Mesophilic Sporeformers Identified in Whey Powder by Using Shotgun Metagenomic Sequencing

Aoife J. McHugh[a,b], Conor Feehily[a,d], John T. Tobin[c], Mark A. Fenelon[c], Colin Hill[b,d], Paul D. Cotter[a,d]*

a Food Bioscience Department, Teagasc Food Research Centre, Cork, Ireland

b School of Microbiology, University College Cork, Cork, Ireland

c Food Chemistry and Technology Department, Teagasc Food Research Centre, Cork, Ireland

d APC Microbiome Ireland, Cork, Ireland

Running Title

Metagenomic analysis of dairy powder sporeformers

Keywords

DNA sequencing, dairy, mesophilic, metagenomics, powder, sporeformers, whey

For correspondence

*paul.cotter@teagasc.ie; Tel. (+353) 25 42694.

Abstract

Spoilage and pathogenic spore-forming bacteria are a major cause of concern for producers of dairy products. Traditional agar-based detection methods employed by the dairy industry have limitations with respect to their sensitivity and specificity. The aim of this study was to identify low-abundance sporeformers in samples of a powdered dairy product, whey powder, produced monthly over one year, using novel culture-independent shotgun metagenomics-based approaches. Although mesophilic sporeformers were the main target of this study, in one instance thermophilic sporeformers were also targeted using this culture-independent approach. For comparative purposes, mesophilic and thermophilic sporeformers were also tested for within the same sample using culture-based approaches. Ultimately, the approaches taken highlighted differences in the taxa identified due to treatment and isolation methods. Despite this, low levels of transient, mesophilic, and in some cases potentially pathogenic, sporeformers were consistently detected in powder samples. Although the specific sporeformers changed from one month to next, it was apparent that 3 groups of mesophilic sporeformers, namely, *Bacillus cereus*, *Bacillus licheniformis*/*Bacillus Paralicheniformis*, and a third, more heterogeneous group containing *Brevibacillus brevis,* dominated across the 12 samples. Total thermophilic sporeformer taxonomy was considerably different to mesophilic taxonomy, as well as the culturable thermophilic taxonomy in the one sample analysed by all four approaches. Ultimately, through the application of shotgun metagenomic sequencing to dairy powders, the potential for this technology to facilitate the detection of undesirable bacteria present in these food ingredients is highlighted.

Importance

The ability of sporeformers to remain dormant in a desiccated state is of concern from a safety and spoilage perspective in dairy powder. Traditional culturing techniques are slow and provide little information without further investigation. We describe the identification of mesophilic sporeformers present in powders produced over one year, using novel shotgun metagenomic sequencing. This method allows detection and identification of possible pathogens and spoilage bacteria in parallel. Strain-level analysis and functional gene analysis, such as identification of toxin genes, were also performed. This approach has the potential to be of great value with respect to the detection of spore-forming bacteria and could allow a processor to make an informed decision surrounding process changes to reduce the risk of spore contamination.

Introduction

Milk and resultant dairy products can become contaminated by bacteria from a number of sources, including production and processing facility contaminants. Soil, bedding, feed, feces and teat surface all harbor bacteria that can transfer to raw milk (1, 2), with milking and housing practices are a potential contributor to raw milk contamination (3). Although many of the bacteria present in milk are killed by traditional processing techniques, bacterial spores can survive heat treatment and desiccation (1, 4). Furthermore, within processing facilities, microbial biofilms formed on equipment surfaces can be persistent. These are frequently resistant to cleaning and cells, including spore-forming bacteria, from these biofilms can slough off to contaminate products (5, 6). Furthermore, spores, regardless of their origin, can withstand further processing and remain in a dormant form in powdered dairy products thereafter (7, 8).

On the basis of culture-based analyses, sporeformers identified frequently in dairy powders include representatives of the genera *Bacillus*, *Geobacillus*, *Anoxybacillus*, *Lysinibacillus*, *Brevibacillus*, *Paenibacillus* (9, 10) as well as *Clostridium* (11, 12). Some of these sporeformers, including *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus amyloliquefaciens*, members of *Bacillus cereus sensu lato* (13) and *Geobacillus* species (14), are associated with spoilage of dairy products. Also, some members of *B. cereus sensu lato* have the potential to cause food poisoning. More specifically, *B. cereus* can cause diarrheal or emetic food poisoning. Diarrheal food poisoning is caused by enterotoxins, i.e., nonhemolytic enterotoxin (Nhe), cytotoxin K (CytK) or hemolysin BL (HBL), with symptom onset occurring 8 to 16 h after ingestion (15, 16).

Emetic food poisoning is caused by emetic toxin cereulide (Ces), which is produced by a nonribsomal peptide synthetase and results in vomiting within a few hours of ingestion (16). Toxin-producing *Clostridium* species, such as *Clostridium botulinum* and *Clostridium perfringens* (7), are also a concern but are less common in dairy powder than bacteria of the class Bacilli(17).

Despite safety and economic concerns, there is little legislation governing the total numbers of permissible sporeformers in dairy powders. However, processors and customers generally set strict guidelines to ensure high standards. U.S. powders destined for international customers have limits of <500 cfu g^{-1} thermophilic sporeformers and <1,000 cfu g^{-1} mesophilic sporeformers (18). These limits reflect the fact that traditional culture-based detection and enumeration methods for spore-forming bacteria rely on a variety of temperature treatments to differentiate between heat-resistant and highly heat-resistant sporeformers, incubation temperatures (to differentiate among psychrotolerant, mesophilic and thermophilic sporeformers), and incubation conditions (to differentiate between aerobic and anaerobic sporeformers) (17, 18). A number of selective and chromogenic agars may also be employed to select for and/or identify pathogenic groups, such as *Clostridium* species and *B. cereus* group (19, 20). Further biochemical or molecular methods can be subsequently utilized to identify the species isolated (1).

The heavy reliance on culture-based assays has been highlighted as a concern in recent years. These methods are labor intensive, requiring many media types, sample treatments, incubation temperatures, and conditions per sample to get an overview of total numbers of different

groups of bacteria present. These need to be followed by additional testing to identify or confirm identity of the species present and, where necessary, further analysis to determine if species present contain toxin genes (21).

Here, shotgun metagenomic DNA sequencing (22) is employed to identify bacteria present in dairy powders, as an extension of the recent application of the technology to identify pathogenic strains in a fermented dairy beverage (23) or spoilage microorganisms in cheese (24). Shotgun metagenomics can generate a considerable volume of data, enabling the detection of culturable and nonculturable microorganisms, with accurate identification to species level and sometimes strain level. It also allows an investigation of specific gene sequences of interest, such as, for example, antimicrobial resistance or toxin-encoding genes (25, 26). However, this analysis remains expensive and poses data analysis-related difficulties due to the frequently large quantity of data generated (22). With a view to beginning to bridge the gap toward the application of this powerful technology to food quality and safety testing, this study employed shotgun metagenomics to test 12 powdered dairy samples produced monthly at the same location over one calendar year to determine the mesophilic spore content of these powders. For the purpose of comparison, one sample (from August [A]) was examined in greater depth to assess the mesophilic spore content (A.M), thermophilic spore content (A.T), as well as the culturable mesophilic spore content (A.M.P), and culturable thermophilic spore content (A.T.P).

Results

Shotgun metagenomics can be used to identify and determine the functional potential of low-abundance sporeformers present in dairy powders.

A total of 12 whey powder samples, produced monthly at the same location over one calendar year, were sampled upon exit from the spray drier prior to packing and collected for spore analysis. Following spore-pasteurization of reconstituted powders, in which samples were incubated at 80°C for 12 min to select for spore-forming bacteria, a total of <400 cfu g^{-1} mesophilic and thermophilic sporeformers were enumerated by plating whey solutions on brain heart infusion (BHI) agar and incubating at 30˚C and 55˚C, respectively, for 24 h. These are within previously described consumer specified limits of <500 cfu g^{-1} thermophilic sporeformers and <1,000 cfu g^{-1} mesophilic sporeformers (18).

To facilitate a more in-depth characterization, sporeformer enrichment was performed and DNA extraction and shotgun metagenomic sequencing was completed. The enrichment approach taken addressed the risk of reads arising from sequencing of DNA from dead cells, but had a drawback of under-representing spores which did not germinate under the conditions employed. The average number of reads after quality filtering and trimming per sample was 1,106,747. Kaiju, Kraken and MetaPhlan2 were all used to assess each package's relative ability to taxonomically assign reads , and results from all three tools are presented. Kraken assigned the greatest percentage of reads at the species level, coupled with the previously reported possibility of high levels of false positives resulting from Kaiju assignment (27) and the fact MetaPhlAn2 works off only a subset of marker genes per species (28), Kraken was employed

preferentially, with a filter threshold of 0.2 to increase precision without detrimentally impacting sensitivity. Furthermore, to reduce the possibility of false positives (27), taxa were included only if present at a minimum of 1% relative abundance in at least one sample, otherwise reads were assigned as “others”.

B. *cereus* was found to be the dominant species in 7 of the 12 monthly mesophilic sporeformer enriched samples, i.e., those from January, February, March, May, July, October and November. *Bacillus thuringiensis*, a member of *B*. *cereus sensu lato*, was the next most abundant species in these samples (Fig 1). Among the other mesophilic sporeformer-enriched samples, *B*. *licheniformis* was present as the dominant species in April and August. It was also present in the September and December samples but not dominant. *B*. *subtilis* was dominant in the September sample but also detected at lower relative abundance in August and December. *B*. *paralicheniformis* was the dominant species in the December sample while also present, but not dominant in April, August and September (Fig 1.) The June sample was dominated by *Brevibacillus brevis* and *Streptococcus thermophilus*. *S*. *thermophilus* was also detected in lower levels in other samples too, despite not being a spore-former. All species identified as dominant were confirmed by Kaiju- and MetaPhlAn2-derived results (Fig S1 & S2 in the supplemental material).

Spearman correlations were utilized to evaluate the population relationships in samples with taxonomic analysis and functional gene analysis. Spearman correlations are used for nonnormally distributed data that is either skewed or ordinal. Statistical analysis was performed on correlations, asymptotic P values were generated using rcorr in the R package

Hmisc and corrected with Benjamini-Hochberg corrections for multiple comparisons. Significant negative correlation ($P < 0.05$) between *B*. *cereus* and *B*. *licheniformis* and between *B*. *cereus* and *B*. *paralicheniformis* were noted. Significant positive correlations ($P < 0.05$) were seen between *B*. *licheniformis* and *B*. *paralicheniformis*, *Bacteriodes vulgatus* and *Bacteriodes fragilis*, *B*. *vulgatus* and others, and *B*. *fragilis* and others. As expected, many sporulation-associated functional gene groups were significantly positively correlated ($P < 0.05$), including those for dormancy and sporulation with those for virulence disease/defense, regulation/cell signaling, iron acquisition/metabolism, and cell wall/capsule formation (Fig S3 in the supplemental material).

FASTA reads were aligned to Bagel 3 bacteriocin database using DIAMOND to determine if bacteriocin production potential could be influencing current and future relationship dynamics. This showed a number of potential type I, type II and type III bacteriocin genes in each sample, with highest relative abundance per sample going to type I bacteriocin genes (Fig 2). Bacteriocin genes of note included two lichenicidin peptide genes present in all samples containing *B*. *licheniformis*, genes for sporulation killing factor skfA and subtilosin are seen in samples containing *B*. *subtilis*, and thuricin genes in samples containing *B*. *thuringiensis* (Fig 2A).

The August sample was selected for a parallel culture-based analysis and was enriched for culturable mesophilic sporeformers by culturing on BHI agar and incubating at 30°C for 48 h, following 48 h enrichment of a spore-pasteurised sample at 30°C. This was labeled August plate-cultured mesophilic sporeformer enriched sample (A.M.P). An additional aliquot of the same spore-pasteurised sample was enriched for thermophilic sporeformers, by incubating at

55°C for 48 h; this was labeled August thermophilic sporeformer enriched sample (A.T). Subsequently this sample was plated on BHI agar and incubated at 55°C for 48 h. This was labeled as August plate-cultured thermophilic sporeformer-enriched sample (A.T.P). The original August mesophilic sporeformer enriched sample was relabeled A.M for the purpose of comparison. In the case of the plate cultured samples (A.M.P and A.T.P), colonies were scraped off, and DNA extracted in all instances and sequenced as previously described. Comparative analysis revealed that the A.M.P and A.M samples had a very similar profile, highlighting that plating did not bias results (Fig 3). However, the taxonomic profile of the A.T sample and A.T.P samples were very different, i.e., the dominant species present in the A.T sample was *Thermoanaerobacterium thermosaccharolyticum*, whereas the equivalent post-plating sample (A.T.P) was comprised of *B*. *brevis*, *Bacteroides* species as well as a variety of other species at low abundance (Fig 3).

Beta diversity analysis highlights that samples cluster according to the dominant species present

Bray-Curtis beta diversity analysis of mesophilic sporeformer reads showed that the 12 samples clustered into 3 distinct groups (individual data points within each group being co-located; Fig 4A). One cluster consisted of samples that contained *B*. *cereus* (i.e., January, February, March, May, July, October, November); a second cluster consisting of samples from months April, August, September and December, which all contain high relative abundance of *B. licheniformis*; and a third corresponded to the June sample, which had high relative abundance of *B*. *brevis* (Fig 4A).

Alpha diversity analysis did not reveal any notable pattern other than the observation that the June, September and December samples had the highest diversities (Fig 4B). As might be expected from taxonomic results, beta diversity analysis of the August sample, which was enriched in a variety of ways, showed that A.M and A.M.P samples were least dissimilar, whereas the A.T and A.T.P samples differed from each other and from A.M and A.M.P (Fig 4C). Among these samples, alpha diversity in A.T.P was higher than all others (Fig 4D).

Toxin gene analysis revealed presence of potentially toxigenic *B. cereus*

Further analyses was performed to determine if the *B*. *cereus* strains detected in some samples might be capable of causing emetic and enterotoxic food poisoning. For this purpose, toxin genes were aligned to sequence reads and verified by aligning them to assembled contigs. The genes used and the genome sequences from which they originated are shown in Table 1. Of the 11 toxin genes screened for among all 12 mesophilic enriched samples, the two nonhemolytic enterotoxin (Nhe) genes; *nhe* L1 and *nhe* L2*,* were detected in all 7 samples previously shown to contain high relative abundances of *B*. *cereus*. The cytotoxin K-encoding gene was detected in 3 samples (from March, October and November). The presence of toxin genes in the sequence reads was verified by alignment and visualization using MAUVE. Toxin gene sequences were aligned to sample contigs, which were assembled using IDBA-UD (Fig S4 in the supplemental material). Among samples in which all 3 toxin genes were present, toxin genes accounted for close to 0.1% of reads and, when just the two *nhe* genes were present, they accounted for close to 0.06% of reads (Fig 5).

Strain level analysis revealed the absence of evidence for persistent contamination

As the samples that contained high relative abundances of *B*. *cereus* appeared to have different toxin profiles, it was decided to carry out PanPhlAn-based strain analysis to more accurately determine if one strain was dominating across these samples, potentially indicating strain persistence in the processing facility, or transient colonization by different strains. Sequence reads for all samples were aligned to a pangenome created from 32 complete *B. cereus* genomes downloaded from the NCBI database. This analysis established that the presence/absence of *B*. *cereus* was consistent with previous Kraken analysis. PanPhlAn showed the 7 samples containing *B*. *cereus* contained reads that clustered with 5 strains of *B*. *cereus* by Euclidean distance, as visualized using GraPhlAn (Fig 6). The percentage match from sequence reads to the 32 genomes used in the pangenome was also examined (Table 2). Overall, as shown in Table 2, none of the strains identified in the 7 samples are a complete match to any of the 32 strains with which they were compared. Similarly, none of the strain identified in the 7 samples appear to be exactly the same, with January and February samples being most alike (Fig 6).

Discussion

Traditionally, the detection and identification of bacterial sporeformers has involved culturing under different temperatures and conditions (17, 18), together with the use of selective agars to identify pathogenic species (19, 20), followed by further biochemical or molecular approaches for confirmation (1). This study highlights the ability of next-generation shotgun sequencing of enriched samples to identify low-level persistent or transient spore contamination in a dairy powder. Although many of the species identified are similar to those identified in previous studies using traditional detection methods (17), the approach taken has the potential to rapidly identify these species while allowing strain level analysis and, as a result, the tracking of persistent microbes in products. It highlights that extremely low levels of potentially pathogenic bacteria can be present, although in this instance, these bacteria are unlikely to be from persistent contamination in the processing facility but rather are thought to represent a transient low-level contamination. Due to the sensitivity of this approach, new guidelines and standards may need to be introduced to ensure that the risks associated with detection of low-level contamination of *B*. *cereus* in powders are adequately reflected.

The combined approach of high-temperature treatment coupled with shotgun sequencing employed in this study was selected for a number of reasons. Prior to high-temperature activation, the spore-forming bacteria present in dairy powders are likely to be in a spore form, from which DNA extraction can be difficult (29). Furthermore, in the absence of enrichment, the possibly high level of DNA remaining from deceased bacteria and the presence of host bovine cells in the powders could also be an issue, as DNA from these sources will also be

sequenced when untargeted shotgun metagenomic sequencing is employed (22), thereby necessitating the use of additional steps, and expensive kits and reagents to deplete DNA from these other sources (30-34). DNA amplification would likely be necessary thereafter due to the low yield of DNA from the low level of sporeformers present in samples. Amplification methods add extra bias, costs, and potential for contamination (34-36). A more targeted, economical approach was used to focus on sporeformers that could potentially germinate in rehydrated powder given favorable temperatures, without the extra bias and cost of kits. It should be noted that low-speed centrifugation utilized in the approach from which solids were discarded prior to DNA extraction could result in loss of some spore-forming bacteria that have a high affinity for denatured milk protein solids.

This targeted, culture-independent approach was compared with corresponding culture-dependent approaches using the August sample as a representative test case. The thermophilic sporeformer enriched August sample (A.T), from which DNA was extracted, was also subject to culturing on BHI agar at thermophilic temperatures (A.T.P) before pooling colonies and extracting DNA. DNA from both A.T and A.T.P samples were sequenced (Fig S5 in the supplemental material) and compared.

The results were different in that the dominant species identified following enrichment (i.e., without culturing on BHI agar) was *T. thermosaccharolyticum*, while this species was not detected in the cultured sample, potentially showing bias due to agar and conditions used. *T. thermosaccharolyticum* has previously been detected in dried vegetables (37), and more recently, *Thermoanaerobacterium* spp. has been detected in the core microbiome of raw milk

(38). *T*. *thermosaccharolyticum* is a thermophilic, anaerobic sporeformer and was previously classified as a member of the *Clostridium* genus, although subsequently reclassified (39). It is a known canned food spoilage organism (40) that produces hydrogen and causes swelling in canned foods. This ability to efficiently produce hydrogen makes it a potential important organism for sustainable biohydrogen production (41, 42). The previous nondetection of the species in dairy powders may relate to an inability to grow on the agar substrates conventionally employed by the dairy industry.

A decision was made to further investigate the potential *B*. *cereus* sequences identified to eliminate the potential that the taxonomic classifier was misassigning other members of *B*. *cereus sensu lato* and *B*. *cereus sensu stricto*, as members of this group are notoriously difficult to differentiate (43). Toxin profiling and PanPhlAn analysis confirmed that the strains had the potential to be pathogenic, while also highlighting differences between them. All except one of the samples that contained *B*. *cereus* showed complete alignment with both Nhe toxin-encoding genes. The exception was the February sample, from which one half of *nhe* L1 gene was absent. This may be due to misassembly or poor coverage of the genome, or may reflect a natural mutation in this strain (Fig S4). It should be noted that alignment to toxin genes infers potential ability to produce toxins, but does not conclusively indicate functional toxin presence. Also, a total of 10^5 to 10^9 cfu of toxin producing *B*. *cereus* is needed to cause food poisoning (15). The highly sensitive, qualitative nature of shotgun metagenomic analysis suggests that further steps will need to be taken to determine the risk associated with the detection of these and other toxin genes in food samples. PanPhlAn analysis showed that the 7 samples containing *B*. *cereus* contained reads that clustered with at least 5 strains of *B*. *cereus* by Euclidean

distance suggesting the strains identified in the samples are different and not as a result of persistent contamination in the powder production facility or elsewhere.

The negative correlations between *B*. *cereus* and *B*. *licheniformis*/*B*. *paralicheniformis* raises the possibility of competition to determine the dominant species and the possible inhibition of the other species. The potential bacteriocin genes detected in all samples may impact the relationship dynamics observed and may be having an antagonistic effect on some species currently or in future food products. Lichenicidin is a two-peptide lantibiotic previously shown to be active against pathogenic gram-positive bacteria, including *B*. *cereus* (44), and we noted alignment to two peptides associated with lichenicidin in all four samples containing *B*. *licheniformis*. However, potential identification of two peptide genes does not suggest the presence of the whole bacteriocin gene cluster or infer correct post translational modifications to produce a functional bacteriocin. Positive correlations between virulence, disease, defense, dormancy, and sporulation highlight the need to be cautious of sporeformers in food products. A greater understanding of their relationships will aid the prevention of spoilage and pathogenic species that cause concern in food processing.

Disadvantages that need to be overcome in order to allow for the routine use of the sequencing technologies employed in this study primarily relate to cost of analysis, which is currently too expensive for large-scale routine use. Additionally, there are challenges relating to assembly of genomes from shotgun metagenomic sequencing (22) and difficulties arising from insufficient accuracies associated, to different extents, with taxonomic classifiers (27). There are some solutions emerging, whereby new lower-cost, rapid sequencers are arriving on the market, with

MinION (45) leading the way towards quick portable detection systems for microorganisms. Through the generation of more reference genome sequences and good-quality shotgun metagenomic sequencing, reference databases and the accuracy of results will improve.

This study highlighted monthly diverging contamination patterns in whey powder production, which converged into 3 distinct mesophilic sporeformer population groups from 12 powder samples produced in the same production facility, and has shown that the way in which the powders are treated post production (namely, incubation temperature post reconstitution) influences which bacteria germinate and become dominant. Shotgun metagenomics is a useful tool to delve deeper into the understanding of sporeformers and their relationships in food processing, although it brings with it its own set of caveats and need for guidelines for use and interpretation of results.

Materials and methods

Sample preparation and enrichment

Twelve (i.e. one per month for a calendar year) 5-g whey powder samples from a single supplier were suspended aseptically at a concentration of 10% (wt/vol) in sterile ¼ strength Ringer's solution (Sigma-Aldrich). Each reconstituted sample was incubated at 80°C for 12 min to select for spore-forming bacteria as previously described (3, 17). An aliquot was then plated onto Brain Heart Infusion (BHI) agar before incubation at mesophilic (30°C) and thermophilic (55°C) suitable temperatures and cfu g^{-1} values were calculated.

Following spore selection, in which 10% (wt/vol) reconstituted samples were incubated at 80°C for 12 min, reconstituted samples were enriched in a manner consistent with that previously employed to select for low abundance mesophilic sporeformers, but with incubation at 30°C for 48 h as opposed to 32°C as previously documented (3, 17). This is shown in Fig S5 in the supplemental material.

To facilitate an investigation into the extent to which plating altered the identity of the populations of sporeformers detected, powder sourced in August [A], was treated as described above but with incubation at 55°C to select for thermophilic sporeformers (A.T). Both of the enriched August samples were plated onto BHI agar, before incubating at the corresponding mesophilic (30°C; A.M.P) and thermophilic (55°C; A.T.P) temperatures for 48 h (Fig S5). DNA was extracted from the surface of these agar plates as described below.

DNA extraction and library preparation

A total of Fifty millilitres of samples that were reconstituted, heat-treated, and enriched by incubation for 48 h at 30°C were centrifuged at 900 x g for 20 min. The resultant pellet was discarded, and supernatant centrifuged at 4500 x g for 20 min. Pellets were washed in 1 ml sterile ¼ Ringer's solution and centrifuged at 13,000 x g for 2 min. Washing was repeated twice more, centrifuging for 1 min each time at 13,000 x g. DNA was extracted from the resultant pellet as described below. For DNA extraction from a combination of all colonies on the surface of agar plates, 5 ml ¼ Ringer's solution was added and colonies were washed off using a sterile Lazy-L spreader (Sigma-Aldrich). An aliquot of 4 ml of the resultant fluid was removed and centrifuged at 4500 x g for 20 min. The resultant pellet was suspended in 1 ml ¼ Ringer's

solution and centrifuged at 13000 x g for 2 min. The pellet was washed twice more in 1 ml of Ringer's solution, and centrifuged at 13000 x g. Before the final centrifugation 200 µl was removed from the mix to a sterile tube. This was centrifuged at 13000 x g for 1 min. This pellet corresponded to 0.8 ml of culture from original 4 ml.

All pellets were resuspended in 150 µl of 45 mg ml^{-1} lysozyme and incubated at 37°C for 30 min. Samples were centrifuged at 13000 x g for 1 min, and the supernatant was removed. From this point, the MoBio PowerFood DNA Isolation kit protocol was followed, including the use of the alternative lysis step for difficult-to-lyse cells. DNA was eluted in 60 µl of 10mM Tris-HCl and stored at -20°C. DNA was quantified and quality checked using the Qubit double-stranded DNA (dsDNA) high sensitivity assay kit (Bio-Sciences, Dublin, Ireland) and by visualising on a 1% (wt/vol) agarose gel.

Samples were prepared for metagenomic sequencing according to Illumina Nextera XT Library preparation kit guidelines and sequenced on an Illumina Miseq sequencing platform at the Teagasc DNA Sequencing Facility with a 2 x 250 V2 kit using standard Illumina sequencing protocols.

Bioinformatics pipeline

Raw metagenomic shotgun reads were checked for the presence of human and bovine reads filtered on the presence of quality and quantity, and trimmed to 170 bp with a combination of Picard tools (http://broadinstitute.github.io/picard/) and SAMtools (46). Kraken with a filter threshold of 0.2 (47) and SUPER-FOCUS (48) were used to determine microbial composition to species level and biological functions, respectively. MetaPhlAn2 (28) and Kaiju (49), were also

utilized to determine microbial composition, for comparison to Kraken results. Eleven *B. cereus* toxin-associated genes (Table 1) were downloaded from the NCBI database and aligned to sequence reads using Bowtie2 (50). Metagenomes were assembled into contigs using IDBA-UD (51) and toxin genes were aligned to these using Bowtie2 (50). Toxin genes were also aligned to contigs using Mauve version 20150226 (52), more specifically progressiveMauve (53), using default parameters (default seed weight, determine local collinear blocks [LCBs], full alignment, iterative refinement, min LCB weight = default, sum-of-pairs LCB scoring). PanPhlAn (54) was utilized to determine *B. cereus* strain profiles in each sample. A total of thirty two complete *B. cereus* genome sequences were downloaded from the NCBI database and a pangenome was generated and compared to all samples. PanPhlAn outputs were visualized using GraPhlAn (55). Spearman correlations with Benjamini-Hochberg corrections for multiple comparisons were made in R using package Hmisc and visualized using corrplot. Diversity was analyzed using the R vegan package and data visualization was performed using ggplot2. Sequence reads were aligned to Bagel 3 bacteriocin database (56) using DIAMOND (57), to determine the bacteriocin potential of the bacteria identified.

Accession numbers

Sequence data have been deposited in the European Nucleotide Archive (ENA) under the study accession number PRJEB24853.

Acknowledgements including funding source

This Research was funded by Department of Agriculture, Food and the Marine (DAFM) under the FIRM project 'SACCP', reference number 14/F/883. Research in the Cotter lab is also funded by SFI award 'Obesiobiotics' (11/P1/1137). Thanks to Orla O'Sullivan, Fiona Crispie and Laura Finnegan of the Food Bioscience department in Teagasc Moorepark.

References

1. Gleeson D, O'Connell A, Jordan K. 2013. Review of potential sources and control of thermoduric bacteria in bulk-tank milk. Irish Journal of Agricultural and Food Research 52:217-227.
2. Doyle CJ, Gleeson D, O'Toole PW, Cotter PD. 2017. Impacts of seasonal housing and teat preparation on raw milk microbiota: a high-throughput sequencing study. Appl Environ Microbiol 83.
3. Miller RA, Kent DJ, Boor KJ, Martin NH, Wiedmann M. 2015. Different management practices are associated with mesophilic and thermophilic spore levels in bulk tank raw milk. J Dairy Sci 98:4338-51.
4. Setlow P. 2003. Spore germination. Current Opinion in Microbiology 6:550-556.
5. Pérez-Rodríguez F, Valero A, Carrasco E, García RM, Zurera G. 2008. Understanding and modelling bacterial transfer to foods: a review. Trends in Food Science & Technology 19:131-144.
6. Faille C, Benezech T, Midelet-Bourdin G, Lequette Y, Clarisse M, Ronse G, Ronse A, Slomianny C. 2014. Sporulation of *Bacillus* spp. within biofilms: a potential source of contamination in food processing environments. Food Microbiol 40:64-74.
7. Doyle CJ, Gleeson D, Jordan K, Beresford TP, Ross RP, Fitzgerald GF, Cotter PD. 2015. Anaerobic sporeformers and their significance with respect to milk and dairy products. Int J Food Microbiol 197:77-87.

8. Gopal N, Hill C, Ross PR, Beresford TP, Fenelon MA, Cotter PD. 2015. The prevalence and control of *Bacillus* and related spore-forming bacteria in the dairy industry. Front Microbiol 6:1418.

9. Ronimus RS, Parker LE, Turner N, Poudel S, Ruckert A, Morgan HW. 2003. A RAPD-based comparison of thermophilic Bacilli from milk powders. Int J Food Microbiol 85:45-61.

10. Sadiq FA, Li Y, Liu T, Flint S, Zhang G, He G. 2016. A RAPD based study revealing a previously unreported wide range of mesophilic and thermophilic spore formers associated with milk powders in China. Int J Food Microbiol 217:200-8.

11. Barash JR, Hsia JK, Arnon SS. 2010. Presence of soil-dwelling Clostridia in commercial powdered infant formulas. J Pediatr 156:402-8.

12. Buehner KP, Anand S, Djira GD. 2015. Prevalence of thermoduric bacteria and spores in nonfat dry milk powders of Midwest origin. J Dairy Sci 98:2861-6.

13. De Jonghe V, Coorevits A, De Block J, Van Coillie E, Grijspeerdt K, Herman L, De Vos P, Heyndrickx M. 2010. Toxinogenic and spoilage potential of aerobic spore-formers isolated from raw milk. International Journal of Food Microbiology 136:318-325.

14. Seale RB, Dhakal R, Chauhan K, Craven HM, Deeth HC, Pillidge CJ, Powell IB, Turner MS. 2012. Genotyping of present-day and historical *Geobacillus* species isolates from milk powders by high-resolution melt analysis of multiple variable-number tandem-repeat loci. Appl Environ Microbiol 78:7090-7.

15. Granum PE, Lund T. 1997. *Bacillus cereus* and its food poisoning toxins. FEMS Microbiology Letters 157:223-228.

16. Ehling-Schulz M, Guinebretiere MH, Monthan A, Berge O, Fricker M, Svensson B. 2006. Toxin gene profiling of enterotoxic and emetic *Bacillus cereus*. FEMS Microbiol Lett 260:232-40.

17. Miller RA, Kent DJ, Watterson MJ, Boor KJ, Martin NH, Wiedmann M. 2015. Spore populations among bulk tank raw milk and dairy powders are significantly different. J Dairy Sci 98:8492-504.

18. Watterson MJ, Kent DJ, Boor KJ, Wiedmann M, Martin NH. 2014. Evaluation of dairy powder products implicates thermophilic sporeformers as the primary organisms of interest. J Dairy Sci 97:2487-97.

19. Weenk GH, van den Brink JA, Struijk CB, Mossel DAA. 1995. Modified methods for the enumeration of spores of mesophilic *Clostridium* species in dried foods. International Journal of Food Microbiology 27:185-200.

20. Tallent SM, Kotewicz KM, Strain EA, Bennett RW. 2012. Efficient isolation and identification of *Bacillus cereus* group. J AOAC Int 95:446-51.

21. Wehrle E, Moravek M, Dietrich R, Burk C, Didier A, Martlbauer E. 2009. Comparison of multiplex PCR, enzyme immunoassay and cell culture methods for the detection of enterotoxinogenic *Bacillus cereus*. J Microbiol Methods 78:265-70.

22. Sharpton TJ. 2014. An introduction to the analysis of shotgun metagenomic data. Front Plant Sci 5:209.

23. Walsh AM, Crispie F, Daari K, O'Sullivan O, Martin JC, Arthur CT, Claesson MJ, Scott KP, Cotter PD. 2017. Strain-level metagenomic analysis of the fermented dairy beverage

nunu highlights potential food safety risks. Applied and Environmental Microbiology doi:10.1128/aem.01144-17.

24. Quigley L, O'Sullivan DJ, Daly D, O'Sullivan O, Burdikova Z, Vana R, Beresford TP, Ross RP, Fitzgerald GF, McSweeney PL, Giblin L, Sheehan JJ, Cotter PD. 2016. Thermus and the pink discoloration defect in cheese. mSystems 1.

25. Bengtsson-Palme J, Boulund F, Fick J, Kristiansson E, Larsson DG. 2014. Shotgun metagenomics reveals a wide array of antibiotic resistance genes and mobile elements in a polluted lake in India. Front Microbiol 5:648.

26. Leonard SR, Mammel MK, Lacher DW, Elkins CA. 2015. Application of metagenomic sequencing to food safety: detection of Shiga toxin-producing *Escherichia coli* on fresh bagged spinach. Appl Environ Microbiol 81:8183-91.

27. Piro VC, Matschkowski M, Renard BY. 2017. MetaMeta: integrating metagenome analysis tools to improve taxonomic profiling. bioRxiv doi:10.1101/138578.

28. Truong DT, Franzosa EA, Tickle TL, Scholz M, Weingart G, Pasolli E, Tett A, Huttenhower C, Segata N. 2015. MetaPhlAn2 for enhanced metagenomic taxonomic profiling. Nat Meth 12:902-903.

29. Wielinga PR, de Heer L, de Groot A, Hamidjaja RA, Bruggeman G, Jordan K, van Rotterdam BJ. 2011. Evaluation of DNA extraction methods for *Bacillus anthracis* spores spiked to food and feed matrices at biosafety level 3 conditions. Int J Food Microbiol 150:122-7.

30. Rudi K, Moen B, Dromtorp SM, Holck AL. 2005. Use of ethidium monoazide and PCR in combination for quantification of viable and dead cells in complex samples. Appl Environ Microbiol 71:1018-24.

31. Forghani F, Langaee T, Eskandari M, Seo KH, Chung MJ, Oh DH. 2015. Rapid detection of viable *Bacillus cereus* emetic and enterotoxic strains in food by coupling propidium monoazide and multiplex PCR (PMA-mPCR). Food Control 55:151-157.

32. Feehery GR, Yigit E, Oyola SO, Langhorst BW, Schmidt VT, Stewart FJ, Dimalanta ET, Amaral-Zettler LA, Davis T, Quail MA, Pradhan S. 2013. A method for selectively enriching microbial DNA from contaminating vertebrate host DNA. PLoS ONE 8:e76096.

33. Yokouchi H, Fukuoka Y, Mukoyama D, Calugay R, Takeyama H, Matsunaga T. 2006. Whole-metagenome amplification of a microbial community associated with scleractinian coral by multiple displacement amplification using phi29 polymerase. Environ Microbiol 8:1155-63.

34. Binga EK, Lasken RS, Neufeld JD. 2008. Something from (almost) nothing: the impact of multiple displacement amplification on microbial ecology. ISME J 2:233-41.

35. Ahsanuddin S, Afshinnekoo E, Gandara J, Hakyemezoglu M, Bezdan D, Minot S, Greenfield N, Mason CE. 2017. Assessment of REPLI-g multiple displacement whole genome amplification (WGA) techniques for metagenomic applications. J Biomol Tech 28:46-55.

36. Thoendel M, Jeraldo P, Greenwood-Quaintance KE, Yao J, Chia N, Hanssen AD, Abdel MP, Patel R. 2017. Impact of contaminating DNA in whole genome amplification kits

used for metagenomic shotgun sequencing for infection diagnosis. J Clin Microbiol doi:10.1128/JCM.02402-16.

37. Postollec F, Mathot AG, Bernard M, Divanac'h ML, Pavan S, Sohier D. 2012. Tracking spore-forming bacteria in food: from natural biodiversity to selection by processes. Int J Food Microbiol 158:1-8.

38. Rodrigues MX, Lima SF, Canniatti-Brazaca SG, Bicalho RC. 2017. The microbiome of bulk tank milk: Characterization and associations with somatic cell count and bacterial count. J Dairy Sci doi:10.3168/jds.2016-11540.

39. Collins MD, Lawson PA, Willems A, Cordoba JJ, Fernandez-Garayzabal J, Garcia P, Cai J, Hippe H, Farrow JA. 1994. The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. Int J Syst Bacteriol 44:812-26.

40. André S, Zuber F, Remize F. 2013. Thermophilic spore-forming bacteria isolated from spoiled canned food and their heat resistance. Results of a French ten-year survey. International Journal of Food Microbiology 165:134-143.

41. O-Thong S, Prasertsan P, Karakashev D, Angelidaki I. 2008. Thermophilic fermentative hydrogen production by the newly isolated *Thermoanaerobacterium thermosaccharolyticum* PSU-2. International Journal of Hydrogen Energy 33:1204-1214.

42. Mtimet N, Guegan S, Durand L, Mathot AG, Venaille L, Leguerinel I, Coroller L, Couvert O. 2016. Effect of pH on *Thermoanaerobacterium thermosaccharolyticum* DSM 571 growth, spore heat resistance and recovery. Food Microbiol 55:64-72.

43. Liu Y, Lai Q, Goker M, Meier-Kolthoff JP, Wang M, Sun Y, Wang L, Shao Z. 2015. Genomic insights into the taxonomic status of the *Bacillus cereus* group. Sci Rep 5:14082.

44. Begley M, Cotter PD, Hill C, Ross RP. 2009. Identification of a novel two-peptide lantibiotic, lichenicidin, following rational genome mining for LanM proteins. Appl Environ Microbiol 75:5451-60.

45. Brown BL, Watson M, Minot SS, Rivera MC, Franklin RB. 2017. MinION™ nanopore sequencing of environmental metagenomes: a synthetic approach. GigaScience 6:1-10.

46. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, Genome Project Data Processing S. 2009. The sequence alignment/map format and SAMtools. Bioinformatics 25:2078-9.

47. Wood DE, Salzberg SL. 2014. Kraken: ultrafast metagenomic sequence classification using exact alignments. Genome Biology 15:R46.

48. Silva GG, Green KT, Dutilh BE, Edwards RA. 2016. SUPER-FOCUS: a tool for agile functional analysis of shotgun metagenomic data. Bioinformatics 32:354-61.

49. Menzel P, Ng KL, Krogh A. 2016. Fast and sensitive taxonomic classification for metagenomics with Kaiju. Nat Commun 7:11257.

50. Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. Nat Meth 9:357-359.

51. Peng Y, Leung HCM, Yiu SM, Chin FYL. 2012. IDBA-UD: a de novo assembler for single-cell and metagenomic sequencing data with highly uneven depth. Bioinformatics 28:1420-1428.

52. Darling ACE, Mau B, Blattner FR, Perna NT. 2004. Mauve: multiple alignment of conserved genomic sequence with rearrangements. Genome Research 14:1394-1403.

53. Darling AE, Mau B, Perna NT. 2010. progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. PLOS ONE 5:e11147.

54. Scholz M, Ward DV, Pasolli E, Tolio T, Zolfo M, Asnicar F, Truong DT, Tett A, Morrow AL, Segata N. 2016. Strain-level microbial epidemiology and population genomics from shotgun metagenomics. Nat Meth 13:435-438.

55. Asnicar F, Weingart G, Tickle TL, Huttenhower C, Segata N. 2015. Compact graphical representation of phylogenetic data and metadata with GraPhlAn. PeerJ 3:e1029.

56. van Heel AJ, de Jong A, Montalbán-López M, Kok J, Kuipers OP. 2013. BAGEL3: automated identification of genes encoding bacteriocins and (non-)bactericidal posttranslationally modified peptides. Nucleic Acids Research 41:W448-W453.

57. Buchfink B, Xie C, Huson DH. 2015. Fast and sensitive protein alignment using DIAMOND. Nat Meth 12:59-60.

Table 1. Toxin genes aligned to sequence reads.

Toxin	Toxin gene	Gene identifier	Genome	Description
Cytotoxin K	*cytK*	gi|30018278:c1092459-1091449	*Bacillus cereus* ATCC 14579 chromosome	BC1110 (cytotoxin K)
Nonhemolytic enterotoxin	*nhe*	gi|30018278:1765248-1766408	*Bacillus cereus* ATCC 14579 chromosome	BC1809 (nonhemoyltic enterotoxin lytic component L2)
Nonhemolytic enterotoxin	*nhe*	gi|30018278:1766446-1767654	*Bacillus cereus* ATCC 14579 chromosome	BC1810 (nonhemolytic enterotoxin lytic component L1)
Hemolysin BL	*hbl*	gi|30018278:c3062258-3060858	*Bacillus cereus* ATCC 14579 chromosome	BC3101 (hemolysin BL binding component precursor)
Hemolysin BL	*hbl*	gi|30018278:c3063761-3062634	*Bacillus cereus* ATCC 14579 chromosome	BC3102 (hemolysin BL binding component precursor)
Hemolysin BL	*hbl*	gi|30018278:c3065018-3063798	*Bacillus cereus* ATCC 14579 chromosome	BC3103 (hemolysin BL lytic component L1)
Hemolysin BL	*hbl*	gi|30018278:c3066399-3065080	*Bacillus cereus* ATCC 14579 chromosome	BC3104 (hemolysin BL lytic component L2)
Cereulide	*cesA*	gi|190015498:c35141-25017	*Bacillus cereus* strain AH187 plasmid pCER270	CesA (cereulide synthetase A)
Cereulide	*cesB*	gi|190015498:c25003-16958	*Bacillus cereus* strain AH187 plasmid pCER270	CesB (cereulide synthethase B)
Cereulide	*cesC*	gi|190015498:c16813-15917	*Bacillus cereus* strain AH187 plasmid pCER270	CesC (ABC transporter ATP binding protein)
Cereulide	*cesD*	gi|190015498:c15900-15094	*Bacillus cereus* strain AH187 plasmid pCER270	CesD (putative permease)

Table 2. Percentage match of *B. cereus* strains present in samples to 32 reference strains.

		Percentage match to thee 32 genomes used in the pangenome						
ENA assembly no.	No. of genes	January	February	March	May	July	October	November
GCA_000007825	5554	14.3	13.9	13.3	15.9	14.4	13.4	13
GCA_000008005	5338	52	53.3	89.4	53	52.4	69.2	85.8
GCA_000011625	5868	46.9	47.5	35.5	42.4	44.5	38.5	34.9
GCA_000013065	5521	67.6	68.6	49.9	59.2	76.7	51.7	48.8
GCA_000021205	5366	14.8	14.7	14.4	16.6	15.4	14.5	14.1
GCA_000021225	5637	65.8	66.1	50.1	59.6	79.3	51.7	49.1
GCA_000021305	5749	12.8	12.5	12.4	14.1	13.2	12.5	12.2
GCA_000021785	5595	47.2	47.4	36.2	43.7	43.5	38.3	35.7
GCA_000022505	5515	46	46.5	37.2	42	43.2	39.5	36.6
GCA_000143605	5586	47.5	48.3	36.4	43.4	44.5	38.7	35.8
GCA_000239195	5374	46.7	46.9	36.8	42.1	43.9	39.6	36.2
GCA_000283675	5575	65.9	66.7	50.2	60.1	79.9	51.2	49.1
GCA_000292415	5427	53.8	55.5	69	53.4	54.5	78.5	67.2
GCA_000635895	5625	13.8	13.9	13.4	15.3	14.2	13.6	13.1
GCA_000724585	5583	37.2	37	40.7	43.5	37	36.5	39.4
GCA_000789315	5781	45.5	45.9	41.5	54.3	47.2	42.7	40.6
GCA_000832385	5721	45	47.4	37	41.7	42.1	38.7	36.4
GCA_000832405	5522	45.8	46.3	36.9	41.7	43	39.1	36.2
GCA_000832525	5653	47	47.2	42.7	55.9	48.5	44.2	41.7
GCA_000832765	5708	46.6	46.9	36.1	43	46.8	41.4	35.5
GCA_000832805	5708	46.4	47	42.1	55.4	48.1	43.6	41.1
GCA_000832845	5332	51.7	52.1	44.3	61.4	53	46.4	43.3
GCA_000832865	6126	41.5	42.4	33.8	38.3	39.1	35.6	33.2
GCA_000833045	5885	46.6	47.2	35.3	42.1	44.3	38.2	34.7
GCA_000835185	5707	46.5	46.8	36.1	43.1	46.7	41.4	35.6
GCA_000978375	5312	14.9	14.9	14.5	16.7	15.2	14.5	14.2
GCA_001277915	5369	14.1	13.9	13.5	15.9	14.2	13.7	13.2
GCA_001518875	5669	14.3	14.4	13.4	16.1	15	13.6	13.2
GCA_001635915	5948	13.1	13.4	12.8	14.6	13.1	12.5	12.5
GCA_001635955	5945	13.2	13.5	12.8	14.6	13.1	12.5	12.6
GCA_001635995	5981	14	14.1	13.2	15.3	14.2	13.2	13.1
GCA_001721145	5362	15.1	15.1	14.5	17.1	15.5	14.6	14.2

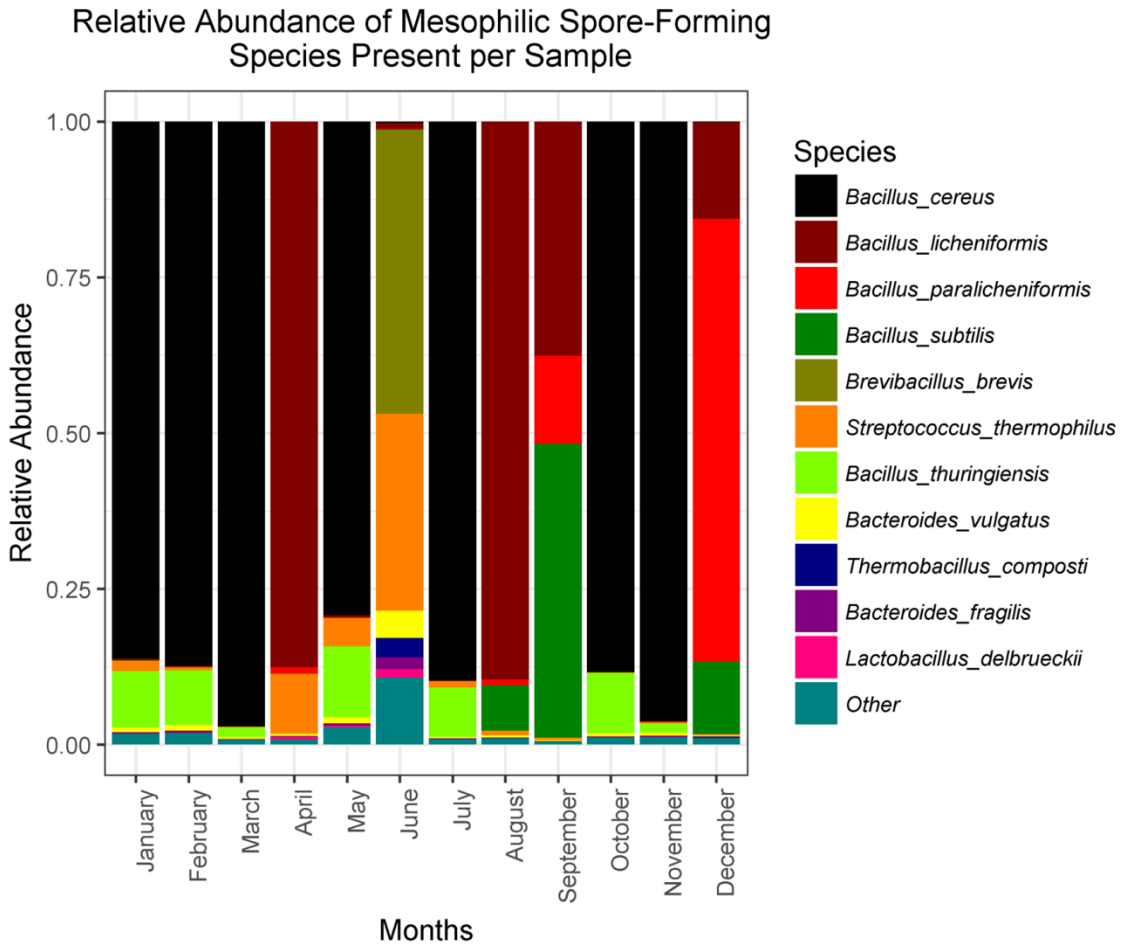
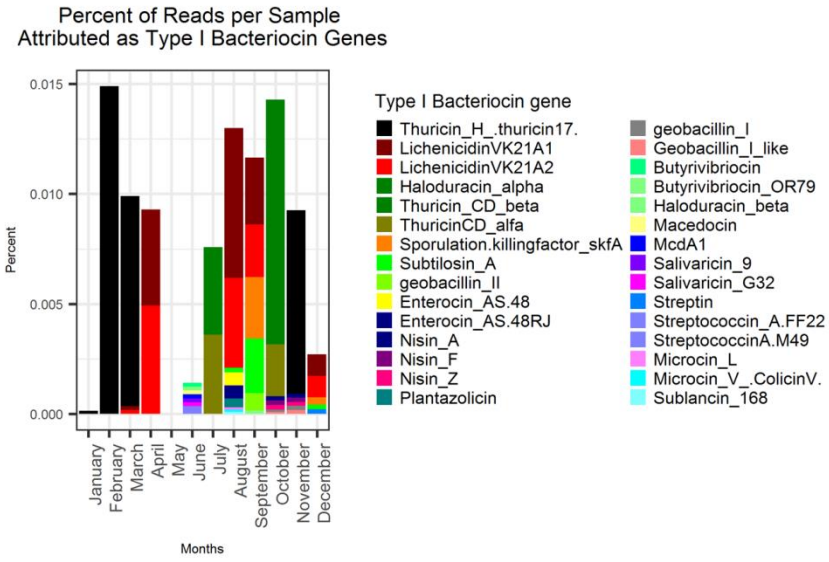
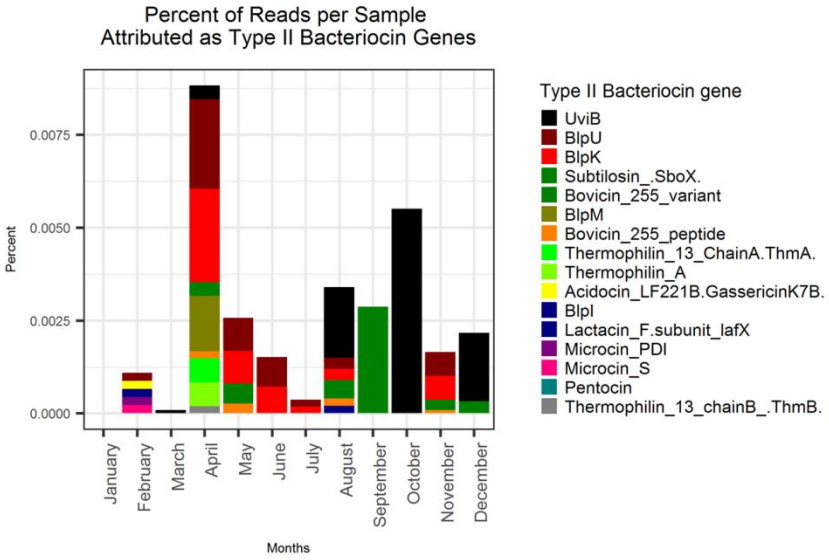


Fig 1. Relative abundance of the most abundant species (shotgun metagenomic reads assigned by Kraken with 0.2 filter threshold and minimum of 1% relative abundance in at least one sample) in samples enriched for mesophilic sporeformers.

A


Percent of Reads per Sample
Attributed as Type I Bacteriocin Genes
Percent
0.015
0.010
0.005
0.000
January
February
March
April
May
June
July
August
September
October
November
December
Months
Type I Bacteriocin gene
Thuricin_H_.thuricin17.
LichenicidinVK21A1
LichenicidinVK21A2
Haloduracin_alpha
Thuricin_CD_beta
ThuricinCD_alfa
Sporulation.killingfactor_skfA
Subtilosin_A
geobacillin_II
Enterocin_AS.48
Enterocin_AS.48RJ
Nisin_A
Nisin_F
Nisin_Z
Plantazolicin
geobacillin_I
Geobacillin_I_like
Butyrivibriocin
Butyrivibriocin_OR79
Haloduracin_beta
Macedocin
McdA1
Salivaricin_9
Salivaricin_G32
Streptin
Streptococcin_A.FF22
StreptococcinA.M49
Microcin_L
Microcin_V_.ColicinV.
Sublancin_168

B


Percent of Reads per Sample
Attributed as Type II Bacteriocin Genes
Percent
0.0075
0.0050
0.0025
0.0000
January
February
March
April
May
June
July
August
September
October
November
December
Months
Type II Bacteriocin gene
UviB
BlpU
BlpK
Subtilosin_.SboX.
Bovicin_255_variant
BlpM
Bovicin_255_peptide
Thermophilin_13_ChainA.ThmA.
Thermophilin_A
Acidocin_LF221B.GassericinK7B.
BlpI
Lactacin_F.subunit_lafX
Microcin_PDI
Microcin_S
Pentocin
Thermophilin_13_chainB_.ThmB.

C

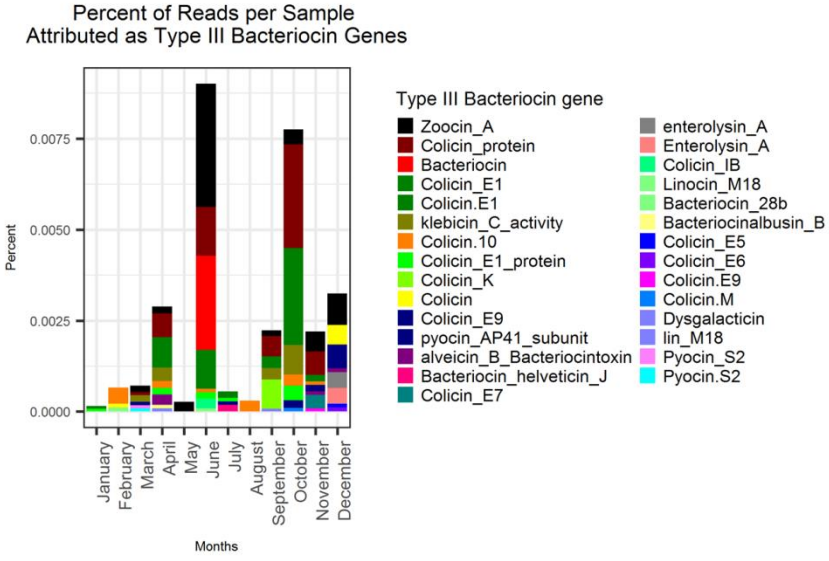

Percent of Reads per Sample
Attributed as Type III Bacteriocin Genes
Percent
0.0075
0.0050
0.0025
0.0000
January
February
March
April
May
June
July
August
September
October
November
December
Months
Type III Bacteriocin gene
Zoocin_A
Colicin_protein
Bacteriocin
Colicin_E1
Colicin.E1
klebicin_C_activity
Colicin.10
Colicin_E1_protein
Colicin_K
Colicin
Colicin_E9
pyocin_AP41_subunit
alveicin_B_Bacteriocintoxin
Bacteriocin_helveticin_J
Colicin_E7
enterolysin_A
Enterolysin_A
Colicin_IB
Linocin_M18
Bacteriocin_28b
Bacteriocinalbusin_B
Colicin_E5
Colicin_E6
Colicin.E9
Colicin.M
Dysgalacticin
lin_M18
Pyocin_S2
Pyocin.S2

Fig 2. Percentage of total reads per sample attributed to bacteriocin genes found in Bagel 3 database. (A) Percentage of reads attributed to type I bacteriocin genes. (B) Percentage of reads attributed to type II bacteriocin genes. (C) Percentage reads attributed to type III bacteriocin genes.

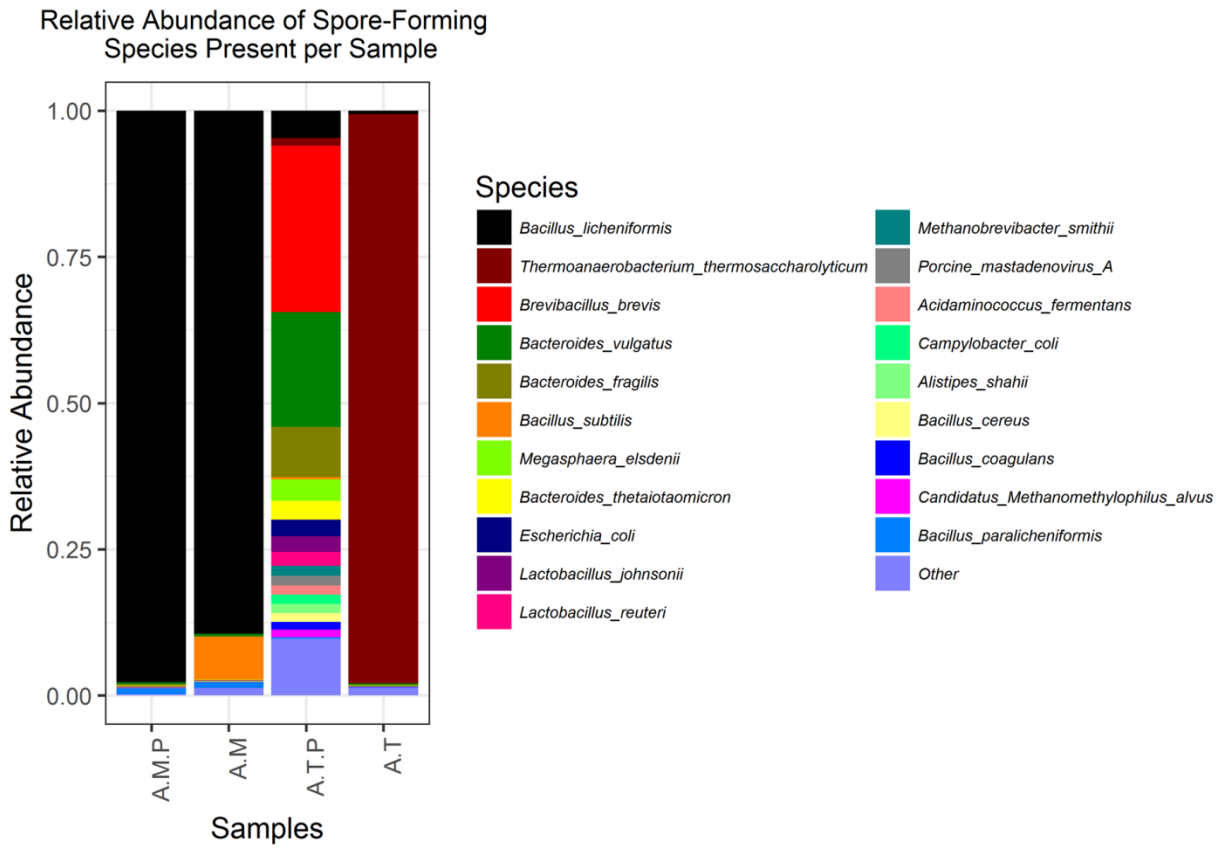


Fig 3. Relative abundance of the most abundant species (assigned by Kraken with a 0.2 filter threshold and with minimum 1% relative abundance in at least one sample) in the August sample subject to different enrichment temperatures and conditions.

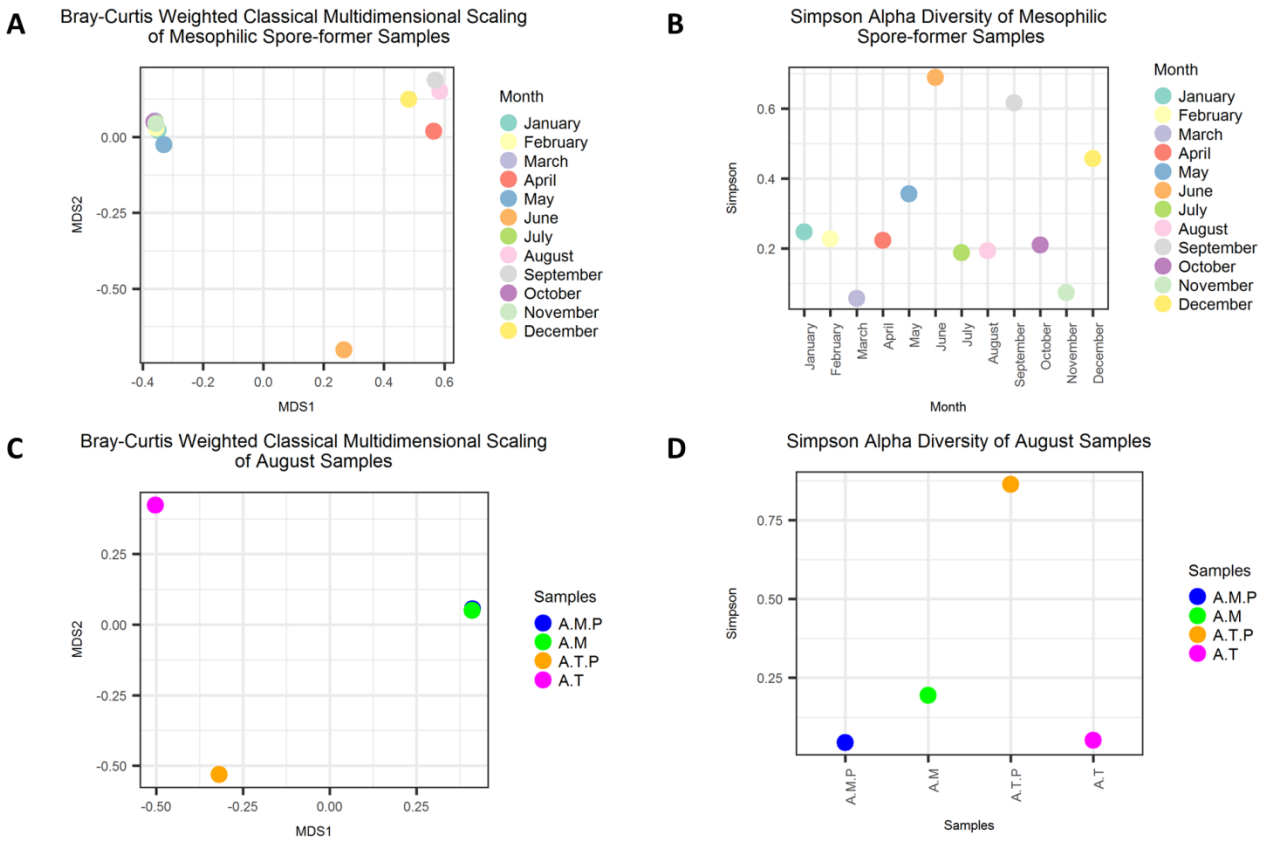


Fig 4. Alpha and beta diversity metrics for mesophilic sporeformer-enriched samples and August samples subject to different incubation temperatures and conditions. (A) Bray-Curtis weighted classical multidimensional scaling (MDS) of mesophilic sporeformer-enriched samples. (B) Simpson alpha diversity of mesophilic sporeformer-enriched samples. (C) Bray-Curtis weighted classical multidimensional scaling (MDS) of August samples. (D) Simpson alpha diversity of August samples in different conditions.

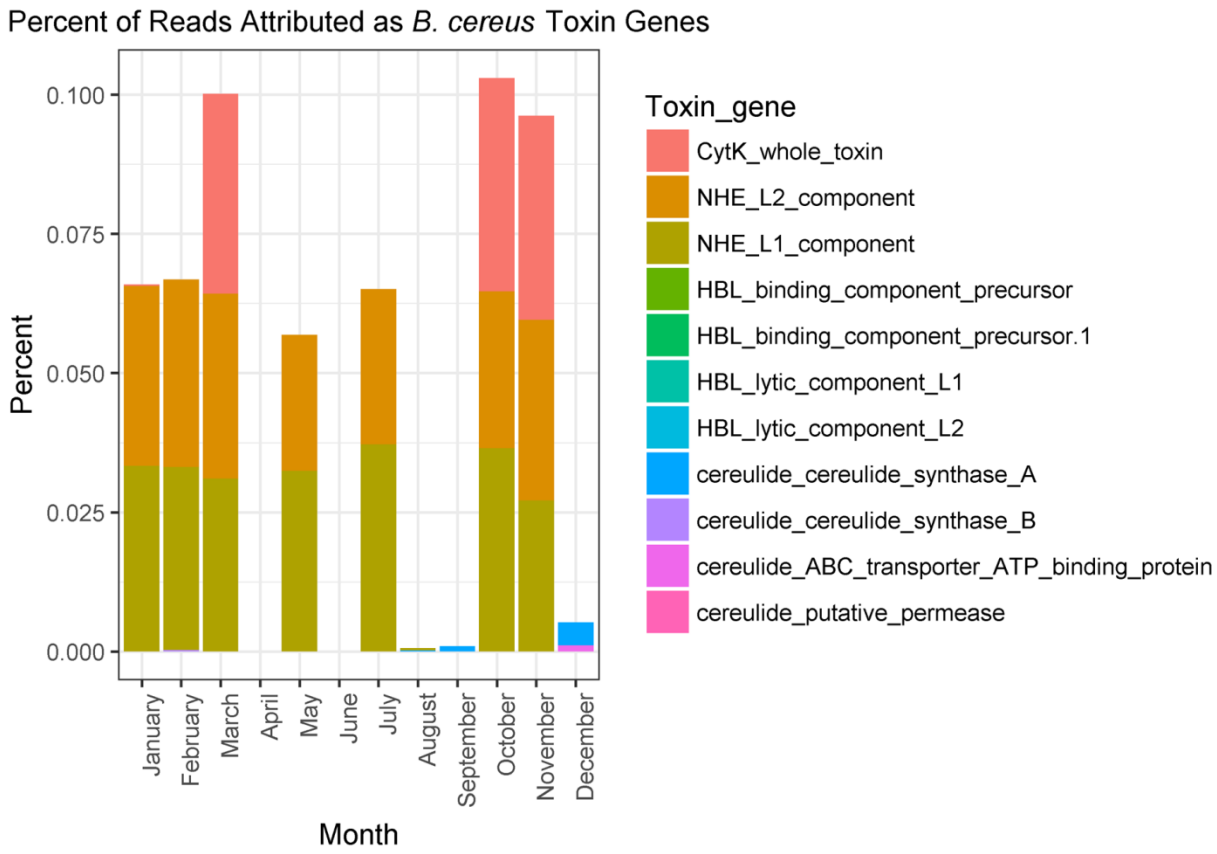


Fig 5. Percentage of total reads attributed to *B. cereus* toxin genes.

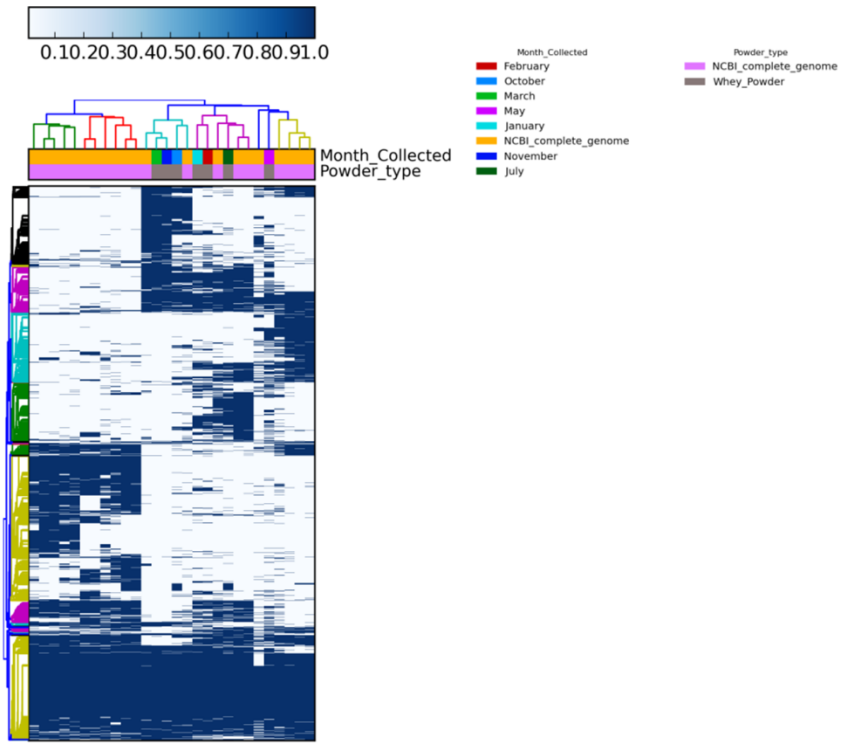


Fig 6. GraPhlAn visualization of PanPhlAn clustering of 7 samples containing *B. cereus* with representative strains of pangenome. The 7 samples contain reads that cluster with at least 5 distinct strains.

Supplemental Figures

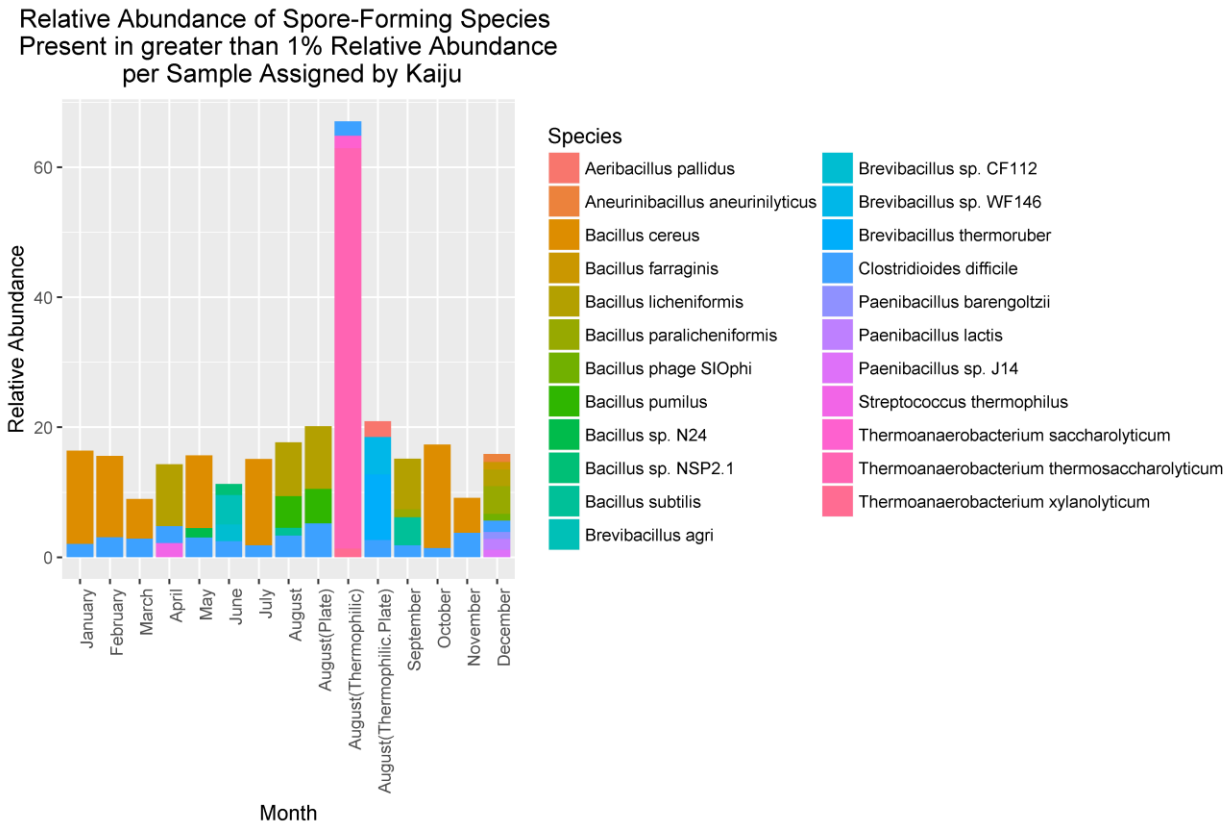


Fig S1. Kaiju species taxonomy results. Species were only included if they were present in >1% relative abundance per sample.

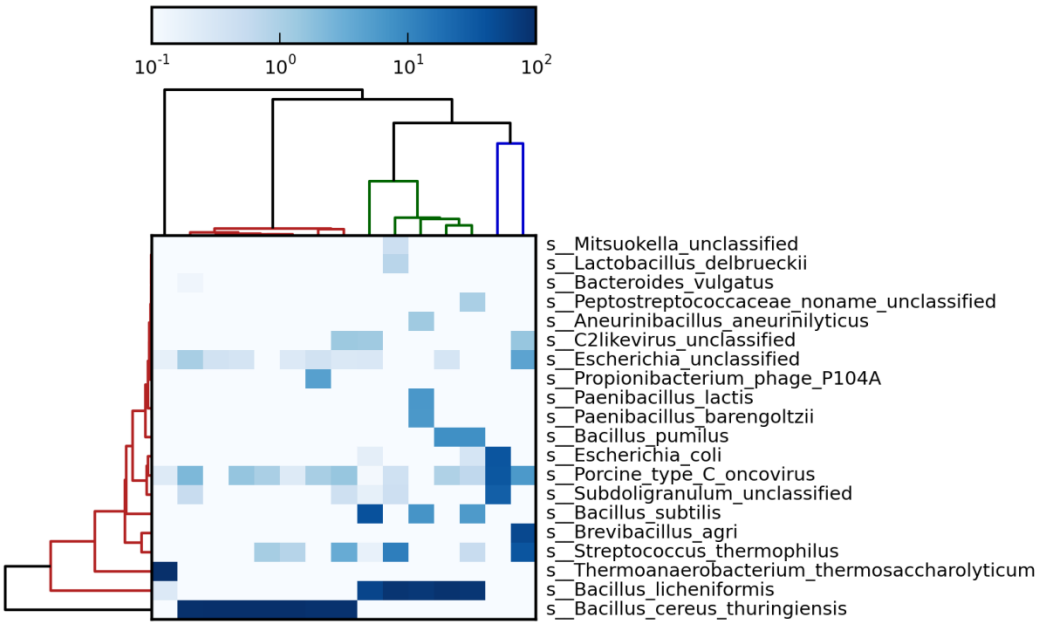


Fig S2. Metaphlan species level classification. From left to right samples are A.T, February, October, March, July, January, November, May, September, April, December, A.M.P, A.M, A.T.P, June.

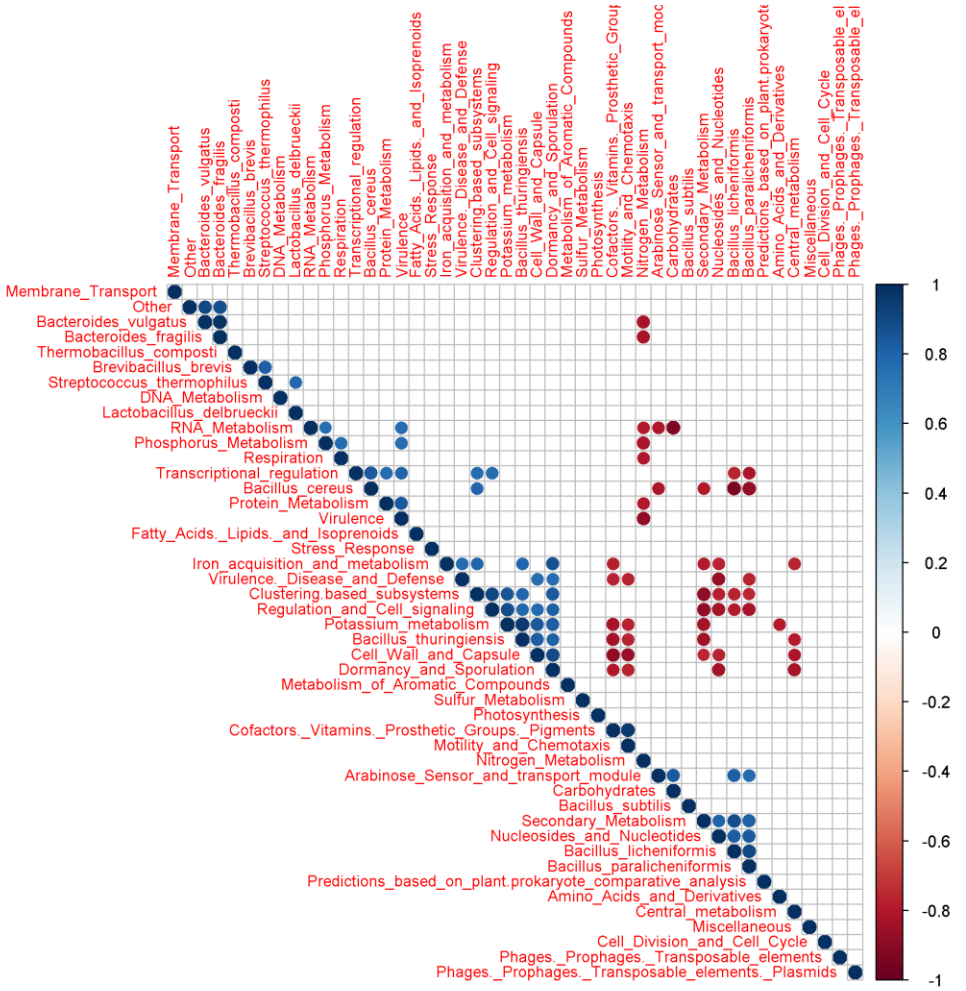


Fig S3. Spearman correlation with Benjamini-Hochberg correction for multiple comparisons between most abundant species identified and SUPER-FOCUS L1 functional groups. Dots represent significant correlations P<= 0.05. Blue being positive correlations and red being negative correlations.

Fig S4. Mauve *B. cereus* toxin genes alignment (3 toxin genes) 7 sets of contigs. Toxin genes on top row, CytK, Nhe L2 Nhe L1, contigs on bottom rows. Samples that align to more than one contig have multiple images. March and November have cytK gene on different contig to two NHE genes, so 2 alignments shown. October has 4 alignments shown as genes are at the edge of contigs.

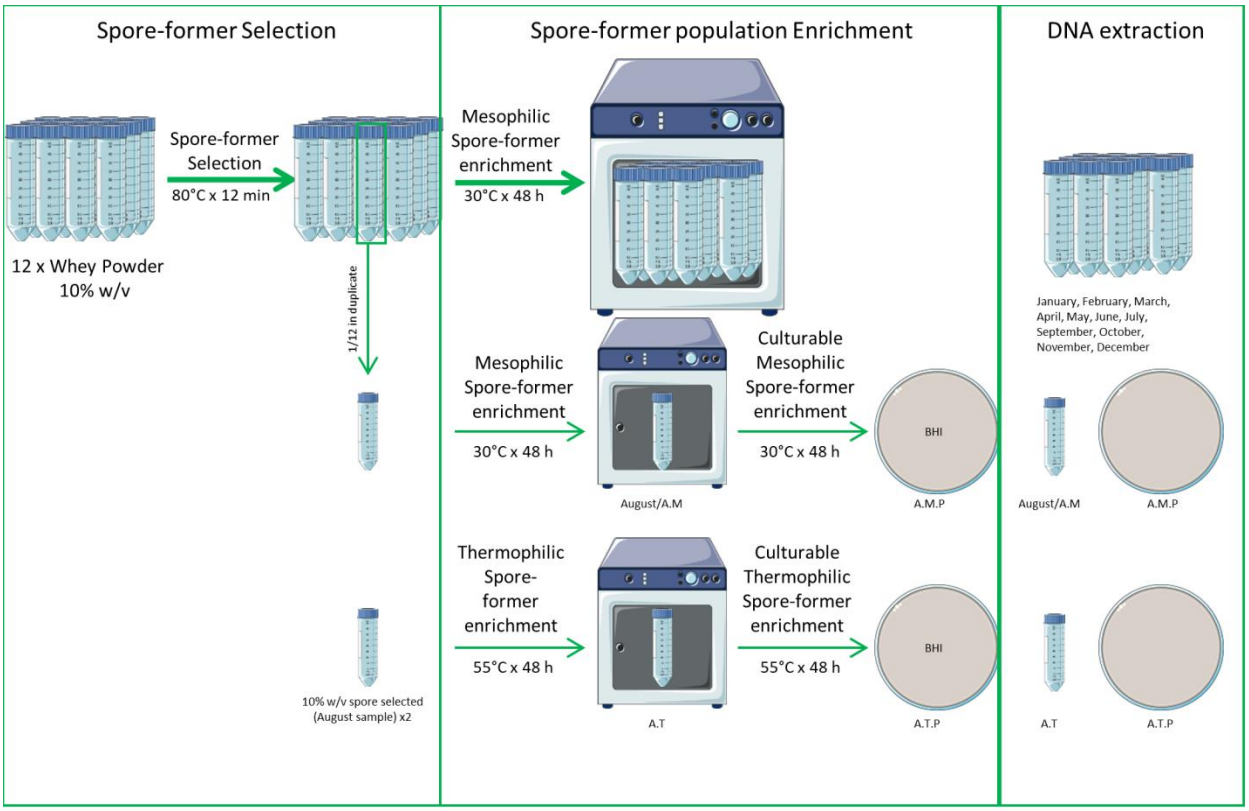


Fig S5. Detailed Schematic of spore selection and enrichment protocols followed by DNA extraction. The schematic shows how each sample was treated for sporeformer selection and mesophilic spore-former enrichment prior to DNA extraction. In addition one sample was also subject to thermophilic spore-former enrichment (A.T), BHI culturable mesophilic spore-former enrichment (A.M.P) and BHI culturable thermophilic spore-former enrichment (A.T.P).