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Occurrence and identification of spore-forming bacteria in skim-milk powders

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ABSTRACT

The different customer and regulatory specifications for mesophilic and thermophilic aerobic and anaerobic spore numbers in skim-milk powder, in addition to some specifications on specific spore-forming bacteria, such as *Bacillus cereus*, can be challenging for the industry to meet. Twenty-two samples of medium-heat skim-milk spray-dried powder from eight sources were analysed in triplicate with 16 bacterial and spore enumeration tests to understand the variety of spore-forming bacteria population. Using 16S rDNA sequencing, the species were identified for 269 isolates that were representative of the various tests. Of the isolates identified, 68% were *Bacillus licheniformis*, a facultative anaerobe that can survive and grow at mesophilic and thermophilic temperatures, making it difficult to eliminate in manufacturing environments. Using whole genome sequencing, 16 of 23 isolates identified as *B. licheniformis* by 16S sequencing were confirmed as *B. licheniformis*, four were identified as *Bacillus paralicheniformis* and three were identified as *Bacillus* sp. H15-1.

1. Introduction

Ireland is one of the leading countries, per capita, in milk and dairy production. From the Bord Bia (Irish Food Board) annual report of Export Performance and Prospects in 2018, the value of dairy products and ingredients exports accounted for one third of the all export categories and were worth more than €4 billion. The safety of dairy products is pivotal in maintaining the reputation of food and guaranteeing the safety of end users and consumers. In terms of microbiological hazards, the threat of bacterial spores in dairy powders has been a cause of concern for many years. Bacterial spores that are generated by some species, known as spore-forming bacteria, which can in some instances be pathogenic, are multilayer-structured dormant endospores that can survive extreme environmental stresses such as desiccation, high pressure, high and low temperatures, UV radiation, and chemical stress (Setlow & Johnson, 2013).

Bacterial spores can survive heating processes, such as most commonly used pasteurisation during milk powder production, and some can even survive ultra-high temperature (UHT) processing, which may lead to spoilage and potential food poisoning. *Bacillus* spp. and *Clostridium* spp. are two of the most often reported spore forming bacteria found in dairy products, associated with food poisoning (Doyle et al., 2015; Gopal et al., 2015; Kumari & Sarkar, 2016; Smelt, Stringer, & Brul, 2013). For example, *Bacillus* spp. or *Clostridium* spp. can produce heat stable protein enterotoxins that lead to diarrhoeal and emetic syndromes (Freedman, Shrestha, & McClane, 2016; Pellett et al., 2016).

Depending on the heat treatment, and incubation temperature applied during the microbial analysis, spore counting methods can be classified as total spore count (TSC, spores are heat-treated at 80 °C for 12 min), highly heat resistant (HHR) spore count (spores are heat-treated at 100 °C for 30 min) and/or as psychrotrophic, mesophilic and thermophilic spore count, for which spores are incubated (aerobically or anaerobically) at 6 °C for 10 days,

30 °C for 48 h and 55 °C for 48 h, respectively (Evelyn & Silva, 2015; Kent, Chauhan, Boor, Wiedmann, & Martin, 2016; Miller, Kent, Boor, Martin, & Wiedmann, 2015a; Sadiq et al., 2016). Because of the lack of standardisation of conditions such as temperature, time, media combinations and atmosphere used in spore tests, there are many different methods that laboratories use, depending mainly on customer specifications for products. This leads to complexity in comparison of the scientific results of spore studies published internationally and in communication of results.

Spores and spore-forming bacteria are ubiquitous in the environment and can easily enter the food chain from farm level. Studies have shown that the occurrence of spores or spore-forming bacteria in raw milk potentially links to the spore levels and type in finished products (Burgess, Lindsay, & Flint, 2010; Doyle et al., 2015; Gupta & Brightwell, 2017; Masiello et al., 2017). Spore forming bacteria have been reported in bulk-tank raw milk and in dairy powders. *Bacillus licheniformis* and *Bacillus pumilus* have been reported as the two most commonly identified species in the bulk-tank raw milk at a combined total of 57% of all 595 spore isolates from 33 farms in the United States (Miller et al., 2015b). In The Netherlands, spores of *Clostridium tyrobutyricum* and *Clostridium beijerinckii* were positively identified in 60% of 96 farm tank milk samples and 40% of the samples were positive for *Paenibacillus* spp. (Driehuis, Hoolwerf, & Rademaker, 2016). In another study (Kent et al., 2016), a total of 55 raw material samples including raw milk, cheese whey and condensed milk were positive at a rate of 100, 98, 80 and 84% for mesophilic total spore count, thermophilic total spore count, mesophilic HHR spore count and thermophilic HHR spore count, respectively. From the 326 bacteria isolates from that survey work, *Bacillus* spp. and *Geobacillus* spp. were the two of most frequently identified genera accounting for 81% and 9% of total isolates collected, respectively.

In the processing plant, the spore population can be affected by the raw milk and by processes applied to the materials including heating, holding-time, and packaging, some of

which can facilitate increasing numbers, or biofilm formation. Heating processes such as pasteurisation, UHT treatment or spray-drying may trigger spore germination (Hanson, Wendorff, & Houck, 2005; Ranieri, Huck, Sonnen, Barbano, & Boor, 2009; Setlow, 2014), leading to out-growth and occurrence of spore-forming bacteria in the processing environment. These spore-forming vegetative cells can be induced into the spore state if the conditions become unfavourable. Additionally, direct contact of product with processing surfaces or packaging material may further contaminate the products with spore reservoirs (Kumari & Sarkar, 2014).

In dairy powders, *Bacillus* spp. (56.4%), *Geobacillus* spp. (19.8%) and *Anoxybacillus* spp. (17.3%) were the predominant spore genera (Miller et al. 2015b). In a study of non-fat dry milk powders (skim milk powder), collected from three processing plants the average spore count level in all the samples was 3.24 ± 0.09 log cfu g^{-1} (Buehner, Anand, & Djira, 2015). In that study, *B. licheniformis* was the most common species, at 63% of the 60 isolates identified.

The quality of milk and dairy products are monitored by many microbiological criteria related to spores or spore-forming bacteria. At a European level the Commission Regulation No 2073/2005 (EC, 2005) states that the presumptive identification of *Bacillus cereus* should be in the range of 50 cfu g^{-1} to 500 cfu g^{-1} in dried infant formulae and dried dietary foods for special medical purposes intended for infants. The United States Dairy Export Council has defined rules, linked to international customer specifications on mesophilic and thermophilic spore counts of less than 1000 cfu g^{-1} and less than 500 cfu g^{-1} in dairy powders respectively, that are destined for use in infant milk formulae (Watterson, Kent, Boor, Wiedmann, & Martin, 2014). For anaerobic spores, the International Commission on Microbiological Specifications for Foods (ICMSF) concluded that sulphite-reducing clostridia (SRC) should be limited to under 100 cfu g^{-1} in dried dairy ingredients

used in powdered infant formula, indicating adequate microbiological hurdles have been applied in the process and good hygiene practice observed (ICMSF, 2013).

The objective of this study was to characterise spores and spore-forming bacteria population in Irish skim-milk powder (SMP) and to identify the different species of spores and heat tolerant bacteria using 16S sequencing and whole genome sequencing (WGS).

2. Materials and methods

2.1. Samples

Twenty-two medium heat skim milk powder samples were obtained from 8 different sources in Ireland. Samples that were outside bacterial specifications had not entered the commercial market. All samples were manufactured in autumn 2016, and obtained from October 2016 to January 2017 for testing. The samples were stored at ambient temperature for 6 to 9 months, away from light, in air-tight packaging before testing.

2.2. Sample preparation for analysis

Independent triplicate 25 g sub-samples of each powder sample were aseptically weighed and transferred to sterile bags. Following that, 225 g of sterile distilled water was added and the bag was left for 20 min at 18–22 °C. The bag was then gently mixed by hand to obtain a homogeneous solution.

2.3. Methods for microbial enumeration

The samples were tested by sixteen microbial methods, as detailed in Table 1. As required by the individual tests, various heat treatments of different combinations of time and temperature were applied to the prepared sample solutions. For thermoduric bacteria, the samples were heated at 63.5 °C for 35 min. For spore count and highly heat resistant (HHR) spore count, the samples were heat-treated at 80 °C for 10 min and at 100 °C for 30 min, respectively. From each duplicate sample, 1 mL of sample or an appropriate serial dilution was pour-plated in duplicate on the appropriate agar for the test, except for presumptive *Bacillus cereus* group bacteria using BACARA^{TM} agar (Biomerieux, Marcy-l'Étoile, France), for which 3 × 0.33 mL of each duplicate sample was spread on the plates. Tryptic soy agar (TSA; Becton Dickinson, New Jersey, US) was used for all bacterial analyses, except that plate count skimmed milk agar (PCSMA; Merck, New Jersey, US) was used for aerobic and non-specific anaerobic spore tests and iron sulphite agar (ISA; Thermo Fisher Scientific, Massachusetts, US) was used to detect SRCs. The agar plates were incubated at the required time, temperature and atmosphere conditions, whereby; anaerobic incubation was achieved using anaerobic jars with Anaerocult (Merck). After incubation, all the colonies on a plate were counted; when there were no colonies present the result was reported as < 1 log cfu mL^{-1}. The results were expressed as cfu mL^{-1} of reconstituted skim milk powder. Spreading colonies were counted as single colonies if less than one quarter of the agar surface was covered; if more than one quarter of the agar surface was covered, the result was discarded.

2.4. Isolation and purification of colonies

Three colonies of variable morphology, where possible, from each microbial test, were isolated and purified. For purification, bacterial isolates were aseptically streaked onto TSA plates which were incubated for 18 h at the temperature of isolation. A single colony was aseptically transferred, using a 10 µL loop, into 10 mL of tryptic soy broth (TSB; Becton

Dickinson) which was incubated for 18 h at the temperature of isolation. Two mL of the growth culture were centrifuged using a benchtop centrifuge at 14,000 rpm for 1 min and the supernatants were discarded. The pellet was resuspended in cryovial solution using Cryoinstant tubes 822075ZA (VWR, Pennsylvania, US) and cryovials were frozen at –20 °C.

2.5. DNA extraction

For each of the 285 isolates selected, a cryobead was added aseptically to 10 mL of brain heart infusion (BHI) broth and incubated for 18 h at the temperature of isolation. DNA was extracted from 2 mL of bacterial culture using the Qiagen DNeasy UltraClean Microbial Kit (Qiagen, Venlo, Netherlands), as per manufacturer's instructions. DNA purity and concentration were measured using a Biodrop µLITE™ (Novex Electrophoresis GmbH, Heidelberg, Germany). The extracted DNA from each isolate was stored in 1.5 mL micro tubes (SARSTEDT, Nümbrecht, Germany) at –20 °C and analysed within 3 months.

2.6. 16S rDNA sequencing

Based on representing an even distribution of the source and test method, 285 bacterial isolates were selected for 16S sequencing. 16S rDNA sequencing was used for the primary species identification. The National Institutes of Health (Maryland, US) primer set 357F/926R (357F - CCTACGGGAGGCAGCAG, 926R - CCGTCAATTCMTTTRAGT) was used to amplify the bacterial 16S rRNA V3-V5 region (Sim et al., 2012). The amplicon size was 570 kb. Partial 16S rDNA sequences were determined by Sanger sequencing (Fellner & Sanger, 1968). Genetic database searching was performed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Where the species is given, the top three 'hits' for species identification were the same. BioNumerics software version 7.6 (Applied-Maths,

BioMérieux Marcy-l'Étoile, France) was used to study the relationship between the closest known species from the BLAST search.

2.7. Whole genome sequencing

From the 16S results, 24 *B. licheniformis* strains were selected for whole genome sequencing (WGS) as this was the predominant species identified and to confirm the identification. The 24 strains selected represented an even distribution of the source and test method.

For WGS, quantification of genomic DNA was performed using a Qubit 2.0 fluorometer (Invitrogen, CA, USA), with the Qubit dsDNA HS assay (ThermoFisher Scientific) according to the supplier's instructions. A 0.2 ng μL^{-1} DNA solution was prepared using molecular grade water. Library preparation tagmentation, library amplification and clean-up were performed with the Nextera® XT DNA sample preparation kit (Illumina, CA, USA). Subsequent quantification of the library was done by means of the Qubit dsDNA HS assay on the Qubit 2.0 fluorometer. Library size distribution and quality were assessed with the 2200 TapeStation (Agilent Technologies, CA, USA). A manual library normalisation was performed and pooling of the libraries was done with 5 µL of each 2.0 nM normalised library. Subsequently, 600 µL of a 12 pM library was made with a 1% PhiX control spike-in. Sequencing was done on an Illumina MiSeq with the v3 chemistry using 2× 300 bp paired-end reads.

2.8. WGS data analysis

The species was predicted from raw read data using Kmerfinder (http://cge.cbs.dtu.dk/services/KmerFinder). A dendrogram was constructed in Bionumerics

7.6 using standard algorithm with Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

Raw sequencing data was assembled by Bionumerics Power Assembler, and assemblies were submitted to Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) with toxin genes, including *lchAA, lchAB, lchAC* from *B. licheniformis*, *lchAA* from *B. licheniformis* DSM 13, *lchAB* from *B. licheniformis* DSM 13, *lchAC* from *B. licheniformis* DSM 13, *cesA* and *cesB* from *B. cereus*. The toxin gene sequences were acquired from the European Nucleotide Archive (https://www.ebi.ac.uk/ena). The results of toxin gene identity values were inputted to GraphPad Prism 7.02 to generate a distribution graph of toxin genes in the sequenced strains.

2.9. Statistical analysis

The spore counts from each sample were converted to log cfu mL^{-1} using Excel. The average and standard deviation of the values at each sampling point were calculated and graphed using GraphPad Prism. Where the numbers were below the detection limit an arbitrary value of 0 log cfu mL^{-1} was applied.

The results of the bacterial and spore numbers were analysed using IBM SPSS Statistics (New York, NY, USA) to generate a Boxplot for each test method, except for the presumptive *B. cereus* group on BACARA plates as the numbers were too low for statistical analysis (see Supplementary material Fig. S1). In the figures, each rectangular box consists of the median (as a horizontal line), the 25^{th} and 75^{th} percentile (as the lower and upper lines of the rectangle, respectively), and the maximum and minimum values as whiskers. The boxplots give more detail on the variation of the bacterial and spore counts between the different samples.

3. Results

3.1. Microbial enumeration

No psychrotrophic spores were detected. From Fig. 1, the range of TBC was from <1 (in samples 12, 21 and 22) to 3.15 log cfu mL^{-1} except for samples 17 and 18, which were not tested. For thermoduric bacteria, the results ranged from <1 (samples 4, 12, 21 and 22) to 2.96 log cfu mL^{-1} (sample 6), while for thermophilic bacteria, the range was from <1 (sample 2) to 3.00 log cfu mL^{-1} (samples 14 and 16). For values on the x-axis which were below the detection limit, an arbitrary value of 0 cfu mL^{-1} was applied. In sample 13, 14 and 16, thermophilic bacteria were significantly ($p < 0.05$) higher than the TBC and thermoduric bacterial numbers. Sample 2 had the highest TBC in all tested samples, exceeding 3 log cfu mL^{-1}.

Spores were detected in all the powders, but the spore type and numbers varied (Fig. 2). Samples 13–18, which were all from the same source, were the highest for thermophilic anaerobic spores, with numbers ranging from 2.5 to 3.0 log cfu mL^{-1}. For bacterial spores in Fig. 2, mesophilic aerobic spores ranged from <1 to 2.47 log cfu mL^{-1}, thermophilic aerobic spores from <1 to 2.91 log cfu mL^{-1}, mesophilic anaerobic spores from <1 to 2.31 log cfu mL^{-1} for, and thermophilic anaerobic spores from <1 to 3.00 log cfu mL^{-1}. In sample 2 and samples 13–17, thermophilic aerobic or anaerobic spores were 1.5 to 2 log higher than the numbers of other bacterial spores for which tests were undertaken. For samples 17–22, not all bacterial spore and SRC tests were done on the powders due to insufficient sample material being available for testing. The highest value of mesophilic SRC was 1.19 log cfu mL^{-1} in sample 8, while for thermophilic SRCs, was 1.16 log cfu mL^{-1} in both sample 6 and sample 7. For mesophilic SRC spores, all the results were below the detection limit except for sample 3,

8, 11, 12 and 13. For thermophilic SRC, spores were detected in sample 5, 6 and 7, with all other tested samples <1 cfu. For values on the x-axis which were below the detection limit, an arbitrary value of log 0 cfu mL^{-1} was applied.

The results of different high heat resistant spore tests are shown in Fig. 3. Samples 13–17 had the highest counts for thermophilic aerobic spores and sample 18 had high thermophilic anaerobic HHR spores.

The minimum and maximum counts observed for HHR spores were <1 to 2.07 log cfu mL^{-1}, for mesophilic aerobic HHR spores, 0.18 to 2.77 log cfu mL^{-1} for thermophilic aerobic HHR spores, <1 to 2.13 log cfu mL^{-1} for mesophilic anaerobic HHR spores and 0.22 to 2.90 log cfu mL^{-1} for thermophilic anaerobic HHR spores. For samples 17–22, not all HHR spore tests were completed on the powders due to insufficient sample being available. For values on the x-axis which were below the detection limit, an arbitrary value of log 0 cfu mL^{-1} was applied.

For samples 13–18, all of which were obtained from the same source, the results were different from other samples. The thermophilic bacteria results were the highest among other tests in Fig. 1 (some data were missing due to the limited powder availability), where thermophilic anaerobic bacteria spore results were the highest in Fig. 2 and thermophilic anaerobic HHR spores in Fig. 3.

In this study, BACARA was used as a selective medium to determine presumptive *B. cereus*. From a total of 20 isolates collected from different colony morphologies on BACARA plates, 5 presumptive *B. cereus* group isolates with typical pink to orange colonies surrounded by an opaque halo, were identified as *B. cereus* group using 16S rDNA sequencing, and the remaining 15 isolates with different morphology without a halo growing on agar surface were identified using 16S rDNA sequencing as *Lysinibacillus* spp., *Enterococcus* spp. and *Bacillus coagulans.*

The detection of SRCs was carried out on ISA plates under anaerobic conditions. The growth of both mesophilic and thermophilic SRCs was observed as typical black colonies on the plates. From all the 22 positive plates, a total of 142 SRC colonies were counted and 8 isolates were obtained from ISA plates identified by 16S rDNA sequencing.

The results of bacterial and spore counts for all the powders from 13 tests are shown as box-plots, giving more detailed information (Supplementary material Fig. S1). The greatest variation was observed for thermophilic anaerobic bacterial spores, ranging from <1 to 3.00 log cfu mL^{-1}, while the highest log median value was for thermophilic bacteria at 2.15 log cfu mL^{-1}.

3.2. 16S sequencing results

Using the 16S sequencing method, the species was identified for 269 of the isolates. The remaining 16 isolates could not be identified from the sequences obtained. The isolates identified were predominantly *Bacillus licheniformis* (68% of total identified isolates), 16% of the isolates were other *Bacillus* species including *B. cereus* group, *B. coagulans* and other *Bacillus* species, 5% were *Clostridia* spp., 3% were *Geobacillus* spp., 2.6% were *Lysinibacillus* spp., 1.8% were *Brevibacillus* spp., 1.5% were *Enterococcus* spp., 1.3% were *Anoxybacillus* spp., and less than 1% were *Aneurinibacillus* species (Fig. 4).

A total of 183 *B. licheniformis* isolates were identified using 16S sequencing, including isolates from most of the methods described previously. Fig. 5 shows a pie-chart of the % of the 183 *B. licheniformis* 16S-sequenced isolates obtained from the different microbiological methods used. There were 121 of the *B. licheniformis* isolates from spore methods, 77 of which were mesophilic spore-formers (38 isolates were from HHR aerobic or anaerobic spore methods), 44 were thermophilic spore-formers (22 isolates were from the HHR aerobic spore method). For bacterial methods, 2 *B. licheniformis* (that were not typical

326 colonies) were isolated from BACARA plates, 7 were from TBC, 27 were from thermophilic
327 bacterial tests, and 22 were from thermoduric bacterial tests.

328 Thirteen isolates of *B. cereus* group were identified by 16S sequencing, 4 from
329 thermoduric bacterial tests, 4 from mesophilic aerobic spore tests and 5 were from typical
330 colonies on BACARA agar plates. There were no *B. cereus* group isolates from thermophilic
331 methods. Six of 11 *Bacillus coagulans* were identified from anaerobic tests. The majority of
332 these isolates came from spore tests on non-selective media (i.e., TSA and PCSMA), mostly
333 under thermophilic conditions but one was collected from a BACARA agar plate. Seventeen
334 isolates from all aerobic tests were identified as *Bacillus* spp.

335 From ISA and other tests, fourteen *Clostridium* spp. isolates were identified. Five
336 isolates were from mesophilic anaerobic spore tests and eight were from mesophilic SRC
337 tests. The remaining one was isolated from BACARA plate. Nine *Geobacillus* spp. isolates of
338 were identified, eight from thermophilic tests (including HHR, aerobic and anaerobic tests).
339 The seven *Lysinibacillus* spp. isolates were all obtained from BACARA agar plates and the
340 four *Anoxybacillus* spp. were isolated from thermophilic methods. Four *Enterococcus* spp.
341 were isolated, two from BACARA agar, one from thermoduric bacteria and the other was
342 isolated from TBC.

343

344 *3.3. WGS results*

345

346 A set of 24 16S sequencing-identified *B. licheniformis* isolates were selected for WGS
347 to confirm the 16S sequencing results. One strain was discarded due to the lack of valid
348 sequencing information. The remaining 23 isolates were identified as *B. licheniformis* (16
349 isolates), 4 as *Bacillus paralicheniformis* and three as *Bacillus* sp. H15-1 (Fig. 6). This shows
350 the relationship between the species identified. Green partitions indicate *B. licheniformis*, red
351 partitions indicate *B. paralicheniformis* and purple partitions are for *Bacillus* sp. H15-1. From

the Fig. 6, the relationship between *B. licheniformis* and *B. paralicheniformis* is more distant than the relationship between *B. licheniformis* and *Bacillus* sp. H15-1.

The presence of a total of eight toxin genes, related to lichenysin synthetase in *B. licheniformis* and cereulide synthetase in *B. cereus,* was assessed in the whole genome sequenced strains. A distribution graph of toxin gene identity against sequenced strains is presented in Supplementary material Fig. S2. Four *B. paralicheniformis* strains (numbers 2, 14, 15 and 16) were distinguishable from the other isolates. These four stains showed a higher identity (>95%) in *lchAA*, *lchAB*, *lchAC*, and a lower percentage (<95%) in *lchAA*_DSM13, *lchAB*_DSM13 and *lchAC*_DSM13 compared with other isolates. The genes *cesA* and *cesB* were not detected in any of the isolates.

4. Discussion

This study was a screening of Irish skim milk powders for various microorganisms in using different test methods. *B. licheniformis* was identified by 16S rDNA sequencing as the most common spore-forming bacteria. If a greater number of colonies were selected from each agar plate, or if different media or incubation conditions were used, it is possible that the variety of organisms may have been different. Similar results where *B. licheniformis* was a commonly isolated spore-forming bacterium from dairy products have been reported (Buehner, Anand, & Garcia, 2014; Gopal et al., 2015; Reginensi et al., 2011; Rückert, Ronimus, & Morgan, 2004; Yuan et al., 2012). The high prevalence of *B. licheniformis* in the dairy industry can be attributed to contamination from external farm sources including soil and silage as well internal sources during dairy processing that allow growth of the organism.

Bacillus licheniformis is a facultatively anaerobic mesophilic or thermophilic spore-forming bacterium that was isolated from a variety of tests used in this study. It is a member of the *Bacillus subtilis* group and can be difficult to distinguish from other members of the *B.*

subtilis group. Of the 269 isolates identified by 16S rDNA sequencing in this study, 189 were identified as *B. licheniformis*. Because of the difficulty in distinguishing *B. licheniformis* from other members of the *B. subtilis* group, whole genome sequencing confirmed the 16S sequencing with regard to differentiating *B. licheniformis* and *B. subtilis*. However, it did show that *B. paralicheniformis* is easily distinguishable using WGS, even though 16S sequencing showed them to be similar. Three of the 23 isolates were identified as *B. paralicheniformis* using WGS.

Depending on the tests, the microbiological counts varied significantly from <1 to 10^3 cfu mL^{-1} in reconstituted skim milk powders. From the results for some samples (for example, sample 14) the counts for some bacterial types are actually higher that the TBC. This is due to the fact that the TBC, although called a 'total' bacterial count, will only detect a certain number of bacteria that grow at the incubation temperature (30 °C) of the TBC. Previous studies of spore counts in dairy powders showed a similar range from below the detection limit to about 10^4 cfu g^{-1} in dry powder (Buehner et al., 2014; Kent et al., 2016; Watterson et al., 2014). In the boxplots, the log mean values in all spore test methods were below 2 log cfu mL^{-1} and the highest was for HHR thermophilic anaerobic spores in this study. It should be noted that from the boxplot charts, all thermophilic methods used showed higher counts than mesophilic methods. This may be a result of sequential heating during pasteurisation, evaporation and spray drying processes which favours growth and survival of thermophyles (Burgess, Flint, & Lindsay, 2014; Cho et al., 2018; Hill & Smythe, 2012).

Whole genome sequencing confirmed that 16 of the 23 isolates obtained by 16S rDNA sequencing were *B. licheniformis*. Four of the isolates were identified as *B. paralicheniformis* by WGS, but as *B. licheniformis* by 16S sequencing. Although the 16S gene sequencing showed a high degree of similarity between the strains of *B. licheniformis*, the strains of *B. paralicheniformis* were distinctly different (Fig. 6). *Bacillus* sp. H15-1, of which 3 isolates was identified from 23 total isolates, was isolated from rusted steel wire rope

404 in 2017 (Xiao et al., 2017) and is a thermophilic bacterium with the ability to produce two
405 hydroxy-pentanone metabolites. Although its species has not been confirmed, from this study
406 *Bacillus* sp. H15-1 is closely related to *B. licheniformis.* Additionally, in another study,
407 *Bacillus* sp. H15-1 was shown to have a similarity value of greater than 99% to *B.*
408 *licheniformis* strains (Lee et al., 2017). Two of the 3 *Bacillus* spp. H15-1 strains were isolated
409 from the same source in this study. Apart from this study, there was no record of *Bacillus* sp.
410 H15-1 found in dairy industry environment.

411 A recent review (Wells-Bennik, Driehuis, & van Hijum, 2016) had discussed the great
412 potential of genetic approaches, such as WGS and 16S marker gene sequencing, in
413 characterising dairy-relevant sporeformers. These molecular tools can provide information on
414 gene absence/presence, gene regulation on protein expression as well as metabolite
415 production, which allow the prediction of phenotypes at genus, species and strain level. In
416 this study, genome analysis was used to contribute to the awareness of bacteria and spores
417 isolated from specific enumeration methods.

418 Currently, there is no standardisation of spore testing methods accepted worldwide
419 (Kent et al., 2016) and variation inherent in the methods applied can lead to great differences
420 in spore counts in milk powders (Wells-Bennik et al., 2019), with an up-to 3 log cfu mL^{-1}
421 difference of spore numbers as determined by different methods. Additionally, many of the
422 different tests will identify the same organism, as was the case with *B. licheniformis*, which
423 grows over a wide range of temperatures and atmospheric conditions. This makes it more
424 difficult to control during processing. It also indicated that using one spore testing method
425 with a specific heating and incubation temperature combination may not show a complete
426 view of all spore formers in a given milk powder. To gain a better understanding of spore-
427 forming bacteria population, several different methods should be applied together.

428 Most food poisoning incidents attributed to *Bacillus* species are associated with
429 *Bacillus cereus*, but from previous studies, some heat-stable toxin production has been shown

in *B. licheniformis* strains (Salkinoja-Salonen et al., 1999; Taylor, Sutherland, Aidoo, & Logan, 2005). Nieminen et al., (2007) identified toxin-producing *B. licheniformis* from 2 of 23 samples from milk of mastitic cows. The toxin-producing properties of the two *B. licheniformis* isolates were similar to those of *B. licheniformis* strains that produce the lipopeptide lichenysin, and were toxic at concentrations of 20–30 μg mL^{-1}. Lichenysin synthesis was found to be universal among the 53 *B. licheniformis* strains examined by Madslien et al., (2013), although the quantities varied considerably, with more than two orders of magnitude between strains. Cytotoxicity was evident at lichenysin concentrations above 10 μg mL^{-1}. Salkinoja-Salonen et al., (1999) isolated toxin-producing isolates of *B. licheniformis* from foods involved in food poisoning incidents, from raw milk, and from industrially produced milk powder. Those toxins had physicochemical properties similar to those of cereulide (a toxin of *B. cereus*), but had different biological activity. The toxin was non-protein in nature, soluble in methanol, and was not sensitive to heat, protease, acid or alkali. The presence of six linchenysin synthesis related genes and 2 genes responsible for cereulide production (*cesA* and *cesB*) were assessed in the 23 strains that were whole genome sequenced. All strains were positive for the lichenysin genes but negative for *cesA* and *cesB*. However, this study determined gene presence, there are many other factors that could influence gene expression, which was not studied.

Selective media such as BACARA agar and Iron Sulphite Agar can give a good indication of potential *B. cereus* group and SRC group members (Doyle et al., 2015; O'Connell, Ruegg, Jordan, O'Brien, & Gleeson, 2016; Tallent, Kotewicz, Strain, & Bennett, 2012). From all pink colonies with an opaque halo collected from BACARA plates in this study, they were all identified as *B. cereus* group by 16S sequencing. BACARA has been shown to be more sensitive and selective than the ISO7932 method using MYP agar (Kabir, Hsieh, Simpson, Kerdahi, & Sulaiman, 2017). Pink or orange colonies surrounded by an opaque halo, which indicates that lecithinase is produced, should be considered as

presumptive *B. cereus* group isolates. The number of presumptive positives was low so the colony counts were not shown and there was insufficient data for further analysis. In this study, thermophilic SRC were observed on ISA plates but none were successfully collected due to their strict anaerobic requirements, which makes them difficult to recover for further characterisation.

It is very important to differentiate between spores and spore-forming bacteria when carrying out an in-depth microbiological study focused on spores, to ensure that enumeration techniques applied do not represent innate vegetative cells, which must be eliminated by heat treatment prior to enumeration to ensure results accurately reflect true spore levels. Only pre-formed spores are able to survive heat processing, due to the durability and protection provided by their multilayer-structure. Sporulation and germination are two dynamic processes, indicating the transformation from vegetative cells to spores and vice versa. Previous studies reported that the germination time of *Bacillus* spp. spores varied from 5 to 60 min under a variety of conditions (Chen, Huang, & Li, 2006; Santo & Doi, 1974; Zhang et al., 2010). Additionally, heat activation (thermal shock) may induce germination in some *Bacillus* spp. spores, which can be explained by the expression of the *ger* gene (Luu et al., 2015; Soni, Oey, Silcock, Permina, & Bremer, 2018). In the manufacture of dairy powders, thermal processes can trigger spore germination under favourable conditions, which may result in toxin production from growth of the vegetative cells and associated presence of the organisms in finished products.

5. Conclusions

B. licheniformis was demonstrated to be the predominant species identified from skim milk powders collected in this study. Various bacterial and spore tests were used to screen the microorganism population under different heating, incubation and atmosphere conditions. A

large diversity of spore-forming bacteria was identified by 16S sequencing, but most were *B. licheniformis*. Compared with WGS, 16S sequencing was not precise enough to distinguish *B. licheniformis* and *B. paralicheniformis*. More focus should be given to *B. licheniformis* in diary powder production and improvements in processing should be made to reduce spore counts.

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Figure legends

Fig. 1. Total bacterial counts (TBC), thermoduric bacterial and thermophilic bacterial counts in all skim milk powder samples surveyed: ◇, TBC; □, thermoduric bacteria; △, thermophilic bacteria.

Fig. 2. Bacterial spores and sulphite reducing clostridia (SRC) counts in all skim milk powder samples surveyed, ◇, mesophilic aerobic bacterial spores; □, thermophilic aerobic bacterial spores; △, mesophilic anaerobic bacterial spores; ×, thermophilic anaerobic bacterial spores; ∗, mesophilic SRCs; ○, thermophilic SRCs.

Fig. 3. Highly heat resistant (HHR) spore counts in all skim milk powder samples surveyed, ◇, mesophilic aerobic HHR spores; □, thermophilic aerobic HHR spores; △, mesophilic anaerobic HHR spores; ×, thermophilic anaerobic HHR spores.

Fig. 4. Species distribution of 269 isolates selected for 16S sequencing.

Fig. 5. A pie chart showing the % of the 183 *B. licheniformis* 16S-sequenced isolates obtained from the different microbiological methods used.

Fig. 6. Whole genome sequence-based dendrogram of 23 isolates that were previously identified as "*B. licheniformis*" using 16S rDNA sequencing: ■, *Bacillus licheniformis*; ■, *Bacillus* sp. H15-1; ■, *Bacillus paralicheniformis*.

Table 1

Details of the methods used for microbial enumeration. [a]

Test #	Name	Heat treatment		Plate incubation		Incubation time (days)	Agar medium	Reference
		Temperature (°C)	Time (min)	Atmosphere	Temperature (°C)			
1	Total bacterial count (TBC)	none	none	aerobic	30	3	TSA	IDF (1991)
2	Thermoduric bacteria	63.5	35	aerobic	30	2	TSA	Wehr et al. (2004)
3	Thermophilic bacteria	none	none	aerobic	55	2	TSA	ISO/IDF (2009)
4	Presumptive *Bacillus cereus*	none	none	aerobic	30	2	BACARA	FDA (1998); ISO (2004)
5	Mesophilic sulphur-reducing clostridia spores	80	10	anaerobic	30	2	ISA	ISO (2003)
6	Thermophilic sulphur-reducing clostridia spores	80	10	anaerobic	55	2	ISA	ISO (2003)
7	Mesophilic aerobic bacterial spores	80	10	aerobic	30	3	PCSMA	Wehr et al., (2004)
8	Mesophilic anaerobic bacterial spores	80	10	anaerobic	30	3	PCSMA	Wehr et al., (2004)
9	Thermophilic aerobic bacterial spores	80	10	aerobic	55	2	PCSMA	Wehr et al., (2004)
10	Thermophilic anaerobic bacterial spores	80	10	anaerobic	55	2	PCSMA	Wehr et al., (2004)
11	Mesophilic aerobic highly heat-resistant spores	100	30	aerobic	30	3	PCSMA	ISO (2013)
12	Mesophilic anaerobic highly heat-resistant spores	100	30	anaerobic	30	3	PCSMA	ISO (2013)
13	Thermophilic aerobic highly heat-resistant spores	100	30	aerobic	55	2	PCSMA	Wehr et al. (2004); ISO (2009)
14	Thermophilic anaerobic highly heat-resistant spores	100	30	anaerobic	55	2	PCSMA	Wehr et al. (2004); ISO (2009)
15	Psychrotrophic aerobic bacterial spores	80	10	aerobic	6	10	PCSMA	Wehr et al. (2004); ISO (2003)
16	Psychrotrophic aerobic highly heat-resistant spores	100	30	aerobic	6	10	PCSMA	Wehr et al. (2004); ISO (2003)

[a] When counting plates, all colonies on a plate were counted; TSA, tryptic soy agar; BACARA, BACARA™ proprietary *B. cereus* culture medium; ISA, iron sulphite agar; PCSMA, plate count skim milk agar.

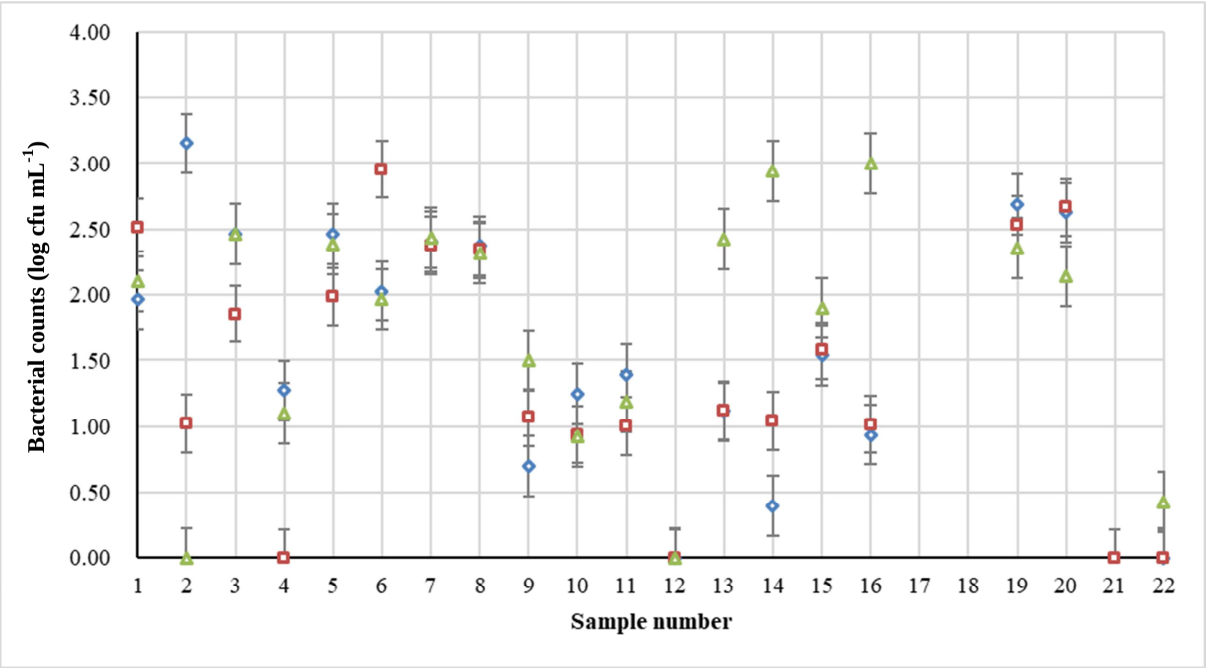


Figure 1.

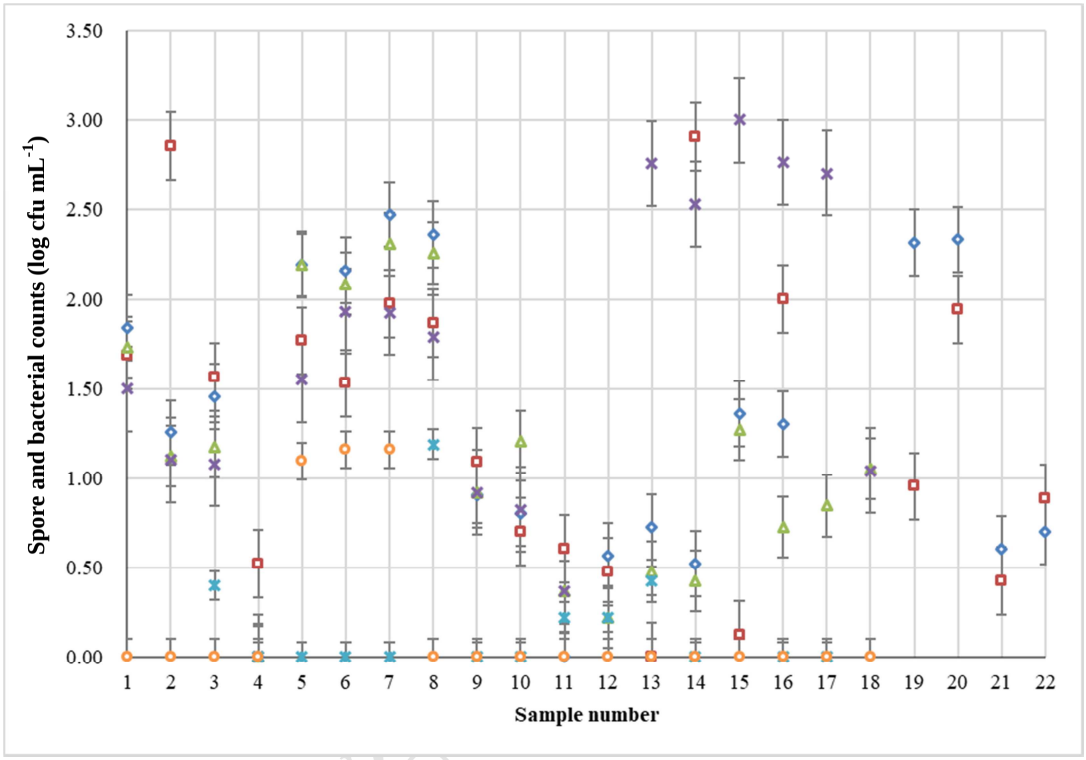


Figure 2.

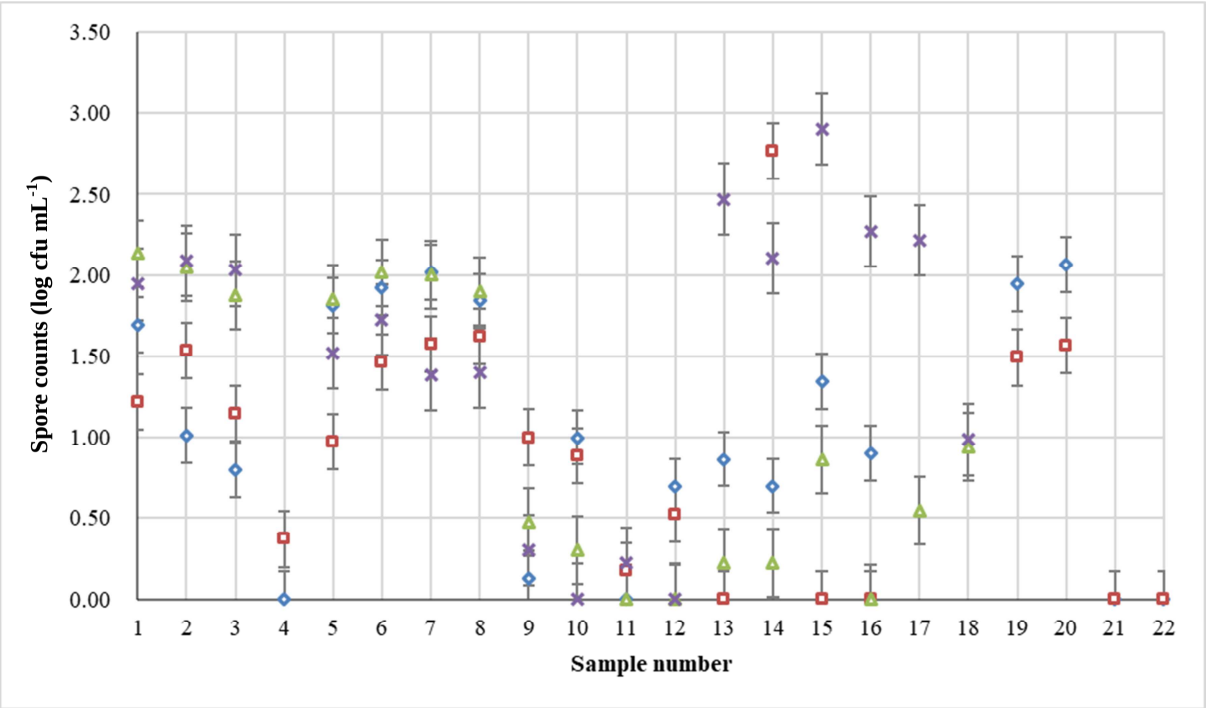


Figure 3.

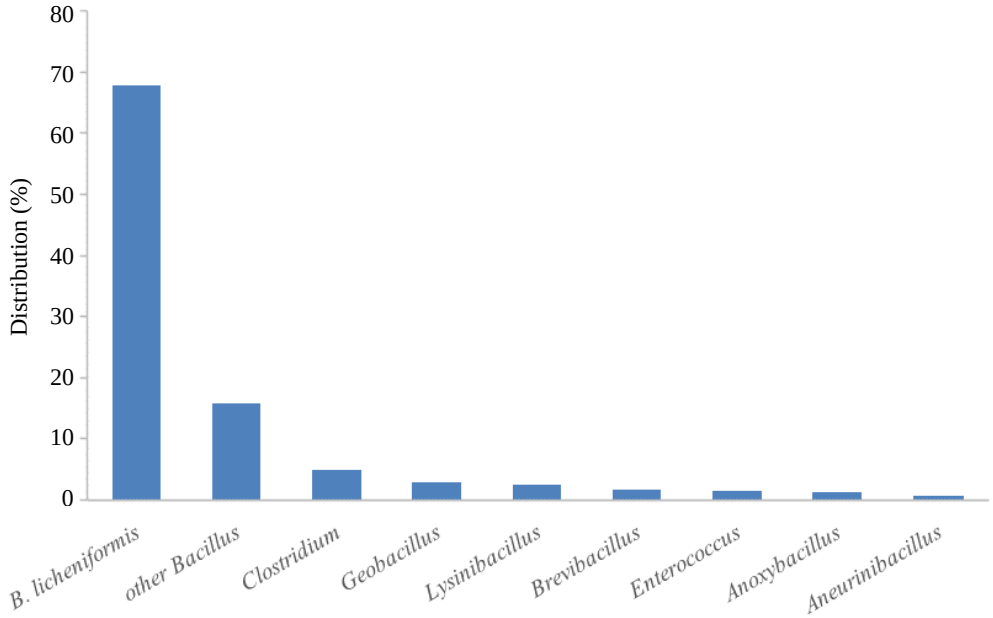


Figure 4

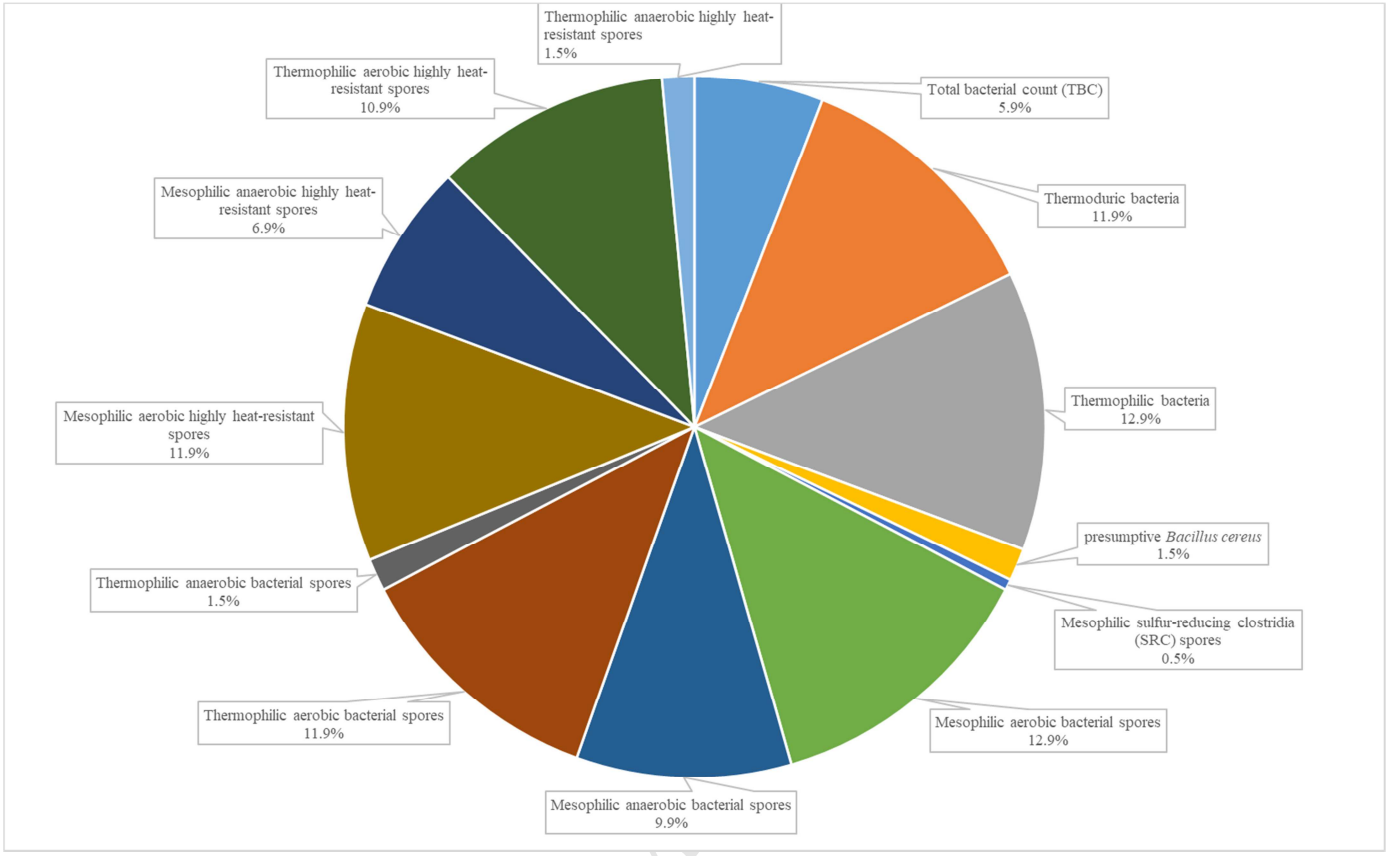
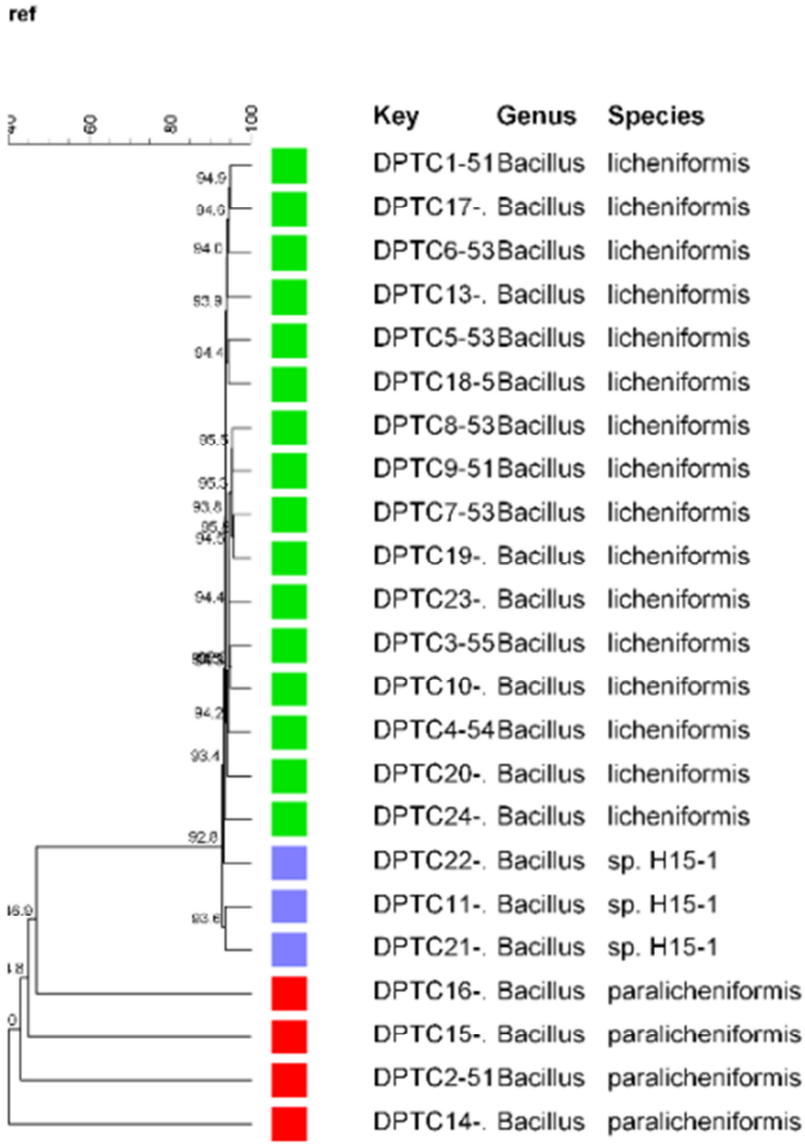


Figure 5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Key Genus Species
DPTC1-51 Bacillus licheniformis
DPTC17-. Bacillus licheniformis
DPTC6-53 Bacillus licheniformis
DPTC13-. Bacillus licheniformis
DPTC5-53 Bacillus licheniformis
DPTC18-5 Bacillus licheniformis
DPTC8-53 Bacillus licheniformis
DPTC9-51 Bacillus licheniformis
DPTC7-53 Bacillus licheniformis
DPTC19-. Bacillus licheniformis
DPTC23-. Bacillus licheniformis
DPTC3-55 Bacillus licheniformis
DPTC10-. Bacillus licheniformis
DPTC4-54 Bacillus licheniformis
DPTC20-. Bacillus licheniformis
DPTC24-. Bacillus licheniformis
DPTC22-. Bacillus sp. H15-1
DPTC11-. Bacillus sp. H15-1
DPTC21-. Bacillus sp. H15-1
DPTC16-. Bacillus paralicheniformis
DPTC15-. Bacillus paralicheniformis
DPTC2-51 Bacillus paralicheniformis
DPTC14-. Bacillus paralicheniformis

Figure 6.