Farm level survey of spore-forming bacteria on four dairy farms in the Waikato region of New Zealand

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Abstract
The aim of our study was to determine the occurrence and diversity of economically important spore-forming bacteria in New Zealand dairy farm systems. Farm dairy effluent (FDE) collected from Waikato dairy farms were tested for the presence of spore-forming bacteria, using a new culture-based methodology followed by genomic analysis. An enrichment step in which samples were inoculated in cooked meat glucose starch broth under anaerobic conditions, aided in the differential isolation of Bacillus and Clostridium species. Furthermore, the use of molecular methods such as ERIC genotyping, 16S rRNA gene sequence analysis identified different spore-forming bacteria present in FDE. C. sporogenes signature PCR gave further information on the phylogenetic relationship of the different Clostridium spp. isolated in this study. In total 19 Bacillus spp., 5 Paenibacillus spp. and 17 Clostridium spp. were isolated from farm dairy effluent. Sequence types similar to economically important food spoilage bacteria viz: C. butyricum, C. sporogenes and members of the Paenibacillus Genus were isolated from all four farms, whereas, sequence types similar to potential toxigenic, B. cereus, C. perfringens, C. butyricum, and C. botulinum were found on at least three of the farms. Sampling of farm dairy effluent provides a good indicator of farm level prevalence of bacterial load as it is used to irrigate dairy pasture in New Zealand. This study highlights the presence of various spore-forming bacteria in dairy waste water and indicates the implementation of good hygienic farm practices and dairy waste effluent management.

KEYWORDS
bacillus, clostridium, dairy farm, spores

1 | INTRODUCTION

Spores from bacteria such as Bacillus and Clostridium spp. may contaminate foods and are often responsible for food quality and safety issues. These spores are heat tolerant and pasteurization has little or no effect on their control. Currently good hygiene management and (thermal) processing largely control spore-forming bacteria during food processing, ensuring quality, and safety of final products. Concerns have been raised regarding the emergence and description of highly heat-resistant endospores (Foschino, Galli, & Ottogali,1990; Klijn et al.,1997) that can survive and grow after commercial sterilization and ultra-high temperature (UHT) processing of milk. Also, mild heat treatments, such as thermization, may intensify problems by activating spore germination in that temperature (Griffiths, Phillips, West, & Muir,1988; Hanson, Wendorff, & Houck, 2005). Clonally related highly heat-resistant spores of Bacillus sporothermodurans have been isolated.
from the farm environment as well as from heat-treated milk highlighting the importance of the environment on spore resistance, survival and germination properties (Gerhardt & Marquis, 1989; Guillaume-Gentil et al., 2002; Scheldeman, Pil, Herman, De Vos, & Heyndrickx, 2005). As new products and technologies have been developed, concerns have also been raised around the impact of spore contamination on quality and safety in minimally processed and shelf stable dairy foods (Guinebretiere, Girardin, Dargaignaratz, Carlin, & Nguyen-The, 2003; Peck, 2006; Ranieri, Huck, Sonnen, Barbano, & Boor, 2009).

Bacillus and Paenibacillus spores can withstand high temperature short time (HTST) pasteurization, a common method used for raw milk processing (Fromm & Boor, 2004; Ranieri et al., 2009). Bacillus is often the predominant genus present postpasteurization in milk stored at 6°C (Huck, Woodcock, Ralyea, & Boor, 2007). Conversely Paenibacillus spp. usually dominate later on during chilled storage (Fromm & Boor, 2004; Ranieri et al., 2009) and have been found to comprise over 95% of the bacterial microflora present in milk after prolonged refrigeration (Ranieri et al., 2009, 2012). Geobacillus stearotherophilus is another species of significance to the dairy industry (Burgess, Lindsay, & Flint, 2010). This can cause long-term persistent contamination of various dairy processing facilities, due to their strong ability to form biofilms on stainless steel surfaces of processing equipment in milk plants (Flint, Bremer, & Brooks, 1997). The metabolic activities of these spore formers can lead to curdling and off-odors, or -flavors (Ageitos, Vallejo, Sestelo, Poza, & Villa, 2007; Dutt, Gupta, Saran, Misra, & Saxena, 2009).

Some of the Clostridia strains are associated with late blowing cheese, C. sporogenes, C. beijerinckii, C. butyricum, and C. tyrobutyricum being the major agents (Cocolin, Innocente, Biasutti, & Comi, 2004; Cremonesi, Vanoni, Silvetti, Morandi, & Brasca, 2012). C. sporogenes and C. butyricum can cause gassy defects of processed cheese. Moreover, if extensive proteolysis occurs during cheese ripening, the release of amino acids and increase in pH will favor the growth of many Clostridium species, especially C. tyrobutyricum (Klijn, Nieuwendorf, Hoolwerf, van der Waals, & Weerkamp, 1995). Silage has been implicated as the principal source of spores of ruminant feed (Vissers, Driehuis, De Jong, & Lankveld, 2007; Vissers et al., 2007a,b). Other factors that may influence silage quality include the starting material, pH, dry matter content, fermentation conditions and microbial content (Rammer, 1996; Vissers et al., 2007a,b; Vissers, Te Giffel, et al., 2007). Current thinking is that raw milk becomes contaminated through consumption of lower grade silage by herd, followed by the survival of spores in the bovine gastrointestinal tract resulting in contaminated faeces. Subsequent fecal contamination of teats and udder surfaces then result in contamination of raw milk particularly if good hygienic practice is not followed (Aureli & Franciosa, 2002). Also, the teats and udders of pasture fed cows can become contaminated through spore-forming bacterial contaminants of soil, particularly Clostridium species (Christiansson, Bertliss, & Svensson, 1999; Slaghuis, Te Giffel, Beumer, & André, 1997).

Whether the contamination of bulk milk with spore-forming bacteria can be eliminated is unknown. Annually, New Zealand dairy companies process around a billion litres of milk, most of which will go overseas as whole milk powder to be used globally as ingredients or for the production of infant formula. If studies to investigate the diversity of spore-forming bacteria and their prevalence on farm are to generate mitigation strategies to control their entry to the food chain, simple tools for their rapid detection and differentiation are required. This study aimed at investigating diversity of spore-forming bacteria in farm dairy effluent (FDE) as cultures from dairy effluent provide an indication of microbial strains that may be cycling on the dairy farm. We developed a new culture based methodology to detect and separate Clostridium and Bacillus species anaerobically. To date, we believe that no study has been undertaken to investigate the occurrence of spore-forming bacteria at a farm level in New Zealand.

2 | MATERIALS AND METHODS

2.1 | Study sites

A cross-sectional pilot study was carried out on four Waikato dairy farms. All the 4 study farms had some component of pasture
grazing; three were pasture-only and in one, the cows had access to HerdHomes®-like facility (http://herdhomes.co.nz). Analysis was carried out on a “whole herd” basis by collecting samples of FDE from the exit point of the milking parlor at final wash down.

2.2 | Sample processing

Farm dairy effluents were collected in July and September 2014 (late winter early spring) and in January 2015 (summer). A one litre grab sample of FDE was collected in a sterile Schott Duran glass bottle from the shed wash collection sump immediately after morning milking. Samples were transported to the laboratory in an insulated box from the shed wash collection sump immediately after morning milking. For ease of recording and to retain confidentiality of individuals, farms were allocated a number.

Samples were centrifuged at 3466 g for 1 hr. The supernatant was removed and pellet suspended in 5 ml of prewarmed Butterfield’s diluents (Fort Richard, New Zealand) and centrifuged at 3466 g for 1 hr. The supernatant was removed and pelleted. Spores were from vegetative cells, the respective suspensions were heated at 80°C for 15 min in a water bath.

2.3 | Bacterial isolates

Clostridium sporogenes NCTC 532 spores were used as a control and to develop the new methodology.

2.4 | New culture-based methodology for isolating mesophilic spore-forming bacteria from environmental samples

2.4.1 | Aerobic spore formers

A 1 ml aliquot from each of the heated sample suspensions described previously was serially diluted in Butterfield’s diluent and plated directly in duplicate on Sheep Blood Agar (SBA) (Fort Richard, New Zealand). Plates were incubated under aerobic conditions at 35°C for 24 hr and then colonies enumerated. This preparation was termed, heated and direct (HD).

2.4.2 | Anaerobic spore formers

A 1 ml aliquot of each of the heated sample suspensions was added to 9 ml of prereduced cooked meat glucose broth (Fort Richard) supplemented with casein (0.03%), L-cysteine (0.0005%), Haemin (0.1%), Vitamin K1 (1%) and yeast extract (0.0005%) for sample enrichment and incubated under anaerobic conditions at 35°C for 3 day. This treatment was termed, heated and enriched (HE). Enriched cultures were removed and transferred to fresh sterile centrifugation tubes. The samples were centrifuged at 6000 g for 15 min and the pellets were re-suspended in 2 ml of Butterfield’s diluent. A 1 ml preparation from each of the enriched suspensions was serially diluted in Butterfield’s diluent and plated in duplicate on Shahidi- Ferguson Perfringens agar (Shahidi & Ferguson, 1991) with 50% egg yolk, polymyxin B (3 mg l⁻¹) and kanamycin (12 mg l⁻¹) (EYA). Plates were incubated under anaerobic conditions at 35°C for 24–48 hr. Enumeration of anaerobic spore formers was not carried out as the samples were enriched in growth medium for 3 days.

All the colonies from both HD, and HE plates (black as well as white colonies on EYA plates) were further sub cultured on Sheep blood agar for obtaining pure cultures. Each of the cultures was further inoculated in thioglycollate broth (Fort Richard) for genomic analysis.

2.5 | DNA extraction

A boiled lysate of each of the cultures was prepared by boiling the culture at 100°C for 10 min and collecting the supernatant after centrifugation at 9838 g for 5 min. The supernatant was used as a source of template DNA for Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR. Genomic DNA was isolated, using the Roche High Pure PCR template preparation kit (Roche diagnostics, Manheim, Germany), according to the manufacturer’s instructions and used for amplification of the 16S rRNA gene from spore-forming bacteria.

2.6 | ERIC PCR genotypic fingerprinting

ERIC PCR was carried out using ERIC 2 primer (Versalovic, Koueth, & Lupski, 1991) and the protocol of Weijtens, Reinders, Uurlings, and Van der Plas (1999). The PCR reaction mixtures (50 μl) had 1 × AmpliTaq Gold (Applied Biosystems, Melbourne, Australia), 1 μmol/L of ERIC 2 primer with sequence 5’- AAG TAA GTG ACT GGG GTG AGC-3’ (Invitrogen, Thermo Fisher Scientific Northshore, City, NZ) and 5 μl of extracted DNA. The PCR protocol was an initial cycle of 5 min at 94°C, 40 cycles of consecutively 1 min at 94°C, 1 min at 25°C and 4 min at 72°C and finally, a cycle of 10 min at 72°C. PCR was carried out in a PTC-100™ Programmable Thermal Cycler (MJ Research Inc, Thermo Fisher Scientific). PCR products of 1500 bp were separated on a 1.5% agarose gel (Fisher Scientific, Loughborough, UK), stained with ethidium bromide (10 mg l⁻¹ Bio-Rad Laboratories, North Harbour, NZ), at 150 V for 4 hr and visualized under UV transillumination using Gel Doc™ XR+ (Bio-Rad Laboratories) and images of band patterns (fingerprints) were captured, using Image LabTM software version 3.0 (Bio-Rad Laboratories). The fingerprints were compared, using Quanti-One software (v. 4.5.2 Bio-Rad), with the background subtraction level set at a rolling disk size 2 and a minimum spacing between bands (tolerance) set at 1% of the lane height. All bands were included in the fingerprint comparison. Each unique ERIC profile for each sequence type was allocated a letter of the alphabet and genomic DNA from representative ERIC profiles was chosen for 16S rRNA gene analysis.

2.7 | 16S rRNA gene amplification and Sequencing

Amplification of the 16S rRNA gene was carried out using forward primer sequence pA 5’-AGAGTTTGATCCTGCTCAAG-3’ (Invitrogen) and reverse primer sequence pH+ 5’-AAGGAGGTGATCCAGCAGCAG-3’ (Invitrogen) as described by Boddinghaus, Wolters, Heikens, and
buffer (Invitrogen) with 1 mmol/L of MgCl₂ (Invitrogen), 1.25 U of Taq polymerase (Invitrogen) and 5 μl of DNA. All the reagents were procured from Life Technologies and nuclease free water was used in all the reactions. PCR was carried out in a PTC-100™ Programmable Thermal Cycler (MJ Research Inc), using the following conditions: 93°C for 3 min; 92°C for 1 min, 55°C for 1 min and 72°C for 2 min for 30 cycles followed by a final extension at 72°C for 3 min. PCR products were visualized on a 0.8% ultrapure agarose (GibcoBRL) gel stained with ethidium bromide (Bio-Rad, 10 mg l⁻¹). The PCR products were purified, using Qiagen DNA extraction kit as per manufacturer’s instructions (Qiagen, Bio-strategy Ltd, New Zealand) and the products sequenced, using an ABI3730 DNA Analyzer (Massey Genome Service, Palmerston North, New Zealand) and the primers described above. 16S rRNA gene consensus sequences were used to investigate the phylogenetic relationship of the spore-forming bacteria obtained from the samples, using the software Geneious version 8.1 by Biomatters. (http://www.geneious.com, Kearse et al., 2012).

2.8 | Phylogenetic analysis

Phylogenetic analysis was carried out, using the software Geneious version 8.1 by Biomatters. A Polar formatted, unrooted phylogenetic tree was created from the converted 16s rRNA gene sequence alignment of all the representative isolates identified during this study and botulinum Group I, II and III to investigate genetic distance between the sequences. 16s rRNA gene sequences of botulinum Groups I, II and III were obtained from Genbank (http://www.ncbi.nlm.nih.gov/genbank/) and PATRIC (Pathosystems Resource Integration Centre, https://www.patricbrc.org). Alignment of all 16s rRNA gene sequences was prepared, using Muscle alignment tool with UPGMA clustering. The most likely tree found using a General Time Reversible model with optimized nucleotide equilibrium frequencies, optimized invariable sites, optimized across site variation, and NON tree-searching operations was bootstrapped with 100 replicates. Branches with less than 50% support were collapsed, using TreeCollapserCL version 4 (Hodcroft, 2013).

2.9 | C. sporogenes genetic signature PCR

PCR primers to hsdMSR (Forward 5’-TGAATCGGAAAACCGATGGAC-3’; Reverse 5’- TGGCCTTGGCTCCTATTCTT-3’), thiHG (Forward 5’- RCGCTTTGTCCKCAACATAT-3’; Reverse 5’- GCCGCTTATGGAGYTAA TTG-3’), lipAS (Forward 5’- TGTGCAAGAATGCTGCTTCT-3’; Reverse 5’- CTTTTCTCCCCAGACTCACA-3’) and dapL (Forward 5’- ACGCTTTGTAGCATCAAA-3’; Reverse 5’- GCCTGGACTTCTG TACTGC-3’) gene sequences were used in this study (Weigand et al., 2015). Each 25 μl PCR mixture contained 0.2 mmol/L of each dNTPs (Invitrogen), 1 μmol/L of forward and reverse primers, 1X reaction buffer (Invitrogen) with 1 mmol/L of MgCl₂ (Invitrogen), 1.25 U of Taq polymerase (Invitrogen) and 2 μl of DNA. All reagents were procured from Life Technologies and nuclease free water was used in all the reactions. PCR amplification was carried out in a T100™ Bio-Rad Thermal Cycler, using the following conditions: 95°C for 3 min; 95°C for 30 s, 53°C for 30 s and 72°C for 90 s for 35 cycles followed by a final extension at 72°C for 10 min. PCR products were visualized on a 2% ultrapure agarose (GibcoBRL) gel stained with ethidium bromide (Bio-Rad, 10 mg l⁻¹).

3 | RESULTS

3.1 | Culture experiments

Mesophilic spore-forming bacteria were isolated from all FDE samples collected from the four farms during all sampling events. The new methodology and conditions described in this study successfully differentiated *Bacillus* and *Clostridium* spp. on culture medium in anaerobic conditions. The use of an enrichment step (using prerduced cooked meat glucose broth vials supplemented with casein (0.03%), L-cysteine (0.0005%), Haemin (0.1%), Vitamin K1 (1%) and yeast extract (0.0005%) prior to plating on Shahidi- Ferguson Perfringens Agar supplemented with 50% egg yolk and polymyxin B (3 mg l⁻¹) and kanamycin (12 mg l⁻¹) under anaerobic conditions aided the isolation of *Clostridium* spp. and inhibited the growth of facultative anaerobic *Bacillus* spp.

Enumeration and isolation of mesophilic aerobic spore-forming bacteria were carried out by direct plating of heat treated sample suspensions serially diluted in Butterfield’s diluent and plated on Sheep Blood Agar and incubated under aerobic conditions at 35°C for 24 hr. Farm 1, did not show any difference in number of colony-forming units ml⁻¹ in winter (20 × 10³ CFU ml⁻¹, July 14) and summer (23 × 10³ CFU ml⁻¹, Jan 15). Farm 2 had 25 × 10⁵ in winter (July 14) and 38 × 10⁴ CFU ml⁻¹ in summer (Jan 15). Farm 3 had 21 × 10⁵ in winter (July 14) and 28 × 10⁴ CFU ml⁻¹ in summer (Jan 15) whereas, Farm 4 did not show huge difference between two seasons; 8 × 10³ July 14 and 28 × 10³ Jan 15 CFU ml⁻¹, respectively (Table 3).

3.2 | ERIC PCR genotypic fingerprinting and 16S rRNA sequencing

ERIC PCR was able to differentiate between isolates cultured from all FDE samples which aided in selecting unique representatives for 16S rRNA gene sequencing (Tables 1 and 2). The maximum identity for the 16S rRNA gene sequences obtained in this study in comparison to type strains ranged from 92 to 100% (Tables 1 and 2) therefore, species were identified on the basis of closest-related taxonomically described species.

3.3 | Occurrence and diversity of aerobic spore-forming bacteria in FDE

In total 19 different mesophilic *Bacillus* spp., five different *Paenibacillus* spp. and six non *Bacillus* spp. (identified by 16S rRNA
gene closest taxonomically described species) were isolated from the four farms (Table 1 and 3). The most commonly isolated Bacillus types were those with 16S rRNA sequences that matched B. licheniformis (maximum identity 97.6%–100%), B. altitudinis (maximum identity 95%–99.6%), B. pumilus (maximum identity 98.5%–99.7%), B. megaterium (maximum identity 97.4%–100%) and B. cereus (maximum identity 97.2%–98.6%). These Bacillus sequence types were found on all farms on at least one of the sampling events. B. cereus was found on all four farms, Farms 1, 2 and 3 in summer and Farm 4 in winter. Paenibacillus sequence-type isolates (maximum identity 95.7%–99.7%) were found on all farms during summer but only on Farm 1 in winter.

Similar to spore counts as mentioned earlier, Farms 1 and 3 showed no major difference in the diversity of Bacillus species isolated.

**Table 1** Diversity of aerobic spore-forming bacteria isolated from four Waikato farms over two seasons

| Farm, Seasons | Eric Type | Number of isolates | Closest-related taxonomically described species | Maximum Identity (%) | GenBank Accession Number |
|---------------|-----------|--------------------|-----------------------------------------------|----------------------|--------------------------|
| Farm 1, Winter | A 7       | B. licheniformis (T); ATCC 14580 | 98.4 | CP000002 |
| B 3 | B. circulans ATCC 4513 | 99.6 | FJ560956 |
| C 2 | B. pumilus ATCC 7061 | 99.6 | AY876289 |
| D 1 | B. thuringiensis IAM 12077 | 99.1 | D16281 |
| E 2 | Paenibacillus lactis strain MB 1871 | 99.7 | NR_025739 |
| F 1 | Virgibacillus proomii LMG 12370 | 99.7 | AJ012667 |
| G 1 | B. clausii (T) DSM 7061 | 99.3 | X76440 |
| H 1 | B. siralis (T) | 99.6 | AFO71856 |
| I 1 | B. thermoamylolavorans TN20 | 98.1 | JQ415992 |
| J 1 | B. mycoides ATCC6462 | 99.2 | AB021192 |
| Farm 1, Summer | A 1 | B. licheniformis (T); ATCC 14580 | 97.8 | CP000002 |
| B 3 | B. pumilus (T); ATCC 7061 | 99.7 | AY876289 |
| C 2 | B. megaterium (T); IAM 13418 | 97.4 | D16273 |
| D 1 | B. circulans (T); ATCC 4513 | 99.4 | AY724690 |
| E 1 | B. cereus (T); ATCC 14579 | 97.4 | AE016877 |
| F 2 | Paenibacillus cookii strain JGR8 | 99.7 | KF873508 |
| G 5 | Paenibacillus cookii (T); LMG 18419 | 99.4 | AJ250317 |
| H 5 | Brevibacillus agri (T); DSM 6348T | 95.1 | AB112716 |
| I 7 | B. altitudinis (T); type strain:41KF2b | 99.6 | AJ831842 |
| J 1 | Solibacillus silvestris SLB046 | 99.5 | AP012157 |
| Farm 2, Winter | A 21 | B. licheniformis (T); ATCC 14580; DSM 13 | 98.8 | CP000002 |
| B 2 | B. circulans ATCC 4513 | 99 | AY724690 |
| C 2 | B. clausii (T); DSM 8716 | 98.7 | X76440 |
| Farm 2, Summer | A 18 | B. licheniformis (T); ATCC 14580 | 99.6 | CP000002 |
| B 4 | B. licheniformis (T); ATCC 14580 | 99.6 | CP000002 |
| C 1 | B. licheniformis (T); ATCC 14580 | 99.6 | CP000002 |
| D 1 | B. licheniformis (T); ATCC 14580 | 99.6 | CP000002 |
| E 1 | B. cereus (T); ATCC 14579 | 97.6 | AE016877 |
| F 1 | B. thuringiensis (T); IAM 12077 | 99.6 | D16281 |
| G 2 | B. subtilis (T); DSM10 | 100 | AJ276351 |
| H 2 | B. cereus (T); ATCC 14579 | 97.6 | AE016877 |
| I 1 | B. mycoides ATCC6462 | 99.2 | AB021192 |
| J 3 | B. subtilis (T); DSM10 | 100 | AJ276351 |
| K 1 | Paenibacillus lactis (T); MB 1871 | 97.5 | AY257868 |
| L 1 | Sporosarcina koreensis (T); F73 | 95.4 | DQ073933 |
| M 1 | B. shackletonii (T); LMG 18435 | 92 | AJ250318 |
| N 1 | B. idriensis (T); SMC 4352-2 | 97.4 | AY904033 |

(Continues)
species isolated from Farms 2 and 4 were found to be more diverse in summer compared with winter sampling, whereas; not much difference was observed with Farm 1 and Farm 3 (Table 1 and 3). On farms 2 and 4 there were only three predominant Bacillus species detected in winter compared with Farms 1 and 3, where at least 10 different Bacillus species were isolated. Interestingly, Bacillus species isolated from Farm 2 in winter were different to Farm 4 and none of those species were common to both the farms (Table 1).

Bacillus strains with a 16S rRNA sequence type most closely matching B. licheniformis were the most prevalent and also gave rise to the most ERIC types per farm, followed by B. altitudinis (Table 3).

### 3.4 | Diversity of anaerobic spore-forming bacteria

In total, 17 different mesophilic Clostridium spp. (identified by 16S rRNA gene closest taxonomically described species), and no non Clostridium spp. were isolated from the 4 farms using the methodology described in this study. Clostridium species isolated from Farms 1, 2 and 3 were found to be more diverse in winter compared with summer sampling. The most commonly isolated Clostridium sequence types were C. bifermentans (maximum identity 97.6%–97.7%), C. perfringens (maximum identity 99.2%–99.8%), botulinum Group I (maximum
TABLE 1  (Continued)

| Farm, Seasons | Eric Type<sup>a</sup> | Number of isolates | Closest-related taxonomically described species | Maximum Identity (%)<sup>a</sup> | GenBank Accession Number |
|---------------|-----------------------|--------------------|-----------------------------------------------|-------------------------------|--------------------------|
| Farm 4, Summer | A                     | 1                  | B. licheniformis (T); ATCC 14580;              | 98                            | CP000002                 |
|               | B                     | 1                  | B. megaterium (T); IAM 13418                   | 97.8                          | D16273                   |
|               | C                     | 9                  | B. pumilus (T); ATCC 7061                     | 99                            | AY876289                 |
|               | D                     | 1                  | B. thuringiensis (T); IAM 12077                | 99.3                          | D16281                   |
|               | E                     | 1                  | B. thuringiensis (T); IAM 12077                | 99.3                          | D16281                   |
|               | F                     | 1                  | B. thuringiensis (T); IAM 12077                | 99.3                          | D16281                   |
|               | G                     | 1                  | Paenibacillus telluris (T); PS38              | 96.1                          | HQ257247                 |
|               | H                     | 1                  | Paenibacillus barengoltzii (T); SAFN-016      | 95.7                          | AY167814                 |
|               | I                     | 1                  | B. stratosphericus (T); 41KF2a                | 99.4                          | AJ831841                 |
|               | J                     | 1                  | B. pseudomycoides; DSM 12442                  | 98.6                          | AM747226                 |
|               | K                     | 2                  | B. altitudinis (T); type strain: 41KF2b       | 99.1                          | AJ831842                 |
|               | L                     | 2                  | B. altitudinis (T); type strain: 41KF2b       | 95                            | AJ831842                 |
|               | M                     | 6                  | B. altitudinis (T); type strain: 41KF2b       | 99.6                          | AJ831842                 |

Sequence similarity to various bacterial type strains is presented.

<sup>a</sup>The similarity has been confirmed by 16S rRNA sequencing and maximum identity is depicted in percentage.

<sup>b</sup>Each unique ERIC pattern per farm per sampling event was allocated a separate letter (A to P), depending on the number of ERIC patterns identified from four farms in each sampling event.

identity 98%–100%) and botulinum Group II (maximum identity 98%–99.9%). Botulinum Group I and Group II like sequence types were isolated from FDE obtained from all farms on at least one sampling event. Sequence types closely related to botulinum Group I (type A and B) representatives were identified on Farms 1 and 4. C. sporogenes sequence type with maximum identity of 93%–100% was identified on all farms in winter and on Farms 1 and 4 in summer. Botulinum Group II sequence type, related isolates were identified on Farms 1 and 2 during winter but only on Farm 1 during summer. C. butyricum sequence types (maximum identity 99%–100%) was found on all farms in winter but only on Farm 2 in summer. No botulinum Group III-related isolates were identified on any of the farms. Isolates with the closest sequence type similarity to C. perfringens were identified on Farms 1, 2 and 3 during summer and only on Farm 3 during winter, whereas, C. butyricum sequence types (maximum identity 96.7%–100%) were identified on all four farms in winter and on Farm 2 in summer. (Table 2).

C. perfringens, C. sporogenes and C. cochlearium (maximum identity 88%–98.8%) were the only species to be represented by two different ERIC types. Most of Clostridium spp. isolated was represented by only one ERIC which may suggest a single source of contamination, however, work needs to be done to confirm it. On the whole, Clostridium spp. were found to be less diverse on the four farms compared with Bacillus spp. but this may be a consequence of culture enrichment undertaken for Clostridium spp. isolation.

3.5 Phylogenetic analysis of Clostridium botulinum-like species

A phylogenetic tree was created, using PhyML tree builder in Geneious version 8.1 from the converted 16S rRNA gene sequence alignments. The tree shows four distinct clustered sets consisting of isolates closely related to botulinum Group I and II, along with few isolates clustering in a completely different set. Four of the isolates clustered in botulinum Group I were found to be closely related to C. sporogenes type strain ATCC 15579 where as one isolate was closely related to C. sporogenes subsp. tusciae which clustered in a different set. The remaining nine isolates clustered under botulinum Group I were found to be closely related to C. botulinum B1 strain Okra. Twelve isolates were grouped in botulinum Group II in which five were closely related to C. butyricum E4, two were closely related to C. botulinum E3 strain Alaska and five isolates were closely related to C. botulinum B strain Eklund as well as C. botulinum E1 strain BONT E Beluga (Figure 1).

Phylogenetic analysis was also performed, using PCR primer sets to four-specific orthologs from the C. sporogenes lineage (Weigand et al., 2015). All farm isolates with 16S rRNA gene sequences closely related to either botulinum Group I and II representatives were tested, using the Sporogenes Signature PCRs (Figure 1 and 2) and the phylogenetic relationship between the Sporogenes Signature Positive isolates compared with Sporogenes Signature Negative, botulinum Group II and Group III isolates are shown in Figure 1.

All four C. sporogenes specific orthologs were amplified from farm isolates with the highest 16S rRNA gene sequence identity (93%–100% maximum identity) to C. sporogenes PA 3679 and ATCC155791 (Sporogenes Signature Positive Clade) except for sample JD1HE21 (Strain number 21 isolated from Farm1, dairy effluent in January) which was negative for thiHG. In addition to the Sporogenes Signature Positive Clade isolates, a dapL amplicon was generated, using C. sporogenes-specific primers from isolates DE1HE10, DE1HE17, DE1HE29, DE1HE22, and DE1HE27. However, no amplicons associated with rest of the three C. sporogenes specific orthologs were observed from these five strains. The phylogenetic relationship of these isolates was much more closely associated with C. botulinum ATCC 3502 type A.
(98.9% 16S rRNA gene maximum identity). The remaining farm isolates with highest sequence identity to the Group I C. botulinum reference strains (Sporogenes Signature Negative Clade) generated a smaller dapL amplicon of approximately 500 bp compared to the predicted amplicon of 546 bp. Farm isolates that had sequence similarity closest to the 16S rRNA gene sequence of the botulinum Group II representatives did not generate any amplicons, using the Sporogenes Signature PCR assays (Figures 1 and 2).

Four bacterial isolates (DE2.1, DE2.9, JDE4.10 and JDE1.21) showing sequence similarity to C. sporogenes type strain and corresponding to Sporogenes Signature Positive Clade, were found on 3 out of the 4 farms either in summer or winter sampling but not over both seasons. Ten isolates

### TABLE 2  Diversity of anaerobic spore-forming bacteria isolated from four Waikato farms over two seasons

| Farm, Seasons | Eric Type | Number of isolates | Isolate ID | Closest-related taxonomically described species | Maximum Identity (%) | Genbank Accession Number |
|---------------|-----------|--------------------|------------|-----------------------------------------------|----------------------|--------------------------|
| Farm 1, Winter | A         | 14                 | DE1. 6,8,10,15,16,17,19,22,27,29,38,44,45,46 | C. botulinum B1 strain Okra | 98.9                | CP000939                |
|               | B         | 5                  | DE1. 20.2,33,37,39,42 | C. botulinum B str. Eklund 17B | 99.9                | CP001056                |
|               | C         | 2                  | DE1. 7.18 | C. butyricum CG56 | 99                  | CP001056                |
|               | D         | 5                  | C. cochlearium JCM 1384 | 98.8               | LC007105              |
|               | E         | 6                  | C. cochlearium DSN5 | 88                 | CP001056              |
|               | F         | 5                  | C. cadaveris JCM 1392 | 99.8               | AB542932              |
|               | G         | 15                 | C. bifermentans ATCC 638 | 97.6               | AB075769              |
| Farm 1, Summer | A         | 21                 | JDE1.6.1,26 | C. perfringens (T); ATCC 13124 | 99.8                | CP000246                |
|               | B         | 2                  | JDE1.21   | C. botulinum B str. Eklund 17B | 99                  | CP001056                |
|               | C         | 1                  | JDE1.14   | C. sporogenes (T); ATCC3584 | 99                  | X68189                  |
|               | D         | 10                 | JDE1.21   | C. senegalence JC122 | 99.8               | NR_125591               |
|               | E         | 9                  | JDE1.21   | C. glycolicum (T); DSM 1288 | 95.4               | X76750                  |
| Farm 2, Winter | A         | 1                  | DE2.33    | C. botulinum B str. Eklund 17B | 99.8                | CP001056                |
|               | B         | 1                  | DE2.37    | C. butyricum RCEB | 99.7               | EU621841                |
|               | C         | 1                  | DE2.11    | C. sporogenes (T); ATCC3584 | 99                  | X68189                  |
|               | D         | 1                  | DE2.29    | C. sporogenes (T); ATCC3584 | 100                | X68189                  |
|               | E         | 1                  | DE2.29    | C. paraputricum ATCC 25780 | 97.1               | X75907                  |
|               | F         | 1                  | DE2.29    | C. senegalence JC122 | 99.4               | NR_125591               |
|               | G         | 3                  | DE2.29    | C. glycolicum (T); DSM 1288 | 95.8               | X76750                  |
|               | H         | 3                  | DE2.29    | C. cadaveris JCM 1392 | 100                | AB542932               |
|               | I         | 20                 | DE2.29    | C. bifermentans ATCC 638 | 97.7               | AB075769               |
| Farm 2, Summer | A         | 12                 | DE3.6     | C. perfringens (T); ATCC 13124 | 99.4                | CP000246                |
|               | B         | 1                  | DE3.6     | C. perfringens (T); ATCC 13124 | 99.2                | CP000246                |
|               | C         | 1                  | DE3.14    | C. butyricum (T); VPI3266 | 100                | AJ458420                |
|               | D         | 1                  | DE3.7     | Swine manure bacterium RT-8B | 97.3               | AY167950                |
|               | E         | 1                  | DE3.7     | C. bifermentans (T); ATCC 638 | 95.6               | AB075769                |
| Farm 3, Winter | A         | 1                  | DE4.9,41,22,23,24 | C. botulinum B1 strain Okra | 99                  | CP000939                |
|               | B         | 5                  | DE4.7,18,27,28,40 | C. butyricum (T); VPI3266 | 96.7               | AJ458420                |
|               | C         | 2                  | DE4.32,27 | C. sporogenes subsp. tusciae | 99.4               | AM237439                |
|               | D         | 1                  | DE4.32,27 | C. tertium (T); DSM 2485 | 99.1               | Y18174                  |
|               | E         | 14                 | DE4.32,27 | C. cadaveris (T); JCM 1392 | 99.7               | AB542932                |
|               | F         | 3                  | DE4.32,27 | Swine manure bacterium RT-8B | 99                  | AY167950                |

(Continues)
from Farms 1 and 4 corresponded to the Sporogenes Signature Negative Clade, including isolate DE4.32 who’s closest sequence type was C. sporogenes subsp. tusciae. Only one Sporogenes Signature Negative isolate was identified from one farm during the summer sampling event and other isolates were found in winter. All the dapL positive isolates were obtained from a single farm (Farm 1) during the winter sampling event (Figure 1).
4 | DISCUSSION

In New Zealand, FDE is used to irrigate dairy pasture as it is a resource full of nutrients and when managed properly increases pasture production. However, dairy effluent also contains bacteria excreted from the dairy cow and hence provides a good indicator of farm level prevalence of different bacteria. Isolation of environmental spore-forming bacteria is notoriously challenging. A major challenge has been to separately isolate Clostridium spp. and Bacillus spp. in anaerobic conditions. This is because Bacillus spp. being facultative anaerobes can grow in anaerobic environment. To date, there is no differential agar-based assay for the detection of SRC species, resulting in a significant percentage of false positives. It is well known that economically significant bacteria such as C. butyricum, C. tyrobutyricum and C. sporogenes are sulphite reducers but there are other species, including Bacillus species, such as B. thuringiensis, B. licheniformis that will also be enumerated as these species are able to reduce sulphite to sulphide. Also, there is no statistical relationship between SRC counts and the presence of pathogens such as C. perfringens and C. botulinum in foods (ICMSF, 2014). The culture and molecular methods described in this study resulted in improved differential isolation of Bacillus and Clostridium species. The use of an enrichment step, using pre-reduced cooked meat glucose broth prior to plating on Shahidi- Ferguson Perfringes Agar supplemented with egg yolk, polymyxin B and Kanamycin under anaerobic conditions aided the isolation of only Clostridium spp. For example, during the study we did not isolate any facultative anaerobic Bacillus strains anaerobically, known to be present in the sample. Furthermore, the use of molecular methods such as ERIC genotyping, 16S rRNA sequence analysis and C. sporogenes Signature PCR gave further information on the phylogenetic relationship of the Clostridium isolates.

Despite regional and methodological differences, the diversity of Bacillus species on the four farms was very similar to that seen internationally. In general, B. licheniformis, B. pumilus, and B. subtilis are the predominant mesophilic spore-forming species (Lukasova, Vyhnalkova, & Pacova, 2001; Sutherland & Murdoch, 1994; Tatzel, Ludwig, Schleifer, & Wallnofer, 1994) and B. cereus being the most common psychrotolerant species (Sutherland & Murdoch, 1994). In this study, the most commonly isolated Bacillus sequence types were B. licheniformis (maximum identity 97.6%–100%), B. altitudinis (maximum identity 95%–99.6%), B. pumilus (maximum identity 98.5%–99.7%), B. megaterium (maximum identity 97.4%–100%) and B. cereus (maximum identity 97.2%–98.6%), with B. licheniformis to be the predominant one. These Bacillus sequence types were found on all farms on at least one of the sampling events. Our results were found to be concordant to a study which showed a remarkable diversity of aerobic spore-forming bacteria in dairy farms and B. licheniformis amongst the most common ones (Scheldeman et al., 2005). In our study, we isolated a range of aerobic spore-forming bacteria from farm dairy effluent used for irrigation...
purposes. McAuley et al., in 2014 also found Bacillus group to be the most prevalent bacteria in all the farms tested and highest occurrence was found in soil which was 93% of the samples tested. Bacillus licheniformis species have been identified as a prominent food borne pathogens in previous studies. In a study, Rowan et al., 2001 found B. licheniformis and B. megaterium to produce diarrhoeal enterotoxin in reconstituted infant milk formula. Similarly B. cereus, a toxin producing Bacillus species have been found in liquid milk and other milk products (Griffiths, 1992). Toxin producing B. cereus strains can cause emetic or diarrhoeal food poisoning, while diarrhoeal toxin is produced as a result of spore germination and outgrowth in the small intestine, the emetic toxin is produced by vegetative cells of B. cereus growing in the preheat-treated milk (Kramer & Gilbert, 1989). However, the present cross-sectional study was carried out investigating the diversity and occurrence of dairy associated spore-forming bacteria at the farm level and determination of toxicity was not in the scope of this study.

B. cereus, B. licheniformis and Paenibacillus spp. are all psychrotrophic thermophilic sporeformers and a particular problem for the dairy industry. They can grow at refrigeration temperatures and often multiply and sporulate in the bulk milk (Murphy, Lynch, & Kelly, 1999). Their spores can then survive various heat treatments and processing and may go on to cause food poisoning, or reduce the shelf life of pasteurized milk and dairy products (Te Giffel, Beumer, Granum, & Rombouts, 1997). B. licheniformis is often the most frequently isolated bacterial contaminant in raw milk (Phillips & Griffiths, 1986; and Crielly, Logan, & Anderton, 1994; Miller et al., 2015). Moreover, some strains of this species have been found to show enhanced growth in skim milk under anaerobic environment (Ronimus et al., 2003).

There is very little data on the occurrence, diversity and seasonal distribution of Clostridium species on dairy farms. Clostridium species are ubiquitous in soil and present in feed is common source of contamination of raw milk. Commonly found species are C. sporogenes, C. butyricum, C. tyrobutyricum, C. disporicum, and C. saccharolyticum. Feligini, Panelli, Sacchi, Ghitti, and Capelli (2014) investigated the occurrence of Clostridium spp. in raw milk from Northern Italy. They found that C. butyricum and C. sporogenes were more abundant in winter, C. tyrobutyricum in spring and C. beijerincki in summer. Similarly, in this study, C. butyricum and C. sporogenes were also found to be abundant in winter than in summer and were representatives from botulinum Groups I and II. From a food safety perspective, C. perfringens is considered significant because of the ability of some strains to induce illness (Grass, Gould, & Mahon, 2013) and have also been recurrently identified as the causative agents of mastitis in bovine and other ruminants (Osman, El-Enbaawy, Ezzeleed, & Hessein, 2009). C. perfringens have been isolated from Australian dairy farms, specifically from milk filters, feces and soil (McAuley et al., 2014). In this study, C. perfringens sequence types were found on all the farms tested on at least one sampling event. Isolates with the closet sequence type similarity to C. perfringens were identified on Farms 1, 2, and 3 during summer and only on Farm 3 during winter.

C. butyricum, and C. sporogenes are known to be associated with spoilage of dairy foods and some strains of C. butyricum can produce botulinum like toxin (Le Bourhis et al., 2007; Toyoda, Kobayashi, & Ahiko, 1990). In this study, C. butyricum sequence types (maximum identity 96.7%–100%) were identified on all four farms in winter and on Farm 2 during summer. C. sporogenes sequence types (maximum identity 93%–100%) were identified on three farms (2, 3, and 4) during winter but only on Farms 2 and 4 during summer.

Currently there is not enough data to determine if seasonal distribution of Clostridium and Bacillus species on dairy farms impacts on the risk of contamination of raw milk and downstream processed products. In 2014 MPI (Clostridia (SRC) in New Zealand Bulk Raw Milk) carried out a survey of sulphite reducing clostridia in NZ bulk raw milk which showed that SRC vegetative cells were detected in only one milk sample out of 150 raw milk samples in December and four raw milk samples in February; the spores concentration in all positive test samples being only 1 spore ml⁻¹. Consuming low levels of C. botulinum spores rarely poses a health issue to individuals older than 1 year old. C. botulinum spores are often consumed without ill effect when eating foods such as honey, carrots, potatoes, or smoked fish. Similarly, lower levels of Clostridial spores in dried milk products do not present a health risk. However, the problem arises when the product is rehydrated and kept in anaerobic conditions at nonrefrigeration temperatures, which allow the C. botulinum spores to germinate and produce toxins.

The use of plastic-wrapped and non-acidified silage as cattle feed has increased the number of botulism outbreaks over the last two decades due to botulinum Groups I-III in dairy cattle (Böhnel & Gessler, 2013; Lindström, Myllykoski, Sivelä, & Korkeala, 2010). In this study, representatives of botulinum Group I and II were identified on all farms, highlighting the presence of these spores in the dairy farm environment. Consumption of silage contaminated with spores by cattle was found to be the main reason of raw milk getting contaminated with spore-forming bacteria (Vissers, Driehuis, Te Giffel, De Jong, & Lankveld, 2006). These spores, survive in the gastrointestinal tract of animals and contaminate the manure by shedding spores in feces which subsequently results in contaminating teats and udder surfaces causing contamination of raw milk during the process of milking (Bergeré, Gouet, Hermier, & Mocquot, 1968). Some of the isolates identified in this study may have potential to spoil food or produce toxin. There is always a possibility that if appropriate farming practices are not conducted, these spores may enter raw milk from the dairy environment and can cause problems as described by Driehuis, 2013. This study did not undertake any risk assessment to investigate the possibility of the contamination of raw milk by these isolates. However, this could be a future scope for another valuable study to understand the contamination level of raw milk in dairy farms.

Good farming practices are perhaps the most significant element in controlling spore numbers in raw milk. The use of good quality silage, cleaning, and maintenance of parlor/milking equipment, as well as a stringent udder cleaning and teat preparation before milking are all considered to be good farming practice. Consequently, the management of dairy waste effluent is also a critical component to consider for the reduction in number and spread of spore-forming bacteria on dairy farms. Furthermore, careful application of dairy waste effluent is particularly important if the farm or neighboring areas are used for wild harvest of foods such as water cress or horticultural operations.
5 | FUTURE DIRECTIONS

To investigate seasonal variation in the number of spore-forming bacteria and their diversity, more samples from different farms will be analyzed during different seasons.

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CONFLICT OF INTEREST

None.

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