up to 90%) in the air-liquid interface biofilms for *B. cereus* were spores. The higher sporulation efficiency in biofilms may be explained by the bacterial response to the high cell density in the biofilms and limited access to nutrients within the biofilm compared to planktonic growth where surplus nutrients are presented (van Gestel et al., 2012). The higher sporulation percentages were observed in biofilms cultured in both TSB and milk, suggesting that the chance of contamination of dairy products with spores of *B. cereus* increases, once *B. cereus* is present as a biofilm.

In addition, spores from biofilms were more heat resistant than spores harvested from the liquid sources, also shown by Simmonds et al., (2003) who measured higher $D_{90°C}$ values of spores isolated from SS surfaces (3.87 to 5.82 min) compared with spores harvested from planktonic culture (1.59 to 2.99 min) for three *B. cereus* strains. In the present study where all the isolates grown in both TSB and milk, especially P4, showed significantly ($P < 0.05$) higher $D_{90°C}$ values for spores from biofilm than from planktonic culture. The enhanced heat resistance of biofilm spores may relate to the increased metal availability from SS which comprises over 70% of iron, and 1% of manganese (Hayrapetyan et al., 2016). However, the mechanism by which surface metals could influence the spore heat resistance of *B. cereus* is unknown. The hydrophobic nature of a SS surface allows the firm attachment of spores (Doyle et al., 1984; Parkar et al., 2001b), and this could be another surface property which may influence the heat resistance of spores. In addition, these results highlight that in

D value calculations for *B. cereus* spores and perhaps for all bacterial spore D value calculations, as spores produced by biofilm are more important than those from planktonic sources.

The higher heat resistance of spores harvested from biofilms than its from planktonic population was also observed when grown in milk, suggesting that biofilms of *B. cereus* in the dairy industry can be a source of heat resistant *B. cereus* spores. Spores harvested from milk showed a slightly higher heat resistance than spores from TSB, although it was not statistically significant ($P > 0.05$). This may be explained by the presence of milk proteins, such as heat-stable casein micelles and/or denatured whey protein (Anema, 2020), which possibly attached/interacted with the surfaces of the spores and therefore protected the spore from heat treatment.

Dipicolinic acid (pyridine-2,6-dicarboxylic acid or PDC and DPA) comprises 5% to 15% of the dry weight of bacterial spores (Setlow, 2006). Within the spore, DPA binds with divalent cations (mainly Ca^{2+}) and this chelated form of DPA is thought to be one component of the spore wall responsible for the heat resistance of spores (Slieman and Nicholson, 2001; Setlow, 2006). In the present study, DPA content was measured in biofilm and planktonic spores after growing in either TSB or milk. Only M4 showed significantly ($P < 0.05$) higher amounts of DPA in the spores from biofilm compared with spores from planktonic culture when grown in TSB, while the DPA content in other isolates was comparable ($P > 0.05$) between spores from biofilm or planktonic culture in either TSB or milk. M1 grown in TSB and M3 grown in milk showed significantly ($P < 0.05$) higher DPA content in their planktonic spores than biofilm spores (Figs. 6. 5 and 6. 6), while the heat resistance of planktonic and biofilm spores was comparable (Figs. 6. 3 and 6. 4), indicating that DPA levels had no effect on the heat resistance of spores for M1 and M3. Hayrapetyan et al., (2016) showed a $D_{95°C}$ value of 17 min and 22 min for two strains of *B. cereus* (NIZO 4080 and ATCC 10987) and suggested that the heat resistance of spores and their DPA content was not always correlated. This is in agreement with Kort et al., (2005) who also reported that DPA levels do not correlate with heat resistance. These previous findings support the present study in that the D values and effect of DPA content on heat resistance are not clearly correlated and may be strain dependent.

The multiple protective layers: inner membrane, cortex, and inner and outer coats observed in bacterial spores are also thought to contribute to heat resistance (Abee et al., 2011). In the present work, TEM identified potential differences in the structure of the spores harvested from biofilm and planktonic populations. The isolates P4, M1 and M3 were chosen as representative isolates for TEM imaging due to their different correlations between the heat resistance and DPA content. All the spores from biofilms of these three isolates showed morphologically intact coats compared with those spores from planktonic culture which were observed to be fragmented for P4 or showed irregular shaped coats for M1 and M3 (Fig. 6. 7 – 6. 12). The coat of a spore is a multi-layered shield comprised of approximately 30 proteins, providing integrity and is fundamental to the spore heat resistance and germination (Driks, 2002). ExsY and CotY are responsible for assembling the coat of the *B. cereus* spore, and a decreased heat resistance occurs in the spores from *exsY* and *cotY* deleted mutants (Johnson et al., 2006). Abee et al., (2011) hypothesized that GerP proteins may play a role in morphogenetic and structural features of the spore coat by affecting the permeability and access to germinating factors. Kutima and Foegeding (1987) demonstrated that coat-defective spores may rapidly lose their heat resistance in the early stage of germination, suggesting that the germination process may differ between spores from biofilm and planktonic culture. The function of the coat in the resistance of spores has been studied for the model spore former, *Bacillus subtilis* (*B. subtilis*). Riesenman and Nicholson (2000) showed that the coat in the spore plays a role in the resistance of *B. subtilis* spores to environmental stresses such as UV radiation and the two morphogenetic proteins, SpoIVA and CotE, are key factors in the structure of the coat for *B. subtilis* (Roels et al., 1992; Little and Driks, 2001; Driks, 2002). The abundance of spore coat proteins was compared between spores harvested from a solid agar plate and liquid medium by Abhyankar et al., (2016), showing that some crust proteins, such as CotX and CotY, were more abundant in the spores harvested from solid media (which may be considered like the biofilm grown on surfaces) than liquid culture. This may indicate that the protein composition in spores from biofilm may differ from those from planktonic culture, which therefore affects the heat resistance. The expression of those genes mentioned above were extracted from the RNA sequencing data (additional to Chapter 5), shown in supplementary file section 6. 8. 5. However, most of the genes related to the coat were upregulated in planktonic cells.

This may be explained by the fact that P5 was used to conduct the transcriptomic analysis which is not a representative model for heat resistance study of spores (no significant difference was found in both TSB and milk growth conditions, Figs. 6. 3 and 6. 4). To further study the expression patterns of genes related to the spore' coat, a better representative isolate such as P4 should be used.

6.5 Conclusions

B. cereus spores are a concern for the dairy industry resulting in the contamination of dairy products with the potential to cause illness and spoilage. Biofilms of *B. cereus* contain higher numbers and more heat resistant spores compared to planktonic cultures. This may correlate with the DPA content in spores, however, the effect of DPA content on heat resistance of spores seems to be strain dependent. The coat differs between biofilm and planktonic spores, suggesting protein patterns in the coat may play a role in the heat resistance of the spore. In this study, three factors, biofilm formation, DPA content and spore structure were considered affecting the heat resistance of spores in milk, highlighting the potential safety and spoilage issues relating to *B. cereus* biofilms. This suggests more severe heat treatments are needed to control *B. cereus* spores from biofilms compared with spores from planktonic cultures.

6.6 Acknowledgement

We would like to thank Yanyu He and Raoul Solomon at the Manawatu Microscopy and Imaging Centre (Palmerston North, New Zealand) for processing the samples for TEM imaging.

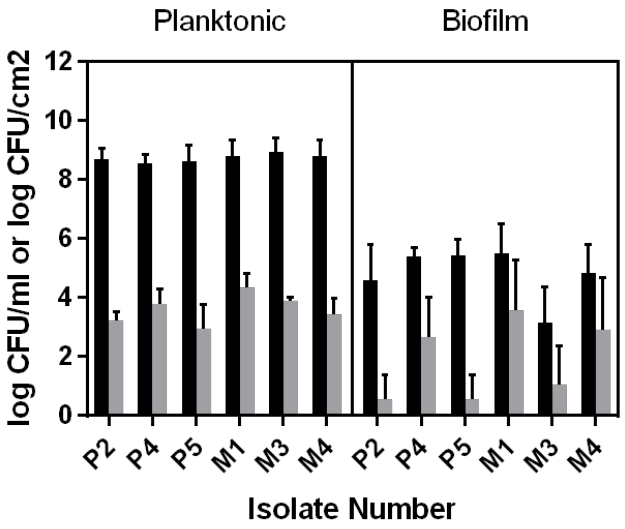
6.7 Copyright information

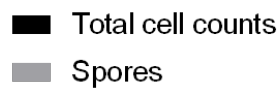
Part of this work has been published in International Dairy Journal, and the Online Statement of Contribution form is attached in Appendix IV.

6.8 Supplementary files

6.8.1 Total cell counts and spores in TSB after one- and two-days incubation

A1

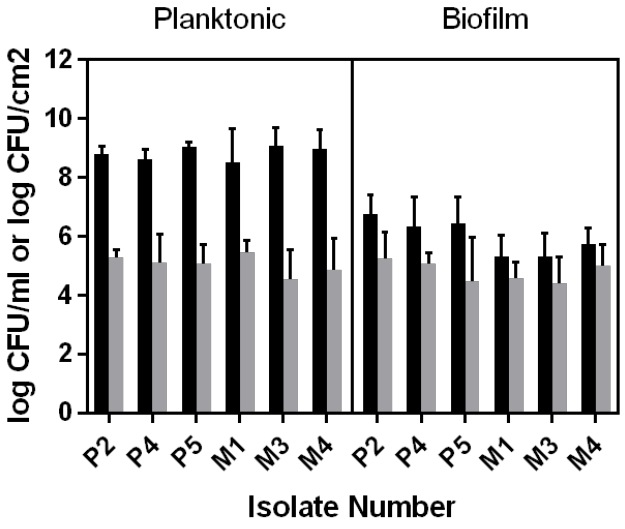


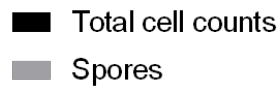


A2

	Planktonic (%)	Biofilm (%)
P2	0.0004	1.88
P4	0.003	2.03
P5	0.0003	0.02
M1	0.005	5.06
M3	0.002	1.67
M4	0.001	6.06*

B1





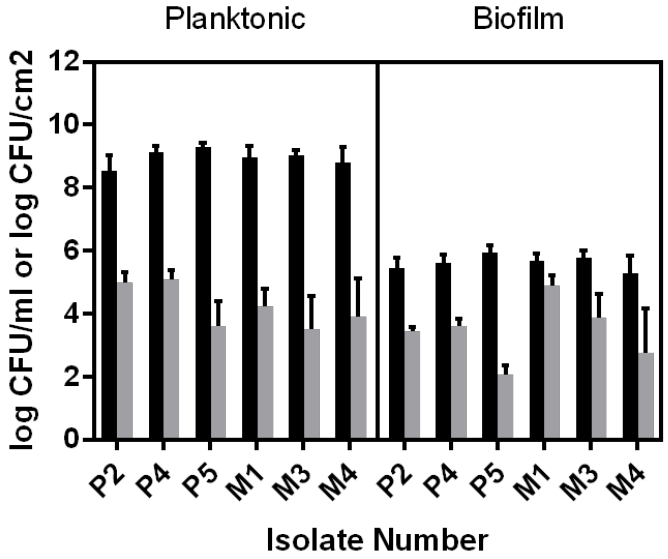
B2

	Planktonic (%)	Biofilm (%)
P2	0.05	11.49
P4	0.19	19.34
P5	0.03	3.78
M1	2.79	30.23*
M3	0.02	33.41*
M4	0.15	23.18*

Figure S6. 1 The sporulation percentages of six *B. cereus* isolates in planktonic culture and biofilms were grown on stainless-steel coupons in TSB after one day (A1) and two days (B1) incubation. The corresponding sporulation percentages were calculated by (spores' numbers / total cell numbers) %, indicated in tables A1 (day one) and B1 (day two). Each column/data in the tables indicates the average number within replicates and error bars represent standard deviation. Statistical analysis compared the significant difference of sporulation in planktonic and biofilm, expressed with "*" ($P < 0.05$).

6.8.2 Total cell counts and spores in milk after one- and two-days incubation

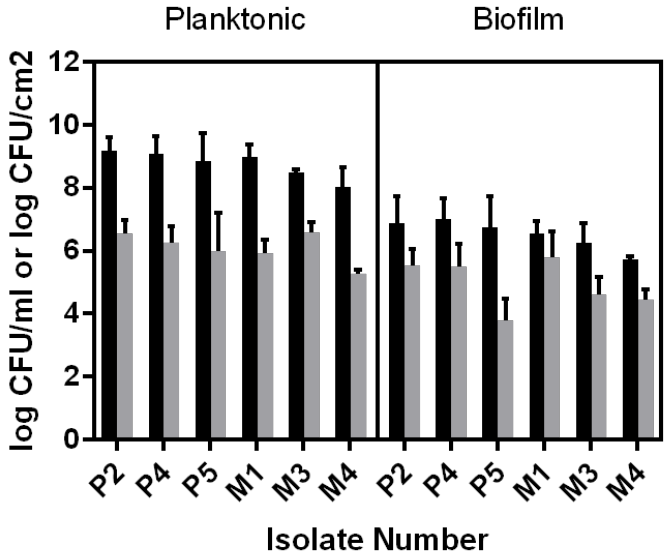
A1



A2

	Planktonic (%)	Biofilm (%)
P2	0.11	1.20
P4	0.01	1.36
P5	0.0004	0.01
M1	0.005	16.75*
M3	0.001	2.03
M4	0.04	1.98

B1



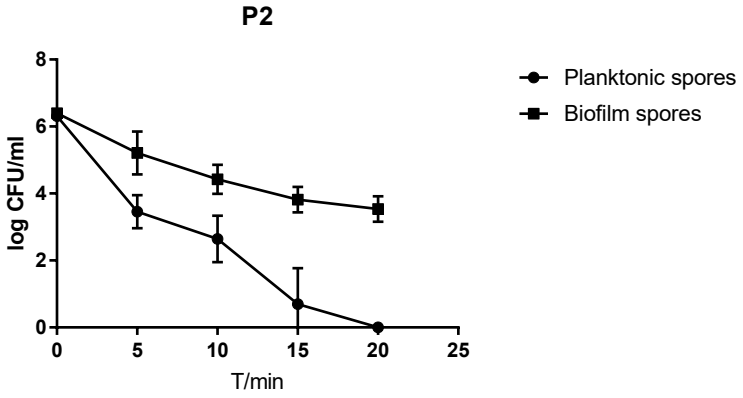
B2

	Planktonic (%)	Biofilm (%)
P2	0.25	12.03
P4	0.16	3.49
P5	0.19	5.05
M1	0.09	25.61*
M3	1.43	2.70
M4	0.49	5.88

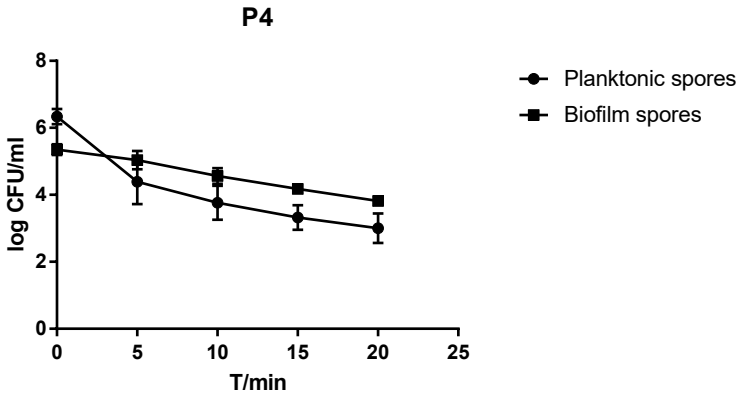
Figure S6. 2 The sporulation percentages of six *B. cereus* isolates in planktonic culture and biofilms were grown on stainless-steel coupons in milk after one day (A1) and two days (B1) incubation. The corresponding sporulation percentages were calculated by (spores' numbers / total cell numbers) %, indicated in tables A1 (day one) and B1 (day two). Each column/data in the tables indicates the average number within replicates and error bars represent standard deviation. Statistical analysis compared the significant difference of sporulation in planktonic and biofilm, expressed with "*" ($P < 0.05$).

6.8.3 Inactivation curves (at 90°C) of spores harvested from TSB

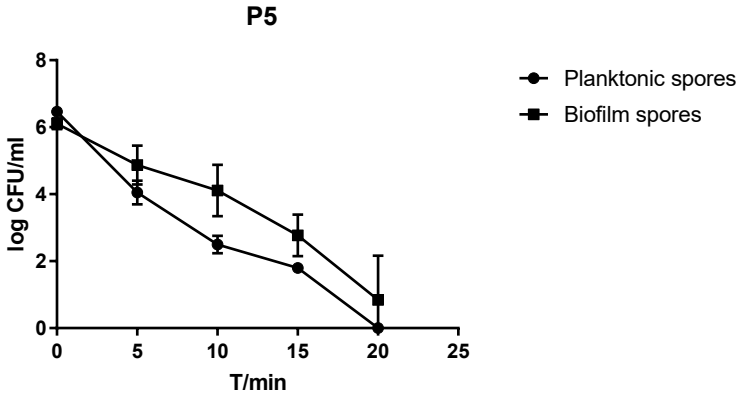
A



B



C



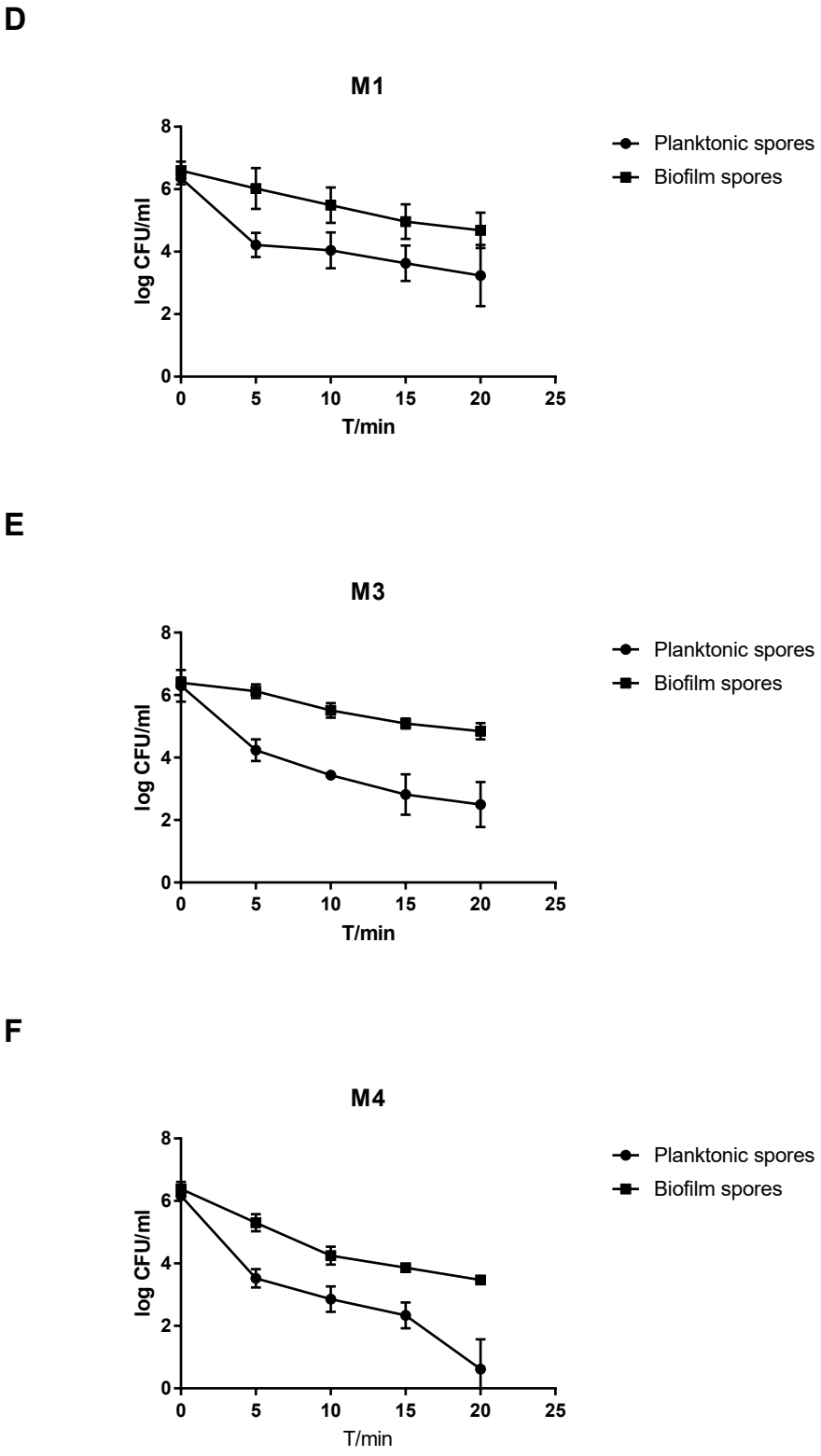
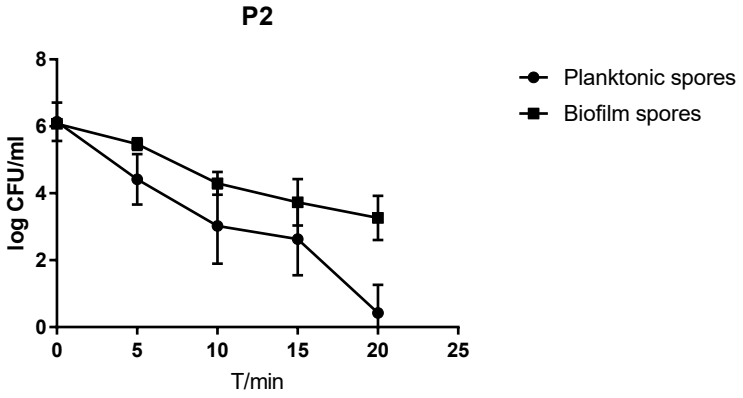


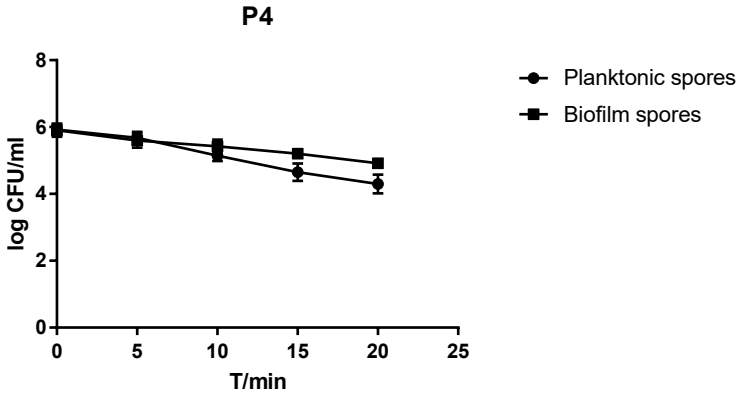
Figure S6. 3 The inactivation curves of planktonic and biofilm spores of six *B. cereus* isolates grown in TSB after three days of incubation. The surviving cells were measured after 5 min, 10 min, 15 min and 20 min after heat treatment at 90°C and were counted after recovery on TSA. The initial spore counts were measured after heating culture at 80°C for 10 min. Each point indicates the average number within replicates and error bars represent standard deviation.

6.8.4 Inactivation curves (at 90°C) of spores harvested from milk

A



B



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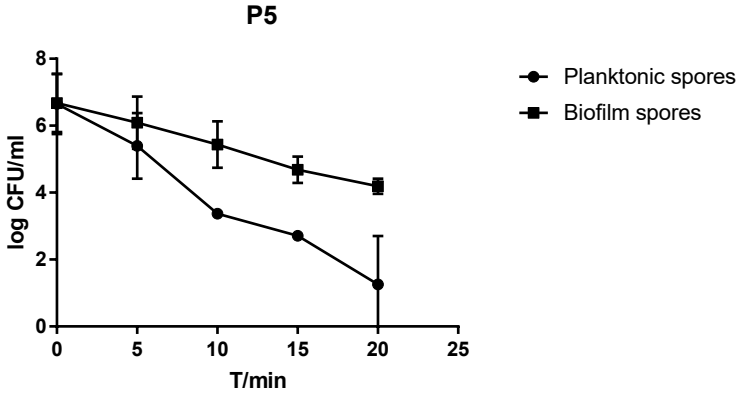

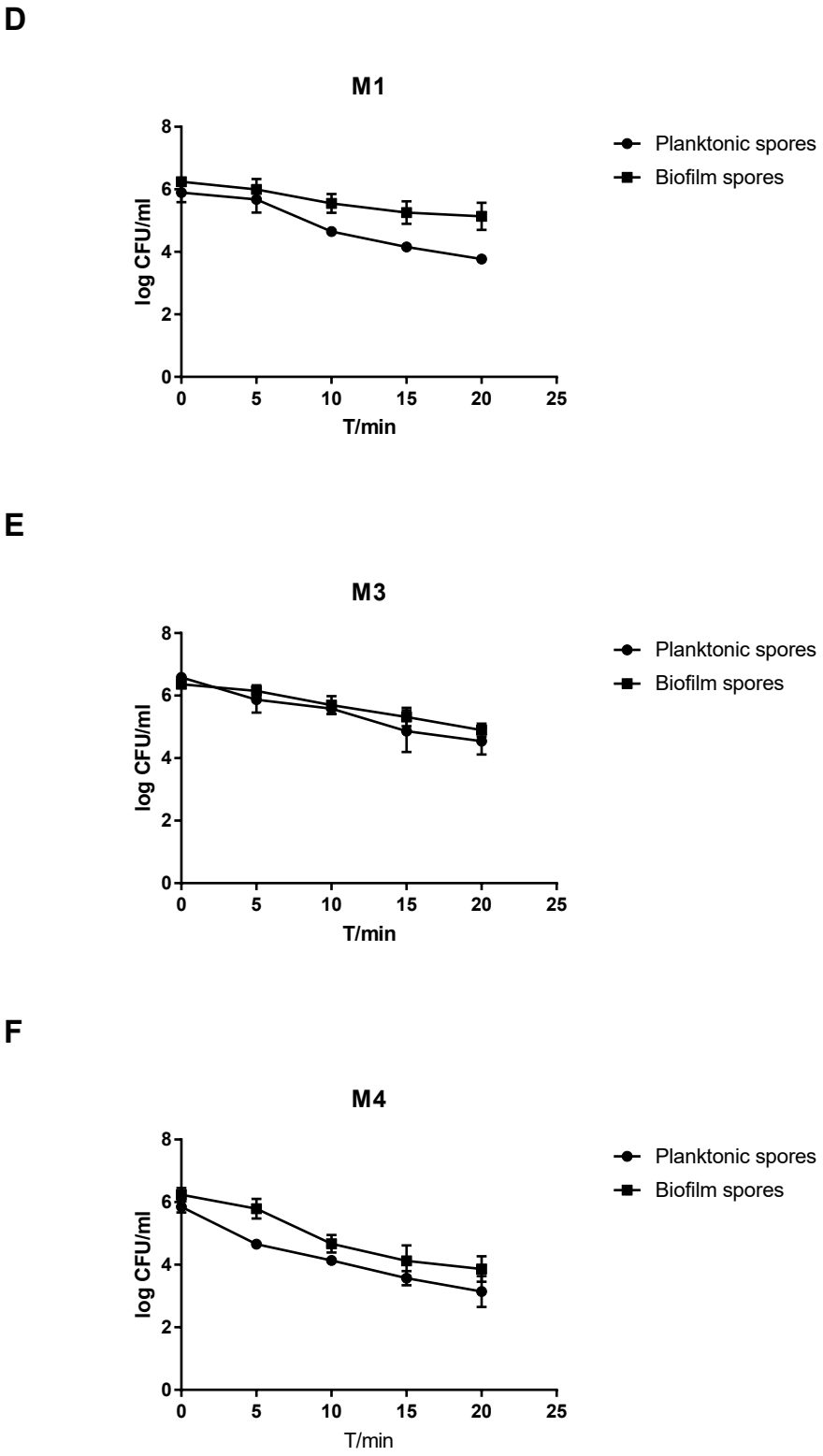

Figure S6. 4 The inactivation curves of planktonic and biofilm spores of six *B. cereus* isolates grown in milk after three days of incubation. The surviving cells were measured after 5 min, 10 min, 15 min and 20 min after heat treatment at 90°C and were counted after recovery on TSA. The initial spore counts were measured after heating culture at 80°C for 10 min. Each point indicates the average number within replicates and error bars represent standard deviation.

6.8.5 Expression of genes related to the spore coat (data from RNA sequencing, additional to Chapter 5)

Table S6. 1 The list of genes related to the spore coat of P5 extracted from RNA sequencing data from section 5, which were differentially expressed in biofilm *versus* planktonic cells.

Gene_id	Protein	Biofilm counts	Planktonic counts	Lfc[a]	FDR[b]
BC_1218	Exosporium protein ExsY	4	11	-1.53	7.55E-06
BC_1222	Spore coat protein CotY	760	2591	-1.77	4.17E-83
BC_4441	Stage IV sporulation protein SpoIVFA	14	20	-0.56	0.019
BC_4440	Stage IV sporulation protein SpoIVFB	13	22	-0.65	0.013
BC_2031	Spore coat protein CotH	13	18	-0.57	0.04
BC_3770	Outer spore coat protein CotE	497	1310	-1.39	3.82E-68
BC_1222	Spore coat protein Coty	760	2591	-1.77	4.17E-83
BC_0823	Spore coat associated protein CotJA	2	18	-2.86	2.75E-20
BC_0821	Spore coat protein CotJC	4	49	-3.49	1.08E-66
BC_4954	Spore coat protein CotS	80	38	1.00	1.25E-11

[a] Log 2-fold change, biofilm *vs* planktonic.
[b] False discovery rate, adjusted P-value.

Chapter 7 Haemolytic enterotoxin (Hbl) production in the biofilms of *B. cereus*

7.1 Introduction

B. cereus is a bacterial pathogen causing two types of foodborne diseases – diarrhoea and emesis (Stenfors Arnesen et al., 2008). Diarrhoea is caused by heat-sensitive enterotoxins, produced in the human gastrointestinal tract (GIT) when large numbers of vegetative cells are ingested (Ceuppens et al., 2013). The production of enterotoxins happens at the onset of the stationary phase of growth and accumulates when high bacterial densities (over 10^5 cells) are reached or when environmental conditions change (Stenfors Arnesen et al., 2008; Ramarao and Sanchis, 2013). The three major enterotoxins produced by *B. cereus* are haemolysin BL (Hbl), nonhemolytic enterotoxin (Nhe) and cytotoxin K (CytK) (Stenfors Arnesen et al., 2008). These enterotoxins form pores in the GIT epithelial cells causing microvilli damage and osmotic lysis of cells, resulting in diarrhoea (Hardy et al., 2001; Minnaard et al., 2001; Fagerlund et al., 2008).

The Hbl toxin comprises protein B (the binding component), L_1 and L_2 (the lytic components), with estimated molecular weights of 37.8, 38.5 and 43.2 kDa, respectively (Beecher and Wong, 1994b). Hbl toxin shows haemolytic, enterotoxic and dermonecrotic activity and vascular permeability (Beecher and Wong, 1994a; Beecher and Wong, 2000). The *B. cereus* Enterotoxin Reverses Passive Latex Agglutination test (BCET-RPLA) semi-quantifies the Hbl toxin produced by *B. cereus*, which detects the L_2 component of Hbl using polyclonal antisera with a detection limit of 2 ng/mL according to the manufacturer (Beecher and Wong, 1994a). Quantitative real-time PCR (RT-qPCR) has been used to detect the transcriptional expression of enterotoxin genes and other virulence regulators of *B. cereus* (van Der Voort and Abee, 2009; Jeßberger et al., 2015).

B. cereus is well known as a biofilm-forming bacterium and is frequently found during food processing (Flemming et al., 2016; Galié et al., 2018). *B. cereus* biofilms can form on various substrates, including stainless-steel which is a common surface widely used in food processing (Simmonds et al., 2003; Ryu and Beuchat, 2005; Hussain and Oh, 2017; Huang et

al., 2021) and glass wool (GW) which creates a large surface area that is used to yield sufficient biomass to investigate the proteomic profile of biofilms (Oosthuizen et al., 2001; Oosthuizen et al., 2002; Vilain and Brözel, 2006). The substratum for biofilm growth affects the number of sessile cells attached, and even sporulation of *B. cereus* (Karunakaran and Biggs, 2011; Hussain and Oh, 2017; Lianou et al., 2020).

The extracellular polymeric substance (EPS) in the biofilm structure provides a protective environment for *B. cereus* and therefore increases the cells' ability to adapt and survive sudden environmental changes compared to planktonic cells (Vilain et al., 2009; Karunakaran and Biggs, 2011; Flemming et al., 2016). Majed et al., (2016) implied that the EPS of *B. cereus* biofilms also contains metabolites, enzymes, bacteriocins and toxins released by cells, however, they did not investigate or specify which toxins were present in the biofilm EPS. The current knowledge about the relationship between toxin production and biofilms of *B. cereus* and other bacteria was reviewed in Chapter 2, highlighted that the production and/or function of toxins in the biofilms of *B. cereus* is unknown (Huang et al., 2020). Joaquín Caro-Astorga et al., (2019) reported the lower expression of Hbl- and Nhe- related genes in biofilms grown on polystyrene than planktonic cells using transcriptomic analysis. Fagerlund et al., (2014) also observed a lower expression of the Hbl gene in biofilms of *B. thuringiensis* than its planktonic counterpart. However, the actual toxin production in biofilms was not investigated. Karunakaran and Biggs (2011) detected the presence of Hbl and Nhe toxin in the EPS from both biofilm and planktonic cells using a sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) based method, implying the possible production of toxin in biofilms of *B. cereus*.

The previous transcriptomic analysis (Chapter 5) indicated that Hbl toxin-related genes (*hblACD*) and the genes involved in the protein secretion pathway were upregulated in biofilms grown on SS compared with the planktonic population (Chapter 5, Table 5. 3 and 5. 4), suggesting biofilm cells may more pathogenic than planktonic cells. This study aimed to investigate Hbl toxin production in the biofilm mode of growth and to understand if the

surfaces on which the biofilm is grown affect toxin production. Both the BCET-RPLA kit and RT-qPCR were used to study the Hbl toxin in the presence of biofilms grown on SS and glass. P5 was used to study the Hbl toxin in the presence of biofilms due to the strong biofilm-forming ability (Chapter 4) and was used for the transcriptomic study in Chapter 5.

7.2 Experimental procedures

7.2.1 Bacterial isolate and culture condition

The preparation of overnight culture is described in Chapter 3, section 3. 1. 2. P5 was used in this study which was identified as *B. cereus* and the strongest biofilm former among all tested isolates in Chapter 4. The TSB was used throughout this study.

7.2.2 Planktonic and biofilm grown conditions

The planktonic culture was grown as described in Chapter 3, section 3. 2. 1. Biofilm was developed on stainless-steel (SS) coupons (refer to Chapter 3, section 3. 2. 3), glass wool (GW) and stainless-steel wool (SSW; refer to Chapter 3, section 3. 2. 4). Approximately 0.5 g of GW and 2 g of SSW were used to develop similar amounts (10^8 CFU/mL) of biofilm cells (refer to Chapter 3, section 3. 7. 1. 3). Both planktonic and biofilm cells were incubated at 30°C with 120 rpm shaking for 24 h before toxin measurement. The number of cells in cultures was counted as described in Chapter 3, section 3. 1. 3.

7.2.3 Toxin production in different types of cultures

7.2.3.1 Hbl toxin in planktonic culture and cell resuspension

To investigate if Hbl toxin produced by P5 is associated with cells or released into the media, the toxin was measured in total planktonic culture, planktonic cell supernatant and the resuspension of planktonic cell pellets in saline after cell growth at 30°C for 24 h. The toxin

in cells grown on the TSA was also measured. A detailed illustration of the procedure can be found in Chapter 3, section 3. 7. 1. 1, Figure 3. 3.

7.2.3.2 Hbl toxin in the biofilm mode of growth

Culture methods used to detect Hbl toxin production by P5 in the presence of biofilms (grown on SS coupons, SSW and GW) are outlined in Chapter 3, sections 3. 7. 1. 2 and 3. 7. 1. 3.

7.2.4 Toxin production by biofilm cells – an independently-grown biofilm

An independently grown biofilm on GW was designed in this study to minimize the effect of planktonic culture on biofilm cells, to estimate the toxin production by biofilm cells solely (refer to Chapter 3, section 3. 2. 5).

7.2.5 Hbl toxin measurement

All the cultures for comparison were normalized to the same number of cells, followed by using the BCET-RPLA detection kit according to the instructions provided by the manufacturer (refer to Chapter 3, section 3. 7. 2). Briefly, normalized cultures were centrifuged (900 × g for 20 min at 4°C) and the supernatant was used for detecting Hbl toxin. The interpretation of results was according to the agglutination pattern formed (Chapter 3, section 3. 7. 2, Fig. 3. 6) and all the pictures of these patterns are included in the supplementary file (section 7. 7).

7.2.6 RT-qPCR assay for toxin gene expression

The RNA from planktonic cells and biofilms grown on SS coupons, SSW and GW was extracted using a Nucleospin RNA Plus kit (Macherey-Nagel, Germany) as described in Chapter 3, section 3. 9. 2 (method 2). Real-time quantitative PCR (RT-qPCR) was used to investigate the relative expression levels of Hbl related genes (*hblA*, *hblC* and *hblD*) between

biofilms and planktonic cells (refer to Chapter 3, section 3. 9. 6). *16S* was used as the reference gene for normalization (Jeßberger et al., 2015).

7.2.7 Statistical analysis

All the Hbl toxin measurements using the BCET-RPLA detection kit were performed with at least three biological replicates, and one representative result is shown in the results and supplementary file for the corresponding agglutination patterns. The data for the RT-qPCR assay was generated from the average value of three independent biological replicates and statistical analysis as detailed in Chapter 3, section 3. 10.

7.3 Results

7.3.1 Toxin production in planktonic culture and cell resuspensions

Planktonic cultures were grown in TSB at 30°C for 24 h and the resuspension of cells grown on TSA contained 10^9 CFU/mL cells. The level of Hbl toxin detected in the supernatant of planktonic culture was like the total planktonic culture (Table 7. 1). The resuspension of planktonic cell pellets and cells grown on an agar plate contained less Hbl toxin than the planktonic culture. These results suggest that the Hbl toxin is mostly released into media rather than associated with cells.

Table 7. 1 Presence of Hbl toxin in planktonic culture and cell resuspensions.

		Undiluted	10-fold diluted	100-fold diluted
Planktonic culture	Total culture	+++	++	++
	Supernatant	+++	++	++
	Resuspension of cell pellets	+++	++	-
Cell grew on TSA	Colony resuspension	+	-	-

"++", "+" and "±" mean the presence of toxin that were detected.
"-" means the absence of toxin that did not been detected.

7.3.2 Toxin production in biofilm growth

7.3.2.1 Biofilm grown on stainless-steel coupons

Three types of culture were assessed for toxin production in the presence of SS coupons inserted into a well-plate: planktonic culture, the liquid including planktonic cells surrounding a SS coupon and resuspension of biofilms swabbed from a SS coupon (Chapter 3, section 3. 7. 1. 2, Fig. 3. 4). Planktonic cultures and the planktonic culture surrounding a SS coupon contained 10^9 CFU/mL cells and were diluted 100 times to achieve 10^7 CFU/mL for toxin detection. The resuspension of biofilm cells from a SS coupon contained 10^7 CFU/mL cells. To compare the toxin production between planktonic and biofilm cultures, two-fold dilutions of the normalized cultures were tested, and the results are shown in Table 7. 2. The amount of Hbl toxin detected in planktonic culture was like the planktonic cells surrounding the SS coupon, showing a positive result until four-fold dilutions of the tested cultures containing 10^7 CFU/mL cells. The Hbl toxin was not detected in the resuspension of biofilm formed on the SS coupon containing 10^7 CFU/mL, suggesting the Hbl toxin is not bound to the biofilms formed on a coupon.

Table 7. 2 Tests for Hbl toxin in planktonic culture, cells surrounding a biofilm on a stainless-steel coupon and biofilm resuspended from a stainless-steel coupon.

		Cell counts in tested culture	Undiluted	Two-fold diluted	Four-fold diluted	Eight-fold diluted
Planktonic culture	Pure culture	10^7 CFU/mL	++	+	±	-
	Cells surrounding the SS coupon	10^7 CFU/mL	++	+	±	-
Biofilm culture	SS coupon resuspended	10^7 CFU/mL	-	-	-	-

"++", "+" and "±" mean the presence of toxin that were detected.
"-" means the absence of toxin that did not been detected.

7.3.2.2 Biofilm grown on glass and stainless-steel wool

Glass wool (GW) and stainless-steel wool (SSW) were used to create a larger surface area compared to coupons for biofilm growth. The biofilm cells attached on GW were confirmed under Differential Interface Contrast (DIC) microscopy, shown in Fig. 7. 1, with an image of GW after 24 h incubation (Fig. 7. 1A) and the effectiveness of using glass beads to detach biofilms from the GW (Fig. 7. 1B).

The planktonic culture (Chapter 3, section 3. 7. 1. 3, Fig. 3. 5A) and planktonic cells surrounding GW or SSW (Chapter 3, section 3. 7. 1. 3, Fig. 3. 5B) contained 10^9 CFU/mL. All the cultures were diluted with saline to achieve 10^7 CFU/mL for toxin detection. Two-fold dilutions of normalized cultures were assessed to provide a semi-quantitative assay for the toxin. The results of Hbl toxin detection in the presence of GW or SSW are shown in Table 7. 3. The planktonic culture surrounding the GW or SSW Chapter 3, section 3. 7. 1. 3, Fig. 3. 5B) contained higher amounts of toxin than the pure planktonic culture (Chapter 3, section 3. 7. 1. 3, Fig. 3. 5A), suggesting the presence of biofilm/wool increase the amounts of Hbl toxin and that the toxin is secreted into the surrounding medium.

7.3.2.3 Substratum effect on Hbl toxin production

Mixed cultures containing planktonic cells and biofilms from either GW or SSW (Chapter 3, section 3. 7. 1. 3, Fig. 3. 5C) contained $10^{9.5}$ CFU/mL cells, and the resuspensions of biofilms detached from either GW or SSW (Chapter 3, section 3. 7. 1. 3, Fig. 3. 5D) contained 10^8 CFU/mL cells. Like previous tests, all the cultures were diluted with saline to achieve 10^7 CFU/mL for toxin detection. Higher amounts of Hbl toxin were detected in the mixed culture containing planktonic and biofilm cells from SSW than the planktonic cells and biofilm cells from GW (Table 7. 4), suggesting a substratum effect on Hbl toxin production, with higher toxin production in the presence of SS compared to GW. In addition, higher amounts of Hbl toxin were detected in the biofilm resuspended cells (Chapter 3, section 3. 7. 1. 3, Fig. 3. 5D)

from SSW than GW (Table 7. 4), suggesting the possibility of toxin binding to the SSW or the biofilm structure.

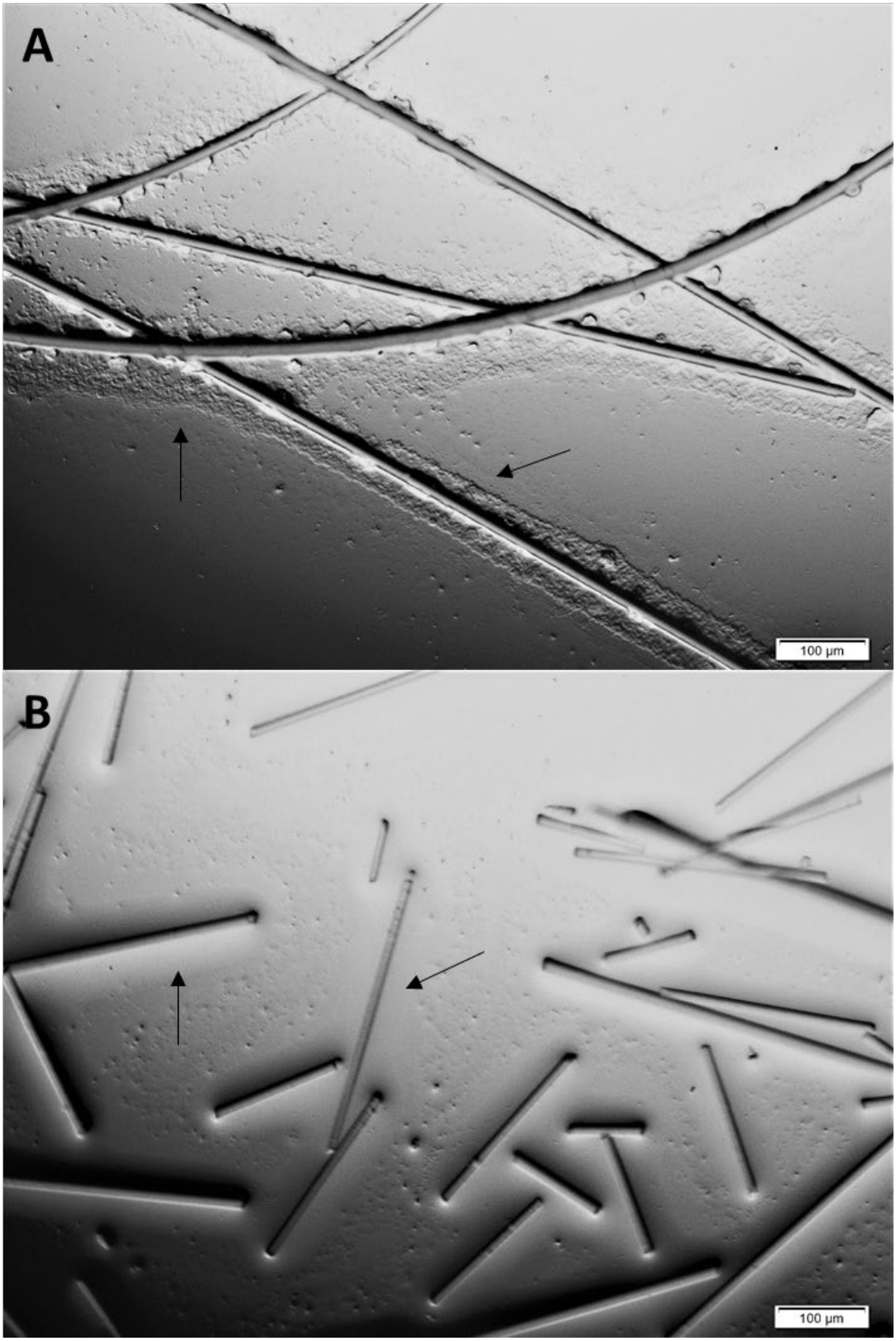

Figure 7. 1 Photomicrographs illustrating biofilm development on glass wool after 24 h (A) incubation at 30°C and detachment from glass wool (B) using glass beads. Arrows in A indicate the biofilm attachment while arrows in B indicate the detachment of biofilms.

Table 7. 3 Presence of Hbl toxin in glass wool or stainless-steel wool biofilm grown system.

		Cell counts in tested culture	Undiluted	Two-fold diluted	Four-fold diluted	Eight-fold diluted
Planktonic culture (Chapter 3, Fig. 3. 5A)	Planktonic cells	10^7 CFU/mL	++	+	±	-
Planktonic culture with the presence of biofilms (Chapter 3, Fig. 3. 5B)	Cells surrounding the GW biofilm	10^7 CFU/mL	+++	++	+	±
	Cells Surrounding the SSW biofilm	10^7 CFU/mL	++	++	+	±

"+++", "++", "+" and "±" means the presence of toxin that were detected.
"-" means the absence of toxin that did not been detected.

Table 7. 4 Effect of substratum on Hbl toxin production.

		Cell counts in tested culture	Undiluted	Two-fold diluted	Four-fold diluted	Eight-fold diluted
Mixed culture (Chapter 3, Fig. 3. 5C)	Planktonic + GW biofilm cells	10^7 CFU/mL	++	+	±	-
	Planktonic + SSW biofilm cells	10^7 CFU/mL	++	++	+	±
Detached biofilm resuspension (Chapter 3, Fig. 3. 5D)	From GW	10^7 CFU/mL	±	-	-	-
	From SSW	10^7 CFU/mL	+++	++	++	++

"+++", "++", "+" and "±" means the presence/levels of toxin that were detected.
"-" means the absence of toxin that did not been detected.

7.3.3 Toxin production by an independently grown biofilm

An independently grown biofilm was designed to estimate the Hbl toxin by biofilm cells with minimum effect by planktonic cells, and the results are shown in Table 7. 5. The attachment of cells after one-hour incubation was confirmed using DIC microscopy and the picture is shown in Fig. 7. 2. The GW was aseptically removed and put into a new sterile empty bottle for further incubation for 24 h after one hour of attachment. The independently grown biofilm culture of P5 contained 10^8 CFU/mL cells showed lower amounts of Hbl toxin than observed in planktonic culture, although the planktonic culture was diluted containing only 10^7 CFU/mL cells (Table 7. 5).

Table 7. 5 Hbl toxin production in an independently grown biofilm.

	Cell counts in tested culture	Undiluted	Two-fold diluted	Four- fold diluted	Eight- fold diluted
Planktonic culture	10^7 CFU/mL	++	+	±	-
Independently grown biofilm culture	10^8 CFU/mL	+	±	-	-

"+++", "++", "+" and "±" means the presence of toxin that were detected.
"-" means the absence of toxin that did not been detected.

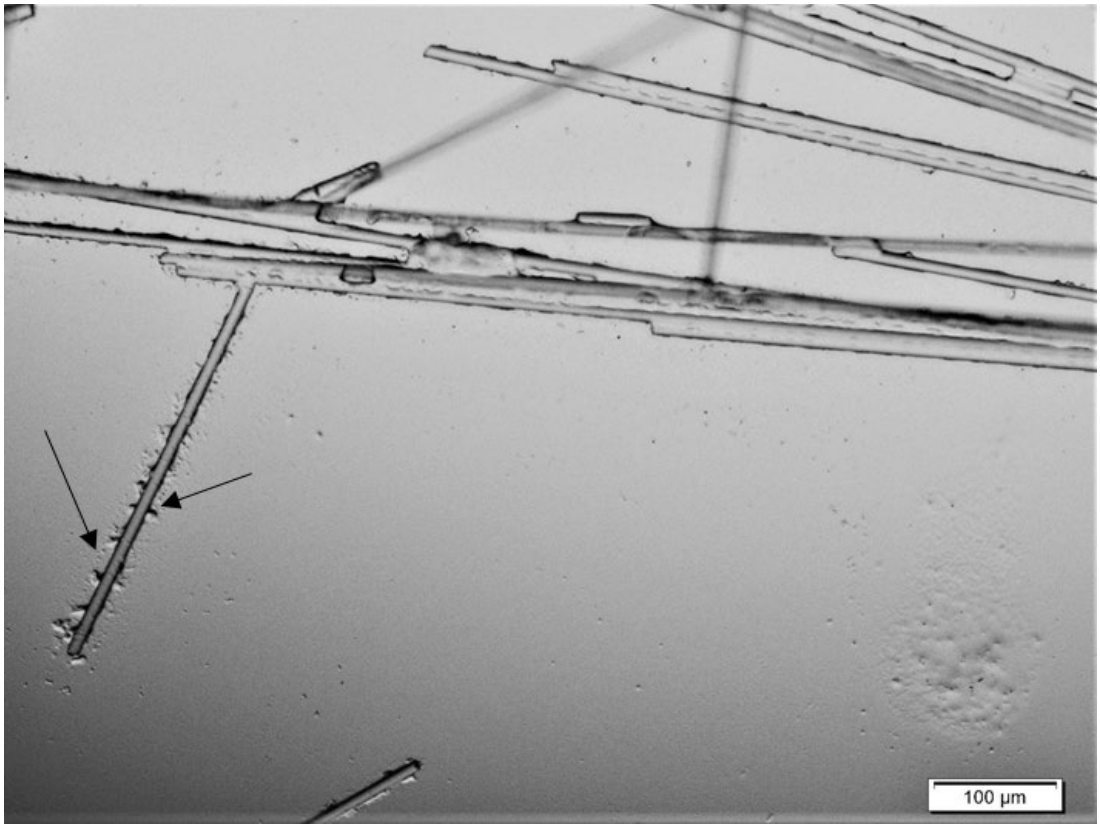

Figure 7. 2 Photomicrograph illustrating biofilm attachment on glass wool after one-hour incubation at 30°C.

7.3.4 Expression of Hbl toxin genes

RT-qPCR was used to investigate the relative expression of the Hbl toxin genes (*hblA*, *hblC* and *hblD*), in biofilm cells grown on three substrates (SS coupon, SSW and GW) compared to planktonic cells, shown in Fig. 7. 3. Significant changes ($P < 0.05$) in the relative expression levels of *hblC* and *hblD* were observed in the biofilms on SS coupons, showing upregulation of 9.1 ± 1.9-fold and 6.1 ± 2.2-fold, respectively, compared to planktonic cells. Similarly, for biofilms grown on SSW, gene expression was upregulated by 4.4 ± 0.2 -fold, 3.1 ± 1.1 -fold and 5.1 ± 0.9 -fold for all three subunit toxin genes, *hblA*, *hblC* and *hblD*, respectively, compared to planktonic cells. For the biofilm on GW, the relative expression levels of all three subunit Hbl toxin genes were like planktonic cells (Fig 7. 3).

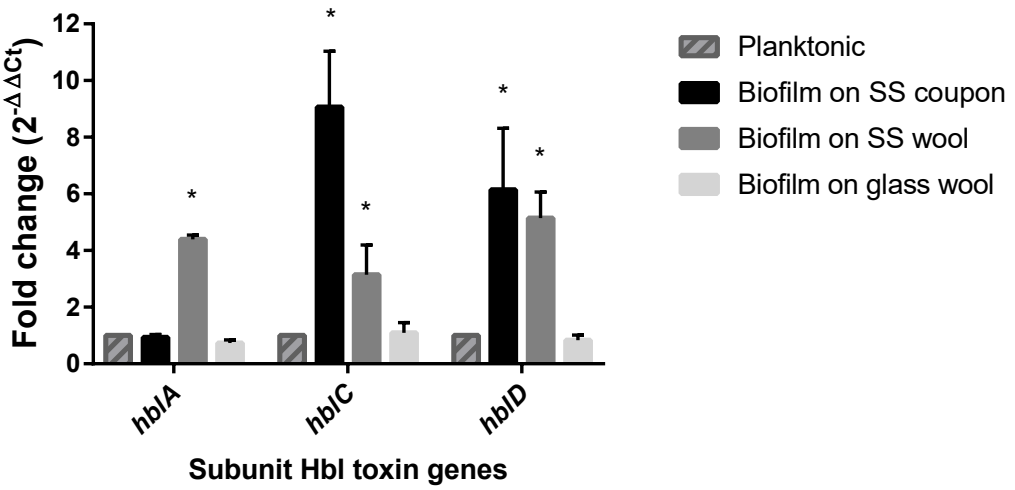

Figure 7. 3 Relative quantitative expression of Hbl toxin genes in biofilm cells grown on three substrates (stainless-steel coupons, stainless-steel wool and glass wool) compared with planktonic cells. Planktonic samples were treated as the calibrator (value = 1); value > 1 presents gene up-regulation while 0 < value < 1 presents down-regulation compared with the gene expression in planktonic cells. The results are expressed as the mean ± SD of three biological preparations. The statistical significance difference is shown with "*" ($p < 0.05$), compared with planktonic cells.

7.4 Discussion

B. cereus strains express a variety of enterotoxic compounds, causing foodborne disease. The biological function of the enterotoxin produced by *B. cereus* is unclear, but it was hypothesized that it enables competition with other microorganisms (Mecsas and Green, 2016). Hbl toxin is one of the enterotoxins produced by *B. cereus* causing diarrhoea. Past work on Hbl toxin production and/or toxin gene expression of *B. cereus* has indicated that toxin production is influenced by strains, their origin, medium composition and growth conditions (Garcia-Arribas and Kramer, 1990; Duport et al., 2004; Ouhib-Jacobs et al., 2009; Li et al., 2016). However, the toxin production in biofilms or influenced by the presence of biofilm growth is unknown (Huang et al., 2020). Therefore, the aim of this study was to understand the effect of biofilms and the substratum for biofilm growth on Hbl toxin production for *B. cereus*.

The planktonic supernatant contained most of the Hbl toxin compared to planktonic culture (TSB culture with cells), while less toxin was found to be associated with planktonic cell pellets or cells swabbed from agar plates (Table 7. 1), indicating that Hbl toxin is not associated with bacterial cells but remains in the surrounding media.

Two types of substrates (GW and SSW) were studied, as SS is commonly used in food processing environment and GW is widely used to produce large numbers of biofilm cells (Oosthuizen et al., 2001; Oosthuizen et al., 2002; Simmonds et al., 2003; Ryu and Beuchat, 2005; Vilain and Brözel, 2006; Hussain and Oh, 2017; Huang et al., 2021). Higher amounts of Hbl toxin were detected in the cultures where a biofilm was present compared to a pure planktonic culture containing the same number of cells using the immunoassay-based toxin detection kit (BCET-RPLA; Table 7. 3). This indicates that Hbl toxin produced by biofilm cells is released into the medium, concurring with results from previous studies suggesting that *B. cereus* enterotoxin proteins and virulence factors (e.g., Hbl, Nhe and CytK) are secreted and not cell wall bound (Jeßberger et al., 2015; Majed et al., 2016). There was no difference in Hbl toxin detection in planktonic cultures grown in a well-plate compared to the planktonic cells surrounding a SS coupon (Table 7. 2), which may be explained by the limited amount of biofilm cells grown on a coupon (approximately 7 log CFU/coupon) resulting in less toxin produced by the biofilm. This may also be due to the low sensitivity of the semi-quantifying method to detect toxin production from the two types of culture by observing the difference in the degree of agglutination.

The substratum effect on Hbl toxin production was observed by comparing the toxin in the mixed cultures containing planktonic and biofilm cells from two substrates (SSW *vs* GW; Chapter 3, Fig. 3. 5C), with higher amounts of Hbl toxin measured in the mixture containing biofilm cells detached from SSW than from GW (Table 7. 4). Although the detached GW/SSW biofilm cell resuspensions (Chapter 3, Fig. 3. 5D) are not comparable to other samples due to the washing steps prior to detachment from the substratum, these were comparable for Hbl toxin production among each other. Results showed that more Hbl toxin

was detected in the resuspension of detached biofilm cell from SSW compared to GW (Table 7. 4), suggesting a possible direct attachment of Hbl toxins to SSW. This may be a result of the high level of chromium oxide and hydrophobic property of SS surfaces (Chmielewski and Frank, 2003). Nomura and Saito (1982) observed that extracellular haemolytic toxin produced by *Aeromonas salmonicida* was stimulated by some bivalent metal ions including Ca^{2+} and Mn^{2+}. On the other hand, magnesium ion has been shown to suppress the production of toxins produced by *Staphylococcus aureus* (Edward et al., 1987). These ions may also affect the enterotoxin production by the biofilm cells by *B. cereus*, however, this needs to be investigated. Interestingly, there was little Hbl toxin detected in resuspended biofilm cells grown on SS coupons (Table 7. 2), which may be due to the limited surface area on coupons. In addition, the swabbed biofilm from SS coupons was mainly at the air-liquid interface which is the main type of biofilm formed by *B. cereus* (Wijman et al., 2007), while the SSW was mostly submerged in the nutrient medium. Consequently, it could be hypothesized that the Hbl toxin production may differ between submerged and air-liquid interface biofilms.

It is difficult to develop a separate biofilm growth mode without the influence of planktonic cells. An independently grown biofilm growth mode was designed in this study to create biofilm growth with minimal effect by planktonic cells, which allowed the cells attached to GW for an hour followed by removing all the liquid/planktonic cells, and it was assumed that the nutrients surrounding GW were sufficient for biofilm growth. The results showed that less Hbl toxin was present in biofilms compared to the planktonic culture (Table 7. 5), suggesting the lower Hbl toxin production in biofilms than planktonic cells. However, limited nutrients (only nutrients surrounding the GW) were used for biofilm cells to grow in this condition, while planktonic cells were grown with surplus nutrients.

Transcriptomic analysis in Chapter 5 showed higher gene expression of the Hbl toxin genes in biofilms grown on SS than planktonic cells, which is consistent with RT-qPCR results in this chapter that demonstrated there was significantly ($P < 0.05$) higher expression of Hbl toxin-related genes (*hblA*, *hblC* and *hblD*) in biofilms grown on both SS coupons and SSW

compared to planktonic cells (Fig. 7. 3). A comparable expression of Hbl toxin genes was measured between biofilm grown on GW and planktonic cells (Fig. 7. 3), which supports the results obtained from the immunoassay (measured L_2 proteins transcribed from *hblC*) that there was a substratum effect on Hbl toxin production. This is a contradictory finding to that reported by Joaquín Caro-Astorga et al., (2019) who showed the downregulation of *HblACD* in biofilm cells compared to planktonic cells of *B. cereus* using RNA sequencing analysis. However, in their study, biofilms were grown on walls of the polystyrene well-plate, whereas in this study *B. cereus* biofilms were grown on GW or SSW, which is a parallel observation to our finding that the substratum for biofilm growth could affect toxin production.

7.5 Conclusions

Hbl toxin production from *B. cereus* in planktonic culture and biofilm on glass and stainless-steel varies. *B. cereus* biofilms can produce Hbl toxin and secrete it into the surrounding medium and the toxin production by biofilm cells is affected by the substratum with more Hbl toxin produced from biofilms on SS compared with GW observed in this study. Together with previous transcriptomic analysis (Chapter 5), higher Hbl toxin production by biofilm cells grown on SS than glass was confirmed by all three methods (BCET-RPLA detection kit, RT-qPCR and RNA sequencing). Understanding the mechanism and factors influencing higher expression and therefore secretion of enterotoxins when *B. cereus* is grown in biofilm may provide new avenues for research in prevention of toxin production of this important foodborne pathogen.

7.6 Copyright information

Parts of this study is intended to be submitted to a journal for publication and the Online Statement of Contribution form is attached in Appendix III.

7.7 Supplementary file _ Agglutination pattern of toxin production using the BCET-RPLA kit

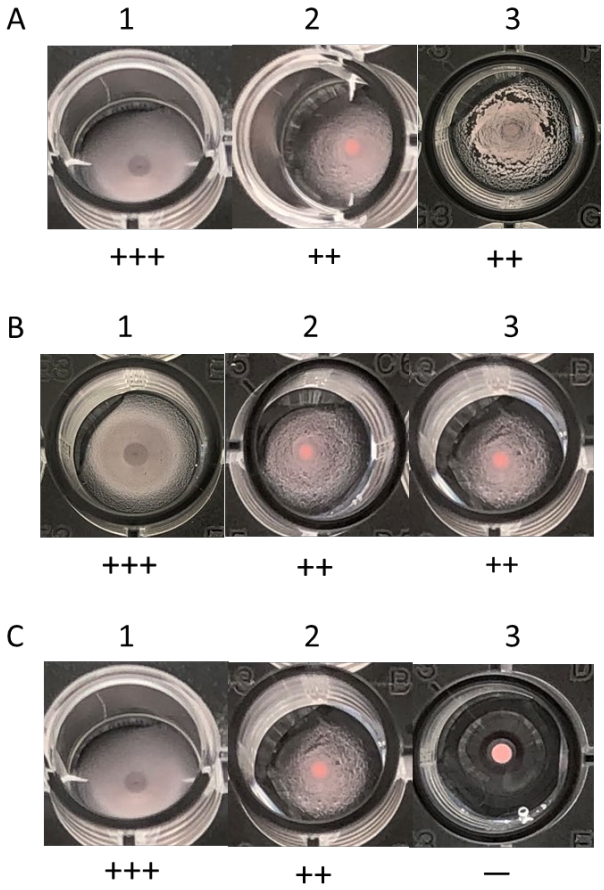

Figure S7. 1 Hbl toxin production in planktonic culture. A, B and C represent the toxin detection within planktonic culture, supernatant of the planktonic culture and resuspension of planktonic cell pellets, respectively. "1, 2 and 3" mean undiluted, 10-times diluted and 100-times diluted culture, respectively.

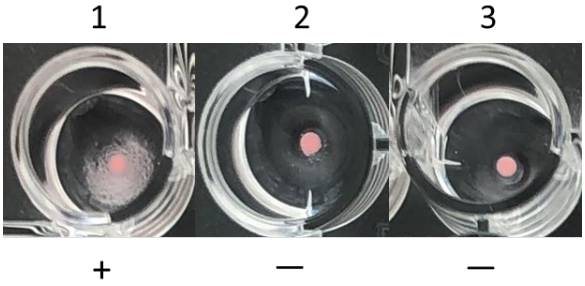

Figure S7. 2 Hbl toxin production in resuspended cells grown on TSA. "1, 2 and 3" mean undiluted, 10-times diluted and 100-times diluted resuspensions, respectively.

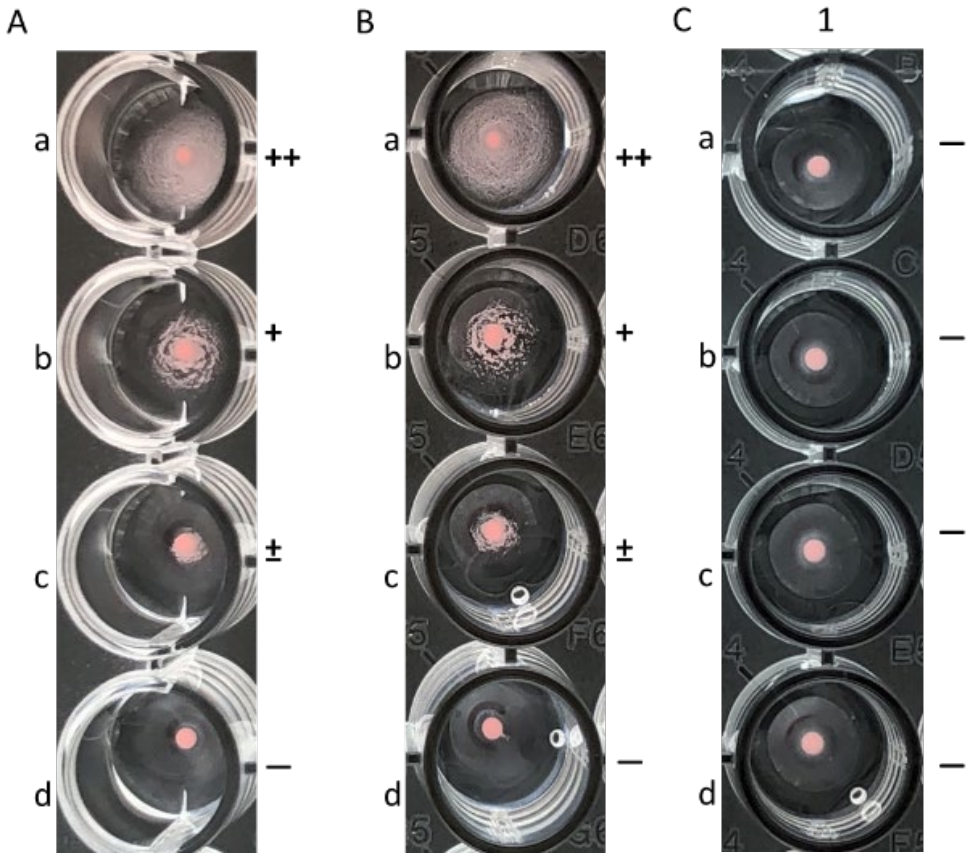

Figure S7. 3 Hbl toxin production in the presence of stainless-steel coupons. A, B and C represent the toxin detection in planktonic culture, SS coupon-influenced planktonic culture and resuspended biofilm cells swabbed from a SS coupon, respectively. All three cultures contained 10^7 CFU/mL cells. Doubling dilutions along each of the rows is shown from "a" to "d".

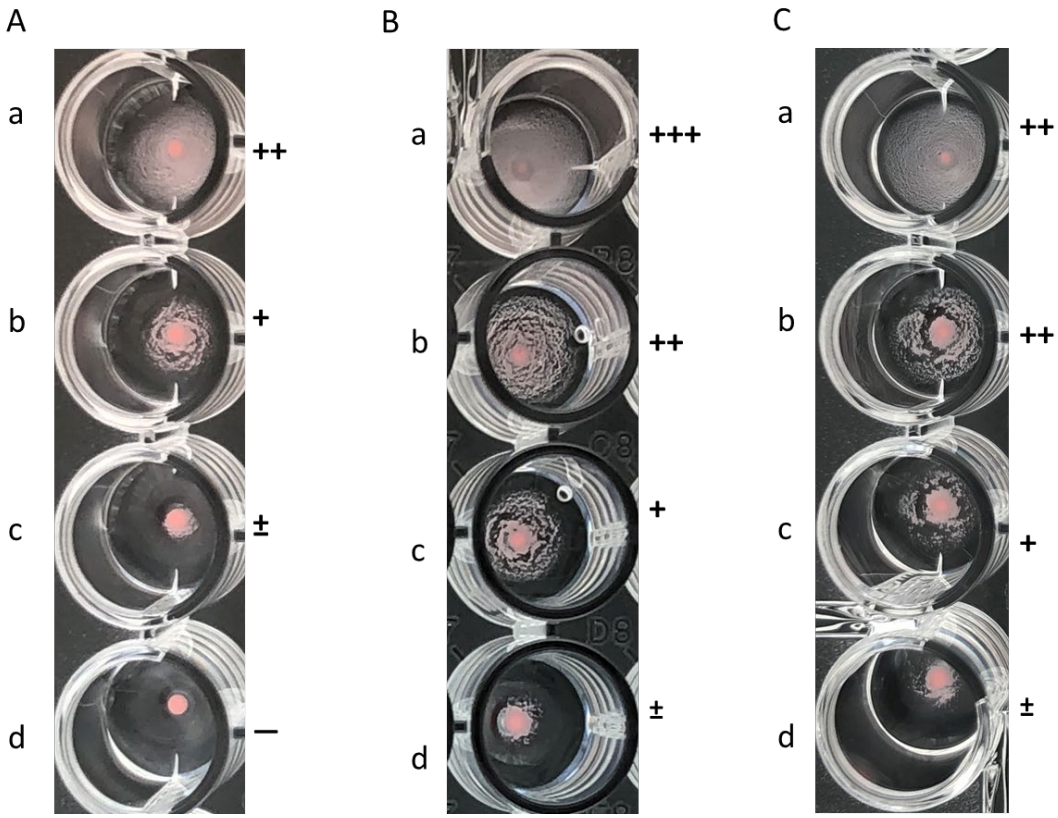

Figure S7. 4 Hbl toxin production in the presence of glass wool or stainless-steel wool. "A" represents the toxin production in pure planktonic culture, while "B" and "C" represent the toxin production in the planktonic culture with the presence of glass wool and stainless-steel wool, respectively. All the tested cultures contained 10^7 CFU/mL. Doubling dilutions along each of the rows from "a" to "d".

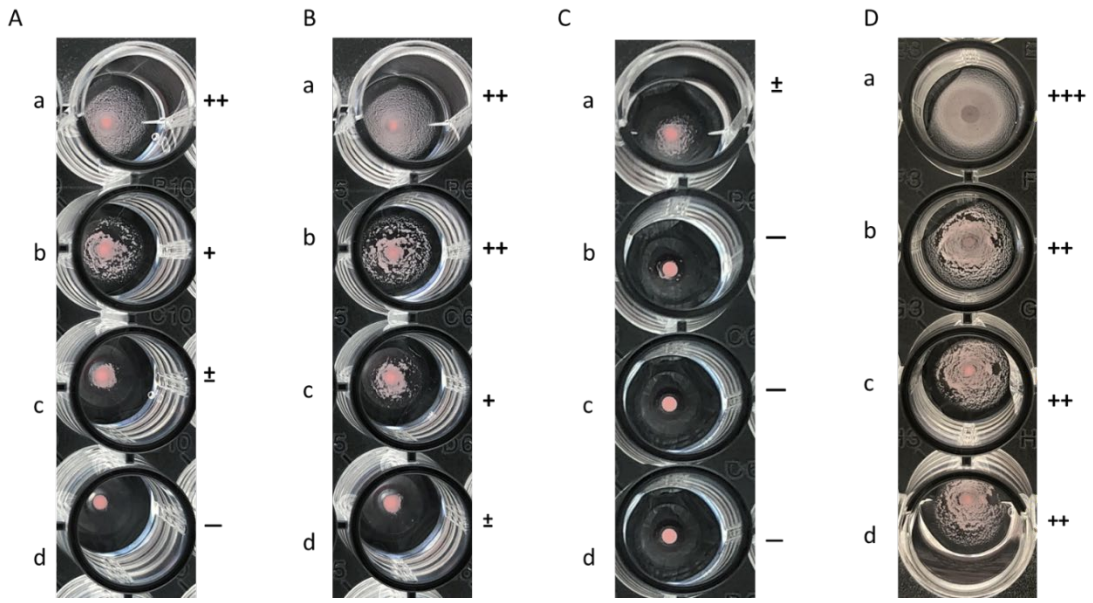

Figure S7. 5 The effect of substratum on Hbl toxin production. "A" and "B" represent the toxin in the mixed culture containing planktonic plus glass wool biofilm cells and planktonic plus stainless-steel biofilm cells, respectively. "C" and "D" represent the toxin in the resuspension of detached biofilms from glass wool and stainless-steel wool, respectively. All the cultures contain 10^7 CFU/mL. Doubling dilutions along each of the rows from "a" to "d".

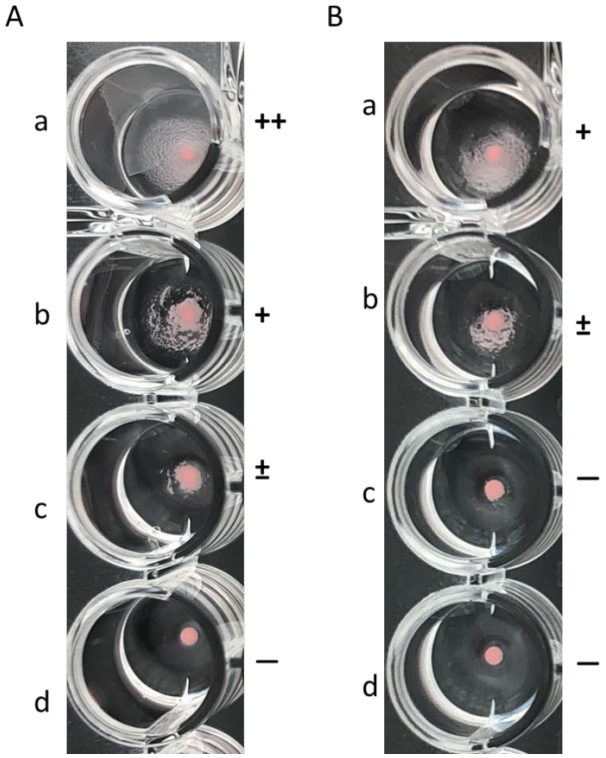

Figure S7. 6 Hbl toxin detection in planktonic culture ("A", contained 10^7 CFU/mL) and an independently grown biofilm ("B", contained 10^8 CFU/mL). Doubling dilutions along each of the rows from "a" to "d".

Chapter 8 Emetic toxin (cereulide) production in the biofilms of *B. cereus*

8.1 Introduction

The haemolytic (Hbl) toxin in the biofilms of a diarrheal toxin producing isolate of *B. cereus* (P5) were studied in Chapters 5 and 7. Unlike the Hbl toxin which is sensitive to heating (55°C for 20 min), the emetic toxin, called "cereulide" (used throughout this chapter), is highly heat- and acid-resistant and responsible for the emesis after ingestion of emetic *B. cereus* contaminated food (Shinagawa et al., 1996; Agata et al., 2002; Ehling-Schulz et al., 2004; Carlin et al., 2006; Bhunia, 2007; Rajkovic et al., 2008). The cereulide toxin is of special concern, as it will stay in the food and processing lines although the *B. cereus* strain producing the toxin may have been eliminated (Rouzeau-Szynalski et al., 2020). Cereulide is a small dodecadepsipeptide toxin (1.2 KDa) and a potassium ionophore which has a structural similarity with the antibiotic valinomycin (Agata et al., 1994), synthesized by a non-ribosomal peptide-synthetase (NRPS), called CesNRPS. Its gene cluster (*ces*) comprises seven genes (*cesHPTABCD*) with variable functions in the synthetic process, such as structural genes, *cesA* and *cesB*, that are two NRPS modules responsible for the assembly of this peptide (Ehling-Schulz et al., 2006a). The toxin can cause the disturbance of mammalian cell membranes and is also known to inhibit mitochondrial activity that can lead to vomiting, liver damage, multi-organ failure and even death (Mahler et al., 1997; Mikkola et al., 1999; Tschiedel et al., 2015). Cereulide production starts at the late exponential phase of *B. cereus* growth and continues throughout the stationary phase, but toxin production is affected by strain variability as well as environmental conditions such as nutrient availability, temperature, and oxygen (Häggblom et al., 2002; Jääskeläinen et al., 2004; Ehling-Schulz et al., 2015; Kranzler et al., 2016).

Higher production and higher gene expression of Hbl toxin were observed in biofilm-grown conditions compared to planktonic growth as outlined in Chapters 5 and 7. These results raise the question, is cereulide production like Hbl production with higher production in biofilm compared to planktonic grown of *B. cereus*? In this study, the emetic reference strain, *B. cereus* F4810/72, was used to investigate the cereulide toxin production in biofilms of *B. cereus* using liquid

chromatography with mass spectrometry (LC-MS/MS). Real-time quantitative PCR (RT-qPCR) was also used to predict the cereulide producing ability of biofilm cells.

8.2 Experimental procedures

8.2.1 Bacterial isolate and culture condition

B. cereus emetic reference strain F4810/72 (DSMZ, Germany) was used in this study. The overnight culture was prepared as described in Chapter 3, section 3. 1. 2. TSB was used throughout this study.

8.2.2 Planktonic and biofilm growth conditions

The planktonic culture was grown as described in Chapter 3, section 3. 2. 1. The biofilms were grown on stainless-steel (SS) coupons, glass wool (GW) and stainless-steel wool (SSW) as described in Chapter 3, sections 3. 2. 3 and 3. 2. 4. The types of cultures measured (Chapter 3, section 3. 8. 1) were like those used for the Hbl toxin study (Chapter 3, section 3. 7. 1), however, 0.5 g, 1 g and 3 g of GW and 1 g, 3 g and 5 g of SSW were used in this study to obtain different amounts of biofilm cells. Both planktonic and biofilm cells were incubated with 120 rpm shaking.

8.2.3 Toxin attachment on glass or stainless-steel wool

The attachment of cereulide toxin on GW or SSW was investigated. The methods and illustration of the experiment are shown in Chapter 3, section 3. 8. 2.

8.2.4 LC-MS/MS quantification of cereulide production

8.2.4.1 LC-MS/MS conditions, standard curves and validation experiments

The detailed LC-MS/MS settings, standard curves for both cereulide and valinomycin (used as internal standard) and validation experiments (correlation effect of cereulide and valinomycin, matrix effect and recovery rate) are included in Chapter 3, section 3. 8. 4. The calibration equation for cereulide toxin quantification is as follows (Chapter 3, section 3. 8. 4. 2, Fig. 3. 8):

$$Y=288057 \times X-20501 \ (R^2=0.9975)$$

where X is the concentration of toxin and Y is the peak absolute area, R^2 determined the coefficient of the linear regression.

8.2.4.2 Sample preparation

Extraction of cereulide from bacterial cultures is described in Chapter 3, section 3. 8. 3.

8.2.5 RT-qPCR assay for toxin gene expression

To compare the cereulide toxin-producing ability between planktonic and biofilm cells, real-time quantitative PCR (RT-qPCR) was used to determine the expression of *cesA* and *cesB*. The RNA extraction was performed using a Nucleospin RNA Plus kit (Macherey-Nagel, Germany) as described in Chapter 3, section 3. 9. 2 (method 2). The detailed experimental procedures of RT-qPCR are shown in Chapter 3, section 3. 9. 6.

8.2.6 Statistical analysis

The data expressed in figures/tables were generated from the average value of at least three independent biological replicates and statistical analysis is detailed in Chapter 3, section 3. 10.

8.3 Results

8.3.1 Toxin production in planktonic growth and cell resuspensions

The toxin was measured in planktonic cell pellets and supernatant to determine the binding relationship between the toxin and cells, and the results are shown in Table 8. 1. The planktonic culture and its resuspended cell pellets contained approximately 8.8 Log CFU/mL cells. The resuspended cell pellets contained most of the toxin (852.23 ± 111.03 ng per mL resuspension) comparable with the total planktonic culture (932.79 ± 115.60 ng per mL culture). The supernatant contained only 88.31 ± 19.43 ng per mL culture, indicating the cereulide toxin was mostly associated

with cells instead of releasing into the surrounding medium. This was also confirmed by measuring the cereulide in the cells grown on TSA containing approximately 8.6 Log CFU/mL cells in the resuspension of swabbed colonies, with 782.23 ± 81.03 ng/mL cereulide toxin detected (Table 8. 1).

Table 8. 1 Cereulide toxin production in planktonic growth and cell resuspensions. One-way ANOVA (Tukey's multiple comparison test) was performed, and different letters within each type of culture indicate a significant difference ($P < 0.05$).

		Cereulide (ng/mL)
Planktonic culture	Total culture	932.79 ± 115.60 [a]
	Supernatant	88.31 ± 19.43 [b]
	Resuspension of cell pellets	852.23 ± 111.03 [a]
Cell grew on TSA	Colony resuspension	782.23 ± 81.03 [a]

8.3.2 Toxin production in biofilm growth

8.3.2.1 Biofilm grown on stainless-steel coupons

The toxin was measured in the presence of biofilms grown on the SS coupons, and the results are shown in Table 8. 2. Cereulide toxin in the planktonic culture surrounding SS coupon contained 1.95 ± 0.74 ng/10^8 cells of cereulide toxin, which was significantly ($P < 0.05$) less than in the planktonic culture without the insertion of the SS coupon (10.59 ± 4.12 ng/10^8 cells), although the cell counts were similar between the two cultures (8.89 ± 0.14 and 8.69 ± 0.15 Log CFU/mL, respectively). Biofilm cells were removed by swabbing the SS coupons (20 coupons in total), the cells were then resuspended in saline and the concentration of cereulide measured. The cells isolated from biofilm contained 22.04 ± 5.07 ng/10^8 cells of cereulide toxin.

Table 8. 2 Cereulide toxin production in the presence of biofilms grown on stainless-steel coupons. The amount of cereulide in cultures or biofilm resuspensions was normalized to the same number of cells (10^8 CFU). One-way ANOVA (Tukey's multiple comparison test) was performed, and different letters within each type of culture indicate a significant difference ($P < 0.05$).

	Cereulide (ng/10^8 cells)	Cell counts (Log CFU/mL)
Planktonic culture in 48-well plate	10.59 ± 4.12 [a]	8.69 ± 0.15
Planktonic influenced by SS coupons	1.95 ± 0.74 [b]	8.89 ± 0.14
SS coupons swabbed	22.04 ± 5.07 [c]	7.77 ± 0.10

8.3.2.2 Biofilm grown on glass and stainless-steel wool

Glass (GW) and stainless-steel wool (SSW) were used to create a larger surface area compared to the SS coupons used in section 8. 3. 2. 1 for biofilm development. The cereulide toxin quantification in the presence of GW and SSW are shown in Tables 8. 3 and 8. 4, respectively. The planktonic cultures surrounding GW contained similar numbers of cells (8.76 ± 0.22 Log CFU/mL) compare with the planktonic culture (8.82 ± 0.09 Log CFU/mL). However, the concentration of the cereulide toxin was significantly ($P < 0.05$) lower in the presence of GW (61.85 ± 4.75 ng/10^8 cells) compared to planktonic cultures with no GW added (143.89 ± 39.04 ng/10^8 cells). Biofilms developed on GW were detached and resuspended in saline followed by toxin quantification to determine the toxin associated with the biofilm complex. The resuspension of detached biofilm from 0.5 g GW contained significantly ($P < 0.05$) higher amounts of cereulide (234.86 ± 2.64 ng/10^8 cells) than the planktonic culture (143.89 ± 39.04 ng/10^8 cells), although only 7.44 ± 0.02 Log CFU/mL cells were detached from 0.5 g GW.

Table 8. 3 Cereulide toxin production in the presence of biofilms grown on glass wool. Approximately 0.5 g glass wool was added to the medium. The amount of cereulide in planktonic or biofilm cultures were normalized to the same number of cells (10^8 CFU). One-way ANOVA (Tukey's multiple comparison test) was performed, and different letters within each type of culture indicate a significant difference ($P < 0.05$).

	Cereulide (ng/10^8)	Cell counts (Log CFU/mL)
Planktonic culture	143.89 ± 39.04 [a]	8.82 ± 0.09
Planktonic influenced by 0.5 g GW biofilm	61.85 ± 4.75 [b]	8.76 ± 0.22
Detached 0.5 g GW biofilm	234.86 ± 2.64 [c]	7.44 ± 0.02

Approximately 1 g of SSW was added in the media to allow biofilm growth, and the toxin quantification is shown in Table 8. 4. Planktonic cultures influenced by the presence of 1g SSW contained comparable amounts of cereulide (176.28 ± 69.15 ng/10^8 cells) with planktonic culture (143.89 ± 39.04 ng/10^8 cells), and the CFU numbers for the two cultures were similar (8.84 ± 0.06 Log CFU/mL and 8.82 ± 0.09 Log CFU/mL, respectively). Detached biofilm cell resuspension from 1

g SSW contained significantly ($P < 0.05$) higher amounts of toxin (871.18 ± 31.57 ng/10^8 cells) than the planktonic culture, although only 7.31 ± 0.01 Log CFU/mL cells were detached from the SSW.

Table 8. 4 Cereulide toxin production in the presence of biofilms grown on stainless-steel wool. Approximately 1 g of stainless-steel wool was added to the medium. The amount of cereulide in cultures or biofilm cultures were normalized to the same number of cells (10^8 CFU). One-way ANOVA (Tukey's multiple comparison test) was performed, and different letters within each type of culture indicate a significant difference ($P < 0.05$).

	Cereulide (ng/10^8)	Cell counts (Log CFU/mL)
Planktonic culture	143.89 ± 39.04 [a]	8.82 ± 0.09
Planktonic culture influenced by 1 g SSW	176.28 ± 69.15 [a]	8.84 ± 0.06
Detached 1 g SSW	871.18 ± 31.57 [b]	7.31 ± 0.01

8.3.2.3 Biofilm grown on larger amounts of glass and stainless-steel wools

Only approximately 7.44 and 7.31 Log CFU/mL biofilm cells were obtained from 0.5 g of GW and SSW, respectively, as shown previously (Tables 8. 3 and 8. 4). Larger amounts GW (1 g and 3 g) and SSW (3 g and 5 g) were used to create larger surfaces for biofilm growth and were expected to obtain comparable numbers of cells with planktonic growth (8.82 ± 0.09 Log CFU/mL), to avoid the overestimated of toxins in the culture by normalizing to "ng/10^8 cells" for comparison. The results are shown in Table 8. 5 and 8. 6. Interestingly, the planktonic culture in the presence of 1 g or 3 g GW (23.11 ± 10.91 and 0.81 ± 0.21 ng/10^8 cells, respectively; shown in Table 8. 5) and the counterpart detached GW resuspensions (37.35 ± 1.86 and 10.42 ± 0.28 ng/10^8 cells, respectively; shown in Table 8. 5) contained less toxin than the results observed with 0.5 g GW (Tables 8. 3), although more biofilm cells were obtained (8.63 ± 0.08 and 8.74 ± 0.03 on 1 g and 3 g GW, respectively).

Comparable amounts of cereulide toxin were measured in the planktonic cells influenced by 3 g and 5 g SSW (31.62 ± 20.14 and 24.82 ± 15.01 ng/10^8, respectively; Table 8. 6), which was significantly lower ($P < 0.05$) than was observed in the presence of 1 g SSW (176.28 ± 69.15 ng/10^8; Table 8. 4), although the numbers of bacterial cells were similar (around 8.8 Log CFU/mL) in all cultures. Significantly ($P < 0.05$) lower amounts of cereulide toxin in biofilm detached resuspensions were measured with 3 g or 5 g SSW (202.26 ± 16.73 and 118.3 ± 3.36 ng/10^8, respectively; Table 8. 6)

compared to 1 g SSW (871.18 ± 31.57 ng/10^8; Table 8. 4), although the numbers of biofilm cells detached increased with larger amounts of SSW as expected.

Table 8. 5 Cereulide toxin production in the presence of approximately 1 or 3 g of glass wool added to the medium. The amounts of cereulide in cultures or biofilm resuspensions were normalized to the same number of cells (10^8 CFU). One-way ANOVA (Tukey's multiple comparison test) was performed, and different letters within each type of culture indicate a significant difference ($P < 0.05$).

	Cereulide (ng/10^8)	Cell counts (Log CFU/mL)
Planktonic influenced by 1 g GW biofilm	23.11 ± 10.91 [a]	8.80 ± 0.26
Planktonic influenced by 3 g GW biofilm	0.81 ± 0.21 [b]	8.66 ± 0.13
Detached 1 g GW biofilm	37.35 ± 1.86 [c]	8.63 ± 0.08
Detached 3 g GW biofilm	10.42 ± 0.28 [d]	8.74 ± 0.03

Table 8. 6 Cereulide toxin production in the presence of approximately 3 and 5 g of stainless-steel wool added to the medium. The amount of cereulide in cultures or biofilm resuspensions was normalized to the same amounts of cells (10^8 CFU). One-way ANOVA (Tukey's multiple comparison test) was performed, and different letters within each type of culture indicate a significant difference ($P < 0.05$).

	Cereulide (ng/10^8)	Cell counts (Log CFU/mL)
Planktonic culture influenced by 3 g SSW	31.62 ± 20.14 [a]	8.85 ± 0.07
Planktonic culture influenced by 5 g SSW	24.82 ± 15.01 [a]	8.80 ± 0.09
Detached 3 g SSW	202.26 ± 16.73 [b]	7.40 ± 0.01
Detached 5 g SSW	118.3 ± 3.36 [c]	7.87 ± 0.12

8.3.3 Toxin attachment on glass and stainless-steel wool

Significantly ($P < 0.05$) less cereulide toxin was measured in the planktonic cells surrounding GW or SSW than planktonic culture alone (Tables 8. 3 to 8. 6). It is speculated that the toxin was associated with or attached to the GW or SSW. To investigate this, the supernatant of a planktonic culture was used as a toxin-containing solution and GW and SSW (approximately 0.5 or 3 g) were added to allow attachment for 30 min and then removed, followed by extracting toxin from the GW or SSW and then quantifying. The residue toxin in the supernatant after attachment was also measured. The results are shown in Table 8. 7. There were significant ($P < 0.05$) losses in the planktonic supernatants after adding either GW or SSW (9.44 ± 0.75 to 16.86 ± 1.1 ng/mL), compared to the supernatant alone

(90.02 ± 9.99 ng/mL). The toxin attached to wools was like the amount of toxin in the original planktonic supernatant, outlined in Table 8. 7.

Table 8. 7 The attachment of cereulide toxin on glass wool or stainless-steel wool. Either 0.5 g or 3 g wool was used as the substrate for the attachment of toxin from exposure to the supernatant from planktonic cells for 30 min. The toxin in planktonic supernatant after attachment and on the wool was measured. One-way ANOVA (Tukey's multiple comparison test) was performed, and different letters within each type of culture indicate a significant difference ($P < 0.05$).

		Cereulide (ng/mL) or (ng/wool)
	Planktonic supernatant	90.02 ± 9.99 [a]
Planktonic supernatant after	Added 0.5g GW	15.51 ± 0.6 [b]
	Added 3 g GW	9.44 ± 0.75 [c]
	Added 0.5 g SSW	16.86 ± 1.1 [b]
	Added 3 g SSW	13.07 ± 0.64 [b]
Toxin on	0.5 g GW after attachment	63.12 ± 9.37 [d]
	3 g GW after attachment	70.27 ± 13.89 [a]
	0.5 g SSW after attachment	103.39 ± 6.94 [a]
	3 g SSW after attachment	108.34 ± 4.59 [a]

8.3.4 Expression of cereulide toxin-related genes

The expression of *cesA* and *cesB* which are responsible for synthesizing cereulide toxin (Ehling-Schulz et al., 2006a) was measured in planktonic cells and biofilm cells grown on three types of substrates (SS coupon, GW and SSW) using RT-qPCR. There was no statistically significant difference in the expression of *cesA* and *cesB* between planktonic and biofilm cells, suggesting a similar toxin synthesis ability for planktonic and biofilm cells (Fig 8. 1).

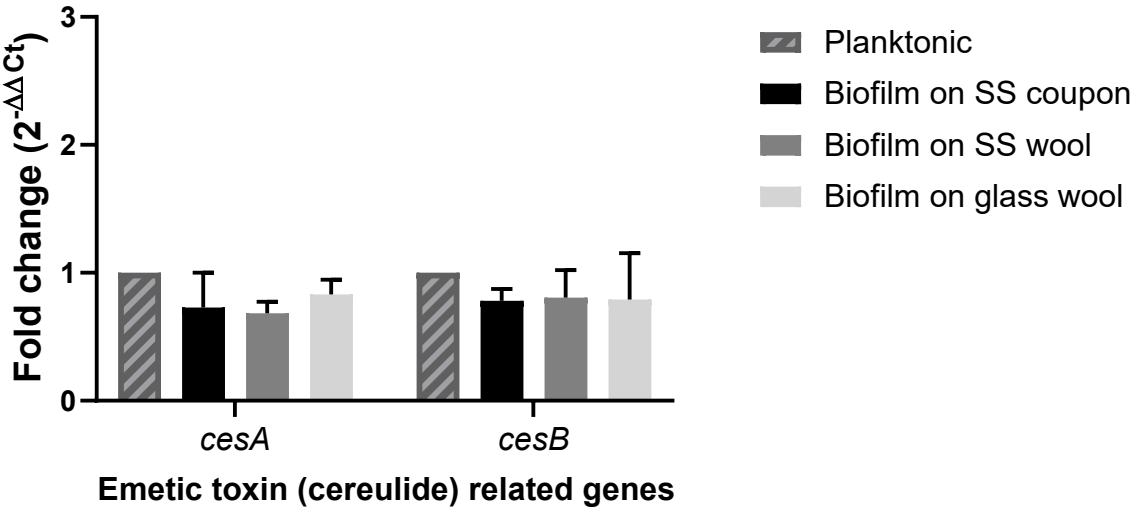

Figure 8. 1 Relative expression of *cesA* and *cesB* expression in planktonic cells and biofilm cells grown on three types of substrates (stainless-steel coupon, glass wool and stainless-steel wool) using an RT-qPCR. Planktonic cells were treated as the calibrator (value = 1); value > 1 presents gene up-regulation while 0 < value < 1 presents down-regulation compared to the gene expression in planktonic cells. The results are expressed as the mean ± SD of three biological preparations.

8.4 Discussion

Cereulide is a potent emetic toxin produced by some *B. cereus* strains, and although *B. cereus* cells may be eliminated during processing, the cereulide toxin can remain behind and is extremely difficult to destroy. This study showed that the cereulide toxin produced by planktonic cells grown in liquid culture or grown on an agar plate is mainly associated with the cells rather than secreted into the surrounding environment. Past studies on cereulide detection generally used the pelleted cultures or collected biomass from agar plates (Altayar and Sutherland, 2006; Yamaguchi et al., 2013; Ulrich et al., 2019), however, they did not specify if the toxin was associated with cells. The current study demonstrated that the emetic toxin is closely associated with cells.

Intensive studies regarding the prevalence of cereulide in food products, development of quantification methods and extrinsic factors influencing toxin production have been conducted and recently reviewed by Rouzeau-Szynalski et al., (2020). In the present study, the toxin measured in the liquid culture in a well-plate (10.59 ± 4.12 ng/10^8) was significantly ($P < 0.05$) less than culture grown in a flask (143.89 ± 39.04 ng/10^8) incubated in the same condition (30°C with 120 rpm

shaking), although these two cultures contained similar numbers of cells (8.69 ± 0.15 and 8.82 ± 0.09 Log CFU/mL, respectively), which is in agreement with Dommel et al., (2011) illustrating that the gene expression and toxicity of cereulide was not simply associated with cell numbers, but also affected by extrinsic conditions. In the present trial, the lower levels of toxin present in a well-plate may be caused by the reduced oxygen availability compared to a shaking flask (Jääskeläinen et al., 2004).

The emetic toxin production in the biofilms of *B. cereus* was unknown. In this study, the resuspension of swabbed biofilm cells from SS coupons contained almost double the amount of toxin (22.04 ± 5.07 ng/10^8) compared to the planktonic culture (10.59 ± 4.12 ng/10^8), however, significantly ($P < 0.05$) less toxin was detected in the planktonic culture in the presence of a SS coupon (1.95 ± 0.74 ng/10^8). This suggests that biofilm cells are either suppressing cereulide production and/or the cereulide toxin is becoming associated with biofilm cells or the substrate. The hydrophobic property of cereulide (Agata et al., 1994) may support the attachment to hydrophobic surfaces such as SS coupons.

Glass wool (GW) and stainless-steel wool (SSW) were used to create larger surfaces compared to coupons for increased biofilm development. Significantly ($P < 0.05$) lower or comparable amounts of the toxin were measured in the planktonic culture surrounding 0.5 g GW (61.85 ± 4.75 ng/10^8) or 1 g SSW (176.28 ± 69.15 ng/10^8), respectively, while, the detached biofilm resuspension from 0.5 g GW and 1 g SSW had significantly ($P < 0.05$) higher amounts of toxin (234.86 ± 2.64 and 871.18 ± 31.57 ng/10^8, respectively) than planktonic cultures (143.89 ± 39.04 ng/10^8), suggesting that cereulide toxin produced by biofilm cells is associated with the biofilm or the substrate. The toxin measured in the detached biofilm resuspension from SSW (871.18 ± 31.57 ng/10^8) was higher than it is from GW (234.86 ± 2.64 ng/10^8), indicating a substrate effect on cereulide production. SS surfaces contain high levels of chromium oxide and iron availability (>70% of SS composition) which has been shown to enhance biofilm formation by *B. cereus* (Rajasekar and Ting, 2011; Hayrapetyan et al., 2015a). These properties of SS material may also affect the cereulide production by biofilms, however, it needs to be confirmed.

Significantly ($P < 0.05$) lower amounts of cereulide toxin were quantified in the cultures (planktonic surrounding wool or detached biofilm resuspension) containing 1 g/3 g GW or 3g/5g SSW compared to 0.5 g GW or 1 g SSW, respectively, although more biofilm cells were counted detached from the larger amounts of wool, suggesting the toxin is not simply associated with biofilm cells or the biofilm complex. Large amounts of wool in the medium may negatively affect the cereulide toxin production, as the wool covered the liquid media potentially resulting in less oxygen availability and disturbing the agitation of the culture. Jääskeläinen et al., (2004) showed that the replacement of the atmosphere with nitrogen significantly reduced the detection of cereulide, and Häggblom et al., (2002) indicated that limited amounts of cereulide toxin were detected in the liquid culture grown in the static conditions compared to those grown on a rotary shaker. These findings support the importance of oxygen and agitation in cereulide toxin production by *B. cereus*.

The attachment of cereulide toxin on either glass or SS surfaces was confirmed (Table 8. 7), supporting the previous observations in this study, which may be explained by the hydrophobic property of cereulide (Agata et al., 1994). The higher metal availability on SS may also support this attachment, as cereulide has ionophoretic properties and binds potassium ions (Mikkola et al., 1999; Teplova et al., 2006), however, this needs to be confirmed. The attachment and accumulation of toxins may increase the risk of *B. cereus* in the food industry where hydrophobic surfaces such as SS are commonly used. The bacterial cells may be removed from processing lines, but the toxin may remain and cause contamination of food products. This leads to the question of whether the toxin can attach to food products without the presence of the *B. cereus* cells. This needs to be clarified in the future.

The cereulide toxin-producing ability of biofilm and planktonic cells of *B. cereus* was compared using the RT-qPCR assay. It showed that the expression of *cesA* and *cesB* genes, which are structural genes encoding for cereulide toxin synthesis, were comparable between three types of biofilm cells (grown on GW, SSW and SS coupons) and planktonic cells, suggesting a similar cereulide toxin-producing ability between the biofilm cells and planktonic cells. The observations made in this study are not due to changes in gene expression.

8.5 Conclusions

Unlike the Hbl toxin (Chapter 7) which is secreted into surrounding media after production by cells, this study shows that cereulide toxin is associated with cells and biofilm structures. Cereulide toxin also attaches to the substrate such as glass and stainless-steel on which the biofilm grows. This study contributes to a better understanding of food safety issues in the industry caused by cereulide toxin produced by *B. cereus* and provides valuable information for developing control methods for cereulide toxin in the food industry to reduce *B. cereus* food poisoning.

8.6 Acknowledgement

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8.7 Copyright information

Part of this work has been submitted to LWT- Food Science and Technology and currently under review by the journal. The Online Statement of Contribution form is attached in Appendix V.

Chapter 9 Final discussion and future work

9.1 Final discussion

B. cereus is found in the environment and is a concern for the food industry as it has three "weapons", spore formation, biofilm formation and toxin production. The metabolically dormant spores and protective biofilm structures are used by *B. cereus* as survival strategies in facing unfavourable conditions, such as heat, dry conditions, changes in pH and nutrient levels (Divanac'h et al., 2012; Flemming, 2016). The ability to grow within a biofilm provides advantages for bacteria compared with planktonic cells, to adapt and survive in diverse environmental conditions. However, biofilms in the food industry are generally unwanted making cleaning and controlling bacterial contamination difficult (Chmielewski and Frank, 2003; Davies, 2003; Flemming, 2016; Galié et al., 2018).

B. cereus can produce toxins, including enterotoxins (haemolytic (Hbl) toxin, non-haemolytic (Nhe) toxin and cytotoxin K (CytK)) and emetic toxin causing diarrhoea and emesis, respectively (Beecher et al., 1995; Granum and Lund, 1997; Granum et al., 1999; Lund et al., 2000). Foodborne illnesses are caused by the toxins produced by the pathogen, sporulation and biofilm formation are likely to exacerbate the risk of the illness. Despite intensive studies into individual aspects of spores (such as heat resistance and sporulation percentages), biofilm formation and toxin production in *B. cereus*, the link between these three features is still unclear. The possible relationship between spore, biofilm and toxin production has been discussed and reviewed in Chapter 2, section 2. 5 – 2. 7. In this PhD project, the spore formation in biofilms and toxin (both Hbl and emetic toxin) production in the presence of biofilms grown on stainless-steel (SS) coupon, stainless-steel wool (SSW) and glass wool (GW) by *B. cereus*, were investigated, as SS is a common material used in food processing and wool provides a large surface area that can be used to grow large amounts of biofilm (Oosthuizen et al., 2001; Oosthuizen et al., 2002; Simmonds et al., 2003; Ryu and Beuchat, 2005; Vilain and Brözel, 2006; Hussain and Oh, 2017).

9.1.1 Isolation and selection of *B. cereus*

This study sourced isolates of *B. cereus* from dairy (M1, M2, M3, M4 and M5) and potato (P2, P4 and P5) sources. "Presumptive" *B. cereus* isolates were detected using *B. cereus* selective agar plates (MYP; mannitol yolk polymyxin), and genotyped using 16S rRNA sequencing, multi-locus sequencing (MLST) and whole-genome sequencing (WGS) to confirm six *B. cereus* species (P2, P4, P5, M1, M3 and M4), one *B. paranthracis* (M2) and one *B. toyonensis* (M5). This is the first report of the *B. cereus* group isolates, *B. paranthracis* and *B. toyonensis*, in New Zealand. The genetic information of these food isolates together with two reference strains (ATCC 14579 and F4810/72) were compared using the average nucleotide identity (ANI) analysis and MLST, showing the diversity of *B. cereus* isolates from food products (Chapter 4, Fig. 4. 1) and novel sequence types (STs; Chapter 4, Table 4. 2). This agrees with studies done by Carter et al., (2018) and Kovac et al., (2016) who showed extensive genetic diversity in the *B. cereus* group isolates including dairy isolates with new STs. Different combinations of toxin- (e.g., *nhe*, *hbl* and *cytK*) and biofilm-related genes (e.g., *comER*, *codY* and *plcR*) were identified within the genomes of the isolates (Chapter 4, Table 4. 3). All of food isolates contained *nhe* genes while only P4, P5, M1, M3 and M5 contained *hbl*, and only P2, P4, P5, M1, M3 and M4 contained *cytK*, indicating the strain-dependent variation in *hbl* and *cytK* (Wijnands et al., 2006a; Park et al., 2009). *B. paranthracis* M2 and *B. toyonensis* M5 contained *hbl* and *nhe*, suggesting a potential food safety risk associated with these species. However, none of food isolates in this study carried the emetic toxin related gene (*ces*).

The biofilm formation (grown on SS coupon and pellicles floating in liquid medium) and sporulation for eight food isolates (P2, P4, P5, M1, M2, M3, M4 and M5) and two reference strains (ATCC 14579 and F4810/72) cultivated in tryptic soy broth (TSB), showed highly variable biofilm and spore formation (Chapter 4, Fig. 4. 2 to 4. 4). This agrees with previous work by Hussain and Oh (2017) who showed that biofilm and spore formation in *B. cereus* is strain-dependent and is influenced by the source of isolates. The shaking seems to favour biofilm formation with the highest numbers of biofilm cells occurring after one day, compared to two or three days in static incubation (Chapter 4, Fig. 4. 2). This may be due to increased oxygen availability and more even distribution of nutrients within the

shaken culture (Oosthuizen et al., 2002; Houry et al., 2010; Moreira et al., 2013). This is the first report comparing the biofilm formation of *B. cereus* in static and shaking incubation.

B. cereus potato isolates (P2, P4 and P5) showed strong biofilm formation out of all isolates. This was particularly true for P5, which demonstrated a significantly ($P < 0.05$) high biofilm formation on SS coupons and strong pellicle formation compared with the other isolates (Chapter 4, Fig. 4. 2 and 4. 4). Different mechanisms may exist between pellicle formation and biofilm formation on the SS coupons, as P2 produced significantly ($P < 0.05$) higher amounts of biofilm on the SS coupons, but was not a strong pellicle former in the same condition. This suggests that increased attention should be given to conditions where pellicle formation is enhanced such as liquid food products filled in containers, stirred storage tanks, pumping of product and transportation.

9.1.2 Biofilm *vs* planktonic: at the transcriptional level

The transcriptomic profiles of biofilm grown on SS surface and planktonic cells grown in liquid were compared using RNA sequence analysis, outlined in Chapter 5. P5 was selected due to the strongest biofilm formation (Chapter 4). Biofilm cells display a different gene expression pattern compared to planktonic cells (Davies, 2003). In this present study, 48.5% (2833 out of 5836) of genes were expressed significantly ($P < 0.05$) differently in biofilms compared to planktonic cells. Most of the genes (1766 out of 2833) were downregulated in biofilms suggesting that biofilm cells are less metabolically active compared to planktonic cells.

Carbohydrate transportation pathways, including the phosphotransferase system (PTS) and pentose phosphate pathway (PPP), were significantly (false discovery rate; $FDR < 0.05$) upregulated in planktonic cells (Chapter 5, supplementary files 5. 7. 4 and 5. 7. 5) which thrive through absorbing surplus nutrients in their surroundings. Biofilms have limited exposure to the environment and rely on a limited nutrient source and consequently are metabolically less active. Genes related to flagellar assembly and chemotaxis were also significantly ($FDR < 0.05$) upregulated in planktonic cells compared to biofilm cells (Chapter 5, supplementary file, section 5. 7. 6). Past studies have suggested that flagellar proteins, such as FlhF and FlhA, are important virulence factors for *B. cereus* (Ramarao

and Lereclus, 2006; Mazzantini et al., 2016), although expressions of *flhF* and *flhA* were not significantly (FDR < 0.05) different in planktonic cells compared to biofilm cells in this study. Senesi and Ghelardi (2010) suggested an essential role of the flagellar proteins in bacteria is secretion, however, the role of flagellar in producing and secreting virulence factors for planktonic cells remains unknown. Chemotaxis belongs to the stress response mechanism by sensing chemical substances and moving to favourable conditions (Den Besten et al., 2009; Ganesh Babu et al., 2011). Two chemotaxis response genes *cheY* (BC_1627) and *cheA* (BC_1628) were upregulated in planktonic cells (Chapter 5, supplementary file, section 5. 7. 6), suggesting that planktonic cells may utilize chemotaxis as a survival strategy when facing stress.

In this study, biofilm cells had increased expression of biotin and siderophore synthesis, as genes related to these synthetic processes were significantly (FDR < 0.05) more highly expressed in biofilms compared to the planktonic population (Chapter 5, Table 5. 5). Biotin is a regulator in glutamate production and is involved in altering the lipid composition of the cell membrane with lower amounts of straight-chain and higher amounts of branched-chain fatty acid in biotin-deficient cells (Sasaki, 1965; Hubbard and Hall, 1968). This suggests that there may be a difference in the composition of the cell membrane between biofilm and planktonic cells, however, the phenotypic consequences are still unknown. Siderophores are used by bacteria to uptake iron and form a stable complex with ferric ions (Bidlack, 1999; Miethke and Marahiel, 2007). The genes related to this biosynthesis were upregulated in biofilms, which may be due to the growth of the biofilm on SS containing iron and may stimulate the formation siderophores. Two siderophores, petrobactin and bacillibactin, can be excreted by *B. cereus* (Wilson et al., 2006), and genes involved were upregulated in biofilms compared to planktonic cells in the present study (Chapter 5, Table 5. 5). The supportive role of petrobactin in increasing microbial virulence has been suggested by Challis (2005). This may play a role in the enhanced virulence of biofilm cells. However, the role and the actual production of biotin and siderophores in biofilms are still unknown.

The transcriptomic analysis also revealed the differential expression of genes related to spore formation and toxin production in planktonic cells and biofilm cells, and these are included in the following sections.

9.1.3 Sporulation and heat resistance of spores in a biofilm

Biofilms of *B. cereus* are a source of spores contaminating the food processing line, although the percentage of spores within biofilms varies between studies and may be influenced by strain differences, growth temperatures and the substratum for biofilm growth (Lindsay et al., 2005; Faille et al., 2014; Hussain and Oh, 2018). Higher sporulation percentages were observed in biofilms grown on SS coupons compared to planktonic populations for all *B. cereus* isolates in this study (Chapter 4). This raises the question, do spores in a biofilm and those isolated from a biofilm demonstrate the same phenotypic characteristics such as heat resistance with planktonic spores?

Six *B. cereus* isolates (P2, P4, P5, M1, M3 and M4) were used to investigate the sporulation efficacy and heat resistance of spores in their biofilms, grown in TSB and milk. Biofilm cells were grown on SS coupons to mimic the food industry environment. The overall trend of higher sporulation percentages observed in biofilms of all six isolates grown in both TSB and milk (Chapter 6, Fig. 6. 1 and 6. 2), may be explained by the bacterial response to the high cell density in the biofilms and limited access to nutrients within the biofilm compared to planktonic growth (van Gestel et al., 2012). In addition, spores harvested from biofilms were, in general, more heat resistant than their liquid culture counterpart, especially for P4 which showed significantly ($P < 0.05$) higher $D_{90°C}$ values for spores isolated from biofilms compared to isolated from planktonic culture when grown in both TSB and milk (Chapter 6, Fig. 6. 3 and 6. 4). This agrees with Hayrapetyan et al., (2016) and Simmonds et al., (2003) who observed that spores from biofilms of *B. cereus* were more heat resistant than spores harvested from the liquid sources. This highlights that more severe heat treatments should be considered in eliminating spores from biofilms, and routine cleaning procedures should be frequent enough to minimize biofilm formation on food processing lines.

The higher sporulation percentage and heat resistance of spores may reflect the inert germination status of spores in biofilms compared to planktonic cells (Chapter 5). Genes related to spore germination proteins including GerABC, SABC and IABC families, were significantly (FDR < 0.05) upregulated in planktonic cells compared to biofilms (Chapter 5, Table 5. 2). This may be explained by the availability of nutrients for vegetative cells growth in planktonic culture.

Sigma B factor plays an important role in the stress response for *B. cereus* (Price, 2001; van Schaik et al., 2004). In this study, genes related to sigma B factor (BC_1004) and its regulators RsbV (BC_1002) and RsbW (BC_1003) were significantly (FDR < 0.05) upregulated in biofilms compared to planktonic cells. Van Schaik et al., (2004) showed that sigma B factor is involved in protecting cells from heat stress, suggesting the possible role of sigma B in the enhanced heat resistance of biofilms compared to planktonic cells observed in this study (Chapter 6), however, further confirmation is required.

The amount of dipicolinic acid (DPA) found in spores is thought to be highly related or responsible for the relative heat resistance of spores (Slieman and Nicholson, 2001; Setlow, 2006). This study showed that spores isolated from biofilm of M4 contained significantly (P < 0.05) higher levels of DPA than planktonic spores when grew in TSB, however, all other isolates contained comparable amounts of DPA between biofilm and planktonic spores (Chapter 6, Fig. 6. 5 and 6. 6). This suggests that the role of DPA in the heat resistance of spores varies between isolates, which is in the agreement with Hayrapetyan et al., (2016) and Kort et al., (2005) who suggested that the heat resistance of spores was not always correlated with their DPA content.

The spore coat may play a role in contributing to the heat resistance of spores, as an intact coat was observed in the spores isolated from biofilms of three isolates (P4, M1 and M3) while the spores isolated from the planktonic population showed a fragmented coat for P4 and irregularly shaped coat for M1 and M3 (Chapter 6, Fig. 6. 7 – 6. 12) in spores grown in both TSB and milk. This corresponds to the heat resistance observed, with significantly (P <0.05) higher $D_{90°C}$ values for spores isolated from biofilms compared to spores from planktonic cultures for P4, M1 and M3. This agrees with

Abee et al., (2011) who proposed that the multiple layers of spores including the spore coat, contribute to the heat resistance. Roels et al., (1992), Little and Driks, (2001), Driks (2002) and Johnson et al., (2006) demonstrated that the *exsY*, *cotY*, *spoIVFA*, *spoIVFB*, *cotH* genes are responsible for the assembly and function of the spore coat. However, the expression of these genes was significantly higher (FDR < 0.05) in planktonic cells compared to biofilm cells based on the transcriptomic data of P5 (Chapter 6, supplementary file 6. 8. 5). The different protein patterns in a coat within spores isolated from biofilm and planktonic population still need to be investigated in the future by using a model spore former, such as P4 used in this study.

9.1.4 Toxin production in the biofilms

9.1.4.1 Haemolytic toxin production in the biofilms

The transcriptome analysis showed that biofilms of *B. cereus* grown on SS resulted in the upregulation of enterotoxin genes (*hbl* and *nhe*) in comparison to planktonic cells (Chapter 5, Table 5. 3). In addition, genes related to the general secretion pathway (Sec) and the two-arginine (Tat) system were also significantly (FDR < 0.05) upregulated in biofilm cells (Chapter 5, Table 5. 4). Bacteria use the secretion system to secrete DNA, carbohydrates and virulence factors into the surrounding environment to compete with other bacteria or to invade host cells causing disease (Mecsas and Green, 2016), and Senesi and Ghelardi (2010) suggested that the Sec pathway is used by *B. cereus* to secrete Hbl and Nhe proteins. These results suggest that biofilm cells could be more pathogenic than planktonic cells, which is not only because of the more highly expressed enterotoxin genes, but also upregulation of the secretion pathway.

In Chapter 7, the production of Hbl toxin from *B. cereus* biofilms was investigated. An immunoassay-based kit, the *Bacillus cereus* enterotoxin test-reverses passive latex agglutination (BCET-RPLA), was used to semi-quantify the Hbl toxin production in bacterial cultures with or without biofilms present. Again, P5 was selected for the Hbl toxin study due to its strongest biofilm formation and its use for the transcriptomics study.

The Hbl toxin was present in the supernatant of the planktonic culture and was absent in the resuspension of the colonies swabbed from an agar plate (Chapter 7, Table 7. 1), indicating Hbl toxin is released into the surrounding medium rather than associated with bacterial cells. Also, higher amounts of Hbl toxin were detected in the planktonic culture surrounding biofilms (SSW and GW) compared to the planktonic culture only (Chapter 7, Table 7. 3) containing the same amounts of cells (9 Log CFU/mL), suggesting the secretion of Hbl toxin by biofilm cells. These results indicate that Hbl toxin production by *B. cereus* cells was released into the medium, concurring with Jeßberger et al., (2015) and Majed et al., (2016) who suggested that enterotoxin is not cell wall-bound and biofilms of *B. cereus* are capable of secreting metabolites such as enterotoxins.

The effect of the substratum on Hbl toxin production was analysed in this study, by comparing the toxin production in mixed cultures containing planktonic and biofilm cells from two substrates (SSW *vs* GW). Higher amounts of Hbl toxin were observed in mixed cultures containing planktonic and biofilm cells from SSW than from GW, also, higher amounts of Hbl toxin were observed in the resuspension of detached biofilm from SS than GW (Chapter 7, Table 7. 4). The substratum effect on Hbl toxin production was confirmed using real-time quantitative PCR (RT-qPCR), where a significantly ($P < 0.05$) higher expression of Hbl toxin genes (*hblA*, *hblC* and *hblD*) was seen in biofilms grown on SS than either planktonic cells or biofilms grown on GW (Chapter 7, Fig 7. 3).

Enterotoxins, including Hbl and Nhe, cause diarrhoea due to the ingestion of *B. cereus* contaminated foods followed by the outgrowth of cells/spores in the small intestine (Ceuppens et al., 2013). In this study, more heat-resistant spores and higher Hbl toxin production/expression were observed in the biofilms of *B. cereus* compared to the planktonic cells. In addition, biofilm cells expressed significantly ($FDR < 0.05$) higher amounts of other virulence factors including pore-forming haemolysins, β-lysine acetyltransferase and immune inhibitor A (InhA) metalloproteases (Chapter 5, Table 5. 3). These factors contribute to protecting bacteria against the host immune system and the degradation of epithelial barrier proteins (Bouillaut et al., 2005; Chung et al., 2006; Guillemet et al., 2010; Hamzeh-Cognasse et al., 2015). Therefore, biofilm formation may support adhesion onto host cells and increase the possibility of illness. The germination and spore formation in epithelial cells

mimicking the small intestine was shown by Wijnands et al., (2007). A similar experiment may be used to investigate toxin production of biofilms of *B. cereus*, in the small intestine to reveal the possible role of biofilms causing diarrhoea.

9.1.4.2 Emetic toxin production in the biofilms

Unlike Hbl toxin which is produced in the small intestine and sensitive to heat (55°C for 20 min) and protease (trypsin, pepsin, and chymotrypsin) (Turnbull et al., 1979; Fermanian et al., 1996; Bhunia, 2007), emetic toxin, also called "cereulide", is produced in food and can survive the passage through the gastrointestinal tract due to its heat, acid and protease resistance (Agata et al., 2002; Carlin et al., 2006). The higher Hbl toxin production and gene expression in biofilms of a diarrheal *B. cereus* isolate (Chapters 5 and 7), raises the question: is cereulide toxin production in the biofilms of *B. cereus* is like Hbl toxin? In Chapter 8, the emetic reference strain F4810/72 was used to study the cereulide toxin production in the presence of biofilms using a liquid chromatography-mass spectrometer (LCMS/MS).

In contrast to the observed secretion of Hbl into the surrounding media, cereulide toxin was detected in the resuspended cell pellets rather than the supernatant (Chapter 8, Table 8. 1), indicating that cereulide was mainly associated with the cells and not the surrounding medium. The attachment of cereulide to biofilm structures and/or biofilm-forming substrates (SSW and GW) was also observed, which may be due to the hydrophobic property of cereulide (Agata et al., 1994). In addition, a substrate effect, as seen for Hbl toxin, was observed for cereulide production, showing significantly higher ($P < 0.05$) amounts of cereulide in the presence of SSW compared to GW (Chapter 8, Tables 8. 3 – 8. 6). The higher metal availability on SS may support this attachment, as cereulide has ionophoretic properties and can bind to potassium ions (Mikkola et al., 1999; Teplova et al., 2006), however, further investigation is needed to confirm the effect of surface characteristics on cereulide production by biofilm cells of *B. cereus*. A comparable expression of both *cesA* and *cesB* was observed between planktonic cells and biofilm cells grown on three substrates (GW, SSW and SS coupon), suggesting a similar toxin-producing ability between the biofilm and planktonic cells.

Larger amounts of wool (1 and 3 g compared to 0.5 g of GW; 3 and 5 g compared to 1 g SSW) were used to obtain comparable amounts of biofilm cells with planktonic cells to avoid the overestimate of cereulide in biofilm cells by normalizing to "ng per 10^8 cells". However, decreased amounts of cereulide were detected in the presence of larger amounts of the wools, although more biofilm cells (8.74 ± 0.03 Log CFU/mL compared to 7.44 ± 0.02 Log CFU/mL on GW; 7.87 ± 0.12 Log CFU/mL compared to 7.31 ± 0.01 Log CFU/mL on SSW) were obtained. It is hypothesized that larger amounts of wool may negatively affect cereulide production, as the wool-covered liquid media results in less oxygen availability and disturbs the agitation of the culture. Jääskeläinen et al., (2004) and Häggblom et al., (2002) showed the importance of oxygen and agitation in cereulide production by *B. cereus*.

These results highlight that toxin production from biofilms of *B. cereus*, could results in a food safety issue and that controlling biofilm formation in the food processing is a key mechanism in food safety.

9.2 Future work

Although this PhD project has produced new information towards understanding the sporulation and toxin production in *B. cereus* biofilms, there are some new questions to be answered in future studies. These are outlined as follows:

1. A limited number of isolates was studied revealing the diversity of *B. cereus.* It is recommended that more isolates from each source (dairy and potato) should be included in future investigations. This will help confirm the findings and conclusions of biofilm and spore formation for the dairy and potato-based industries. The presence of toxin genes was identified in the eight food isolates, showing the potential for toxin production rather than the extent of toxin production. A further investigation of gene expression or actual toxin measurement on more strains will help determine how widespread toxin production is in food isolates of *B. cereus* from dairy and potato sources. More isolates should be included in studies of Hbl toxin and cereulide production in a biofilm of *B. cereus*, as only one isolate (P5 and F4810/72, respectively) was involved in each toxin study.
2. *B. paranthracis* and *B. toyonensis*, reported in New Zealand for the first time in this study, showed sporulation, biofilm formation and the presence of toxin genes, suggesting a potential

food safety risk, which requires further study. The characteristics of these two species, such as growing conditions and risk assessment may be useful to the food industry.

3. In this study, P2 was not a strong pellicle former, although it produced large amounts of biofilm (approximately 7 Log CFU/cm^2) on SS coupons. This leads to the hypothesis that the mechanism behind pellicle formation for *B. cereus* is different from that forming other types of biofilms. Okshevsky et al., (2018) revealed that the genes required for submerged biofilm and pellicle formation are different and these genes are involved in motility and amino acid metabolism. However, the mechanism behind these genes in regulating pellicle formation is unknown. A comprehensive comparison between pellicles and air-liquid interface biofilms or submerged biofilms by *B. cereus* may include transcriptomic analysis, although isolating pellicles is difficult. Studies relating to pellicles mainly rely on observation and no accurate quantification method has been developed. The pellicle is normally regarded as a type of air-liquid interface biofilm, although it is floating and lacks a solid surface for attachment (Branda et al., 2005; Chabane et al., 2014). Pellicle formation suggests that it is important to monitor and control the biofilm formation by *B. cereus* in liquid containers, such as tanks during transportation and other similar environments.
4. A more accurate method is needed for quantifying the DPA content in spores. The Spectro fluorimeter used to estimate the DPA in biofilm and planktonic spores in this study are based on the fluorescence of a complex of $Tb^{3+}$and DPA (Jamroskovic et al., 2016). An LC-MS/MS method was introduced by Wang et al., (2017) to quantify the release of DPA. This precise quantification method would contribute to a better understanding of the role of DPA in the heat resistance of spores regardless of other influencing factors, such as cell densities and dilution factors.
5. The role of the spore coat was speculated as a contributing factor to the greater heat resistance of spores isolated from biofilm than planktonic populations in this study. Further investigation of the role of proteins/genes involved in the spore coat in heat resistance, using transcriptomic and proteomic analysis and RT-qPCR are required. This can be determined using the representative spore former, P4, that showed a significant ($P < 0.05$) increase in the heat resistance in spores

harvested from biofilms compared with planktonic cultures prepared in both TSB and milk and showed visible differences in the structure of the spore coat (fragmented for planktonic spores while intact coat for biofilm spores).

6. A comparison of the resistance of spores from biofilm and planktonic cells to other stress factors such as sanitizers and acid is required. Ryu and Beuchat (2005) concluded that spores and/or vegetative cells in biofilms were protected against sanitizers, such as chlorine and chlorine dioxide. The efficacy of sanitizers in the inactivation of spores in biofilms in a food industry environment is useful information for the food industry. A hypothesis for diarrhoea caused by *B. cereus* has been proposed by Ceuppens et al., (2013), suggesting that spores pass gastric passage followed by survival, germination and enterotoxin production in the small intestine. This indicates the importance of acid resistance of spores and the effect of biofilms in the survival of the spores. Together with the results observed in the Hbl toxin study, diarrhoea may be caused by spores contained in biofilm formed in the small intestine leading to enterotoxin production.
7. How the surface properties of biofilm-forming substrates affect the heat resistance of spores is a useful future investigation. In this study, the spores attached to SS showed higher resistance than those in liquid culture. This may be caused by the metal or the hydrophobicity of SS. Modification of SS by impregnating metals with different ions and coatings to study the effect of surface characteristics on fouling was introduced by Santos et al., (2004). A similar method may be used in the future to investigate the effect of ions or the hydrophobicity of substrates on the heat resistance of biofilm spores. This may lead to the development of new modified surface material for the food industry to reduce the contamination by the heat-resistant spores in biofilms of *B. cereus*. The heat resistance of spores attached to other surface materials, such as glass or plastic, may also be investigated in the future.
8. More Hbl toxin was detected by biofilms formed on SS than on GW or in liquid culture. It was concluded that substratum affected Hbl toxin production. Therefore, the effect of surface characteristics on toxin production may be a worthwhile study. The possible attachment of Hbl toxin and cereulide found on SSW requires further study. Stainless-steel surfaces contain high levels of chromium oxide and ion availability. Free ions can affect *B. cereus* biofilm formation

(Hayrapetyan et al., 2015), which may also affect the enterotoxin production by the biofilm cells, however, how cations affect the toxin production by *B. cereus* is unknown.

9. It is difficult to grow a biofilm without the influence of planktonic cells. An independently grown biofilm was designed in this study, which was an attempt to minimize the effect of planktonic cells on toxin production by biofilms. Lower amounts of Hbl toxin were observed in the independently grown biofilm compared to planktonic culture, which may be because this independently grown biofilm was grown with limited nutrients as only medium surrounding the wool could be utilized by the biofilm cells. A better independent biofilm model is necessary to investigate toxin production by biofilm cells. A drip flow biofilm reactor may be considered in the future (Goeres et al., 2009).
10. More accurate quantification methods are necessary to measure Hbl toxin production. Western blotting using specific antibodies followed by purification and protein sequencing was used to quantify Hbl toxin production (Abdulmawjood et al., 2019). Unfortunately, the antibodies targeted to Hbl toxin are not commercially available. Moreover, the immunological detection of enterotoxin does not necessarily imply the presence of biologically active enterotoxins and the cell culture (cytotoxicity) assay using live tissue cells (such as Vero and Chinese hamster ovary cells), is suggested as the most sensitive method for *B. cereus* enterotoxin detection (Moravek et al., 2006). Ramm et al., (2020) introduced a cell-free protein synthesis method coupled with a transcription/translation reaction, to characterize the tripartite Nhe which is another enterotoxin produced by *B. cereus*. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used by Ramm et al., (2020) to confirm the synthesized Nhe toxin, together with fluorescently labeled protein analysis to confirm the band on a gel. A similar method may be applied for Hbl toxin, as these two enterotoxins have the similar gene and protein structures (Granum and Lund, 1997). All the methods mentioned above may be included in future work, although they are still semi-quantitive. Investigating Nhe toxin production in the presence of biofilms is important, as the cytotoxicity of *B. cereus* strains is thought to be dominated by the Nhe toxin, proposed by Moravek et al., (2006) who showed the toxic activity of *B. cereus* strains producing both Hbl and Nhe was like a sole Nhe producer.

11. The Hbl toxin production in the biofilms formed in a model small intestine environment should be investigated to reveal the possible role of biofilms in causing diarrhoea. Biofilms were shown in this study to contain more spores than planktonic cells. The spores were more heat resistant in the biofilm and they produced more Hbl toxin than planktonic cells. This suggests the need to study the link between spore formation and Hbl toxin in biofilms in the small intestine where enterotoxin causes diarrhoea (Ceuppens et al., 2013). This would help us to understand the role of biofilms in causing diarrhoea by *B. cereus*.
12. More may be learned through further gene expression studies. For example, the transcriptomic analysis of F4810/72 may reveal gene expression patterns for biofilm and planktonic cells of emetic *B. cereus* compared to the diarrhoeal strain used in this study. Transcriptomic studies on biofilms grown on different substrates may reveal the underlying mechanism behind Hbl toxin production seen on different surfaces in this study.
13. The attachment of cereulide on glass and SS surfaces observed in this study, leads to the question of whether the attachment of emetic toxin varies on different food products. The bacterial cells may be removed from the processing line, but the toxin may remain and contaminate food. This is of particular interest for starchy foodstuffs such as rice which is strongly associated with *B. cereus* emetic toxin intoxication (Dietrich et al., 2021).

9.3 Final conclusions

The objective of this research was to understand the spore formation and toxin production in the biofilms of *B. cereus*. This project identified six *B. cereus* isolates from either dairy or potato sources, with diversity in their biofilm- and spore-forming abilities with various combinations of toxin genes. The transcriptomic analysis of P5 showed that 48.5% of the gene content was significantly differentially expressed in biofilms grown on SS compared to planktonic cells, with planktonic cells showing upregulated genes related to germination, carbohydrate pathways and flagellar assembly, while biofilm cells showed upregulated genes involved in the secretion pathways and biosynthesis of biotin and siderophores. The higher sporulation percentage and increased heat resistance of spores

were observed for six *B. cereus* food isolates in biofilms compared to planktonic populations. Three possible explanations were proposed for these observations - (1) the spore coat structure (2) upregulated germination in planktonic cells and (3) upregulated sigma factor B expression in biofilm cells. In biofilms of *B. cereus,* Hbl toxin was mainly secreted into the surrounding environment while cereulide was mostly associated with the cells. Higher Hbl toxin was observed in the presence of biofilms grown on SS than planktonic culture using the BCET-RPLA kit and was supported by the higher expression of *hblACD* and upregulated secretion pathways in biofilm cells. Higher Hbl toxin production and higher gene expression were also observed in the biofilms grown on SS compared to those grown on glass, suggesting an influence of the substrate on Hbl toxin production. Unlike Hbl toxin, cereulide toxin was shown to be associated with biofilm cells/structures and attached to the substrates (SS and glass surface). The expression level of cereulide related genes (*cesA* and *cesB*) was comparable between biofilms and planktonic cells, suggesting a similar toxin-producing ability between the two types of cells. This project has demonstrated the importance of biofilms of *B. cereus* in food safety due to spore heat resistance and toxin production.

Appendix - Online Statement of Contribution

Appendix I Online Statement of Contribution _ Chapter 2

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In which chapter is the manuscript /published work:	Chapter 2

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(●) The manuscript/published work is published or in press

- Please provide the full reference of the Research Output:

Huang, Y., Flint, S.H., Palmer, J.S., 2020. Bacillus cereus spores and toxins – The potential role of biofilms. Food Microbiology. Vol: 90. Page: 103493. Doi: 10.1016/j.fm.2020.103493.

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Appendix II Online Statement of Contribution _ Chapter 4

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Huang, Y., Flint, S.H., Yu, S., Ding, Y., Palmer, J.S., 2021. Phenotypic properties and genotyping analysis of Bacillus cereus group isolates from dairy and potato products. LWT-Food Science and Technology. Vol: 140. Page: 110853. Doi: 10.1016/j.lwt.2021.110853.

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Appendix III Online Statement of Contribution _ Chapter 5 and Chapter 7

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Appendix IV Online Statement of Contribution _ Chapter 6

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Huang, Y., Flint, S.H., Palmer, J.S., 2021. The heat resistance of spores from biofilms of Bacillus cereus grown in tryptic soy broth and milk. International Dairy Journal. Vol: 123. Page: 105169. Doi: https://doi.org/10.1016/j.idairyj.2021.105169

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Appendix V Online Statement of Contribution _ Chapter 8

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LWT - Food Science and Technology

- The percentage of the manuscript/published work that was contributed by the candidate: 80.00
- Describe the contribution that the candidate has made to the manuscript/published work:

Conceptualization; Formal analysis; Investigation; Methodology; Writing – original draft.

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