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Identification and Characterisation of Spore-Forming Bacteria in Bovine Raw Milk Collected from Four Dairy Farms in New Zealand

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Abstract: Contamination of milk and dairy products with pathogenic and spoilage bacteria may result in huge economic loss due to recalls of products. This study aimed to identify spore-forming bacteria from raw milk and characterise those for toxin production and their spoilage ability. Bovine raw milk collected from dairy farms in the Manawatu region of the North Island of New Zealand was tested for the presence of both aerobic and anaerobic spore-forming bacteria using standard culture-based techniques, as well as genomic analysis. The spore-forming bacteria were investigated for the presence of toxin genes and their spoilage potential. A low number of aerobic spore-forming bacteria were detected in raw-milk samples collected from the four farms in summer and winter. The 16S rRNA sequence types similar to important food spoilage bacteria like *C. beijerinckii*, *C. sporogenes*, *B. licheniformis* and members of the *Paenibacillus* genus, as well as potentially toxigenic bacteria such as *B. cereus* and *C. perfringens* were isolated. Genes responsible for important toxin production were present in some of the tested spore-forming bacteria. This pilot study highlights the presence of various spoilage and pathogenic spore-forming bacteria in raw milk from these farms. A low number of spore-forming bacteria indicates the implementation of good hygienic farm practices and management to reduce the contamination of raw milk with spore-forming bacteria.

Keywords: bovine raw milk; clostridium; bacillus; spores; toxin; spoilage

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1. Introduction

Contamination of milk and milk products is a big challenge for dairy industries. Contamination of food with spore-forming bacteria needs to be well controlled as their spores are heat tolerant and heat processing may have little or no effect on their destruction. Alongside this, spores may germinate under favourable conditions and may cause spoilage and/or produce toxins. Most important examples are *Clostridium* and *Bacillus* spp., which may contaminate foods and are often accountable for food safety and quality issues [1–3]. Various sources throughout the dairy chain, e.g., water, soil, dirty udders, milking equipment and faeces have been attributed to the contamination of raw milk [4–6]. The presence of highly heat-resistant spore-forming bacteria such as *Bacillus thermoamylovorans* and *Bacillus sporothermodurans* [7–9] that can survive the commercial sterilisation and ultra-high temperature (UHT) processing of milk, and grow during favourable conditions, also needs to be addressed. On the other hand, mild heat treatments (such as thermisation) may exaggerate contamination issues by activating spore germination [10]. Development of minimally processed and shelf-stable dairy foods have raised concerns about quality and safety in relation to spore contamination [11–13].

Bacillus spp. have been linked to spoilage of both raw and processed milk and dairy products [14]. These include a range of *Bacillus* species such as *B. licheniformis*,

B. coagulans and *B. subtilis* [15]. The production of extracellular proteases and lipases are predominantly linked to the spoilage of raw and processed dairy and dairy products. These enzymatic spoilages are characterised by off flavours and structural changes in milk [16]. Production of lecithinase enzyme by the *B. cereus* group and *Paenibacillus polymyxa* has also been linked to spoilage activity, causing a 'bitty cream' defect in pasteurised milk [17]. The genus *Bacillus* often predominates post-pasteurization when milk is stored at 6 °C [14], whereas *Paenibacillus* spp., another aerobic spore former, usually dominate during chilled storage [13,18] and have been found to be dominant (~95%) in milk after continued refrigeration [13,19]. Spores of *Bacillus* and *Paenibacillus* species can tolerate the common method of raw-milk processing such as high-temperature short-time (HTST) pasteurization [13,18]. The major spore-forming *Bacillus* spp. such as *B. licheniformis*, *B. cereus* and *B. sporothermodurans* were found to contaminate and spoil UHT or sterilised milk [20–22]. Research has been undertaken which suggests that the primary contamination source of dairy products with these bacteria is the raw milk collected from dairy farms [23].

Another group of spore-forming bacteria which comprises anaerobic *Clostridium* spp. such as *C. perfringens*, *C. sporogenes*, *C. beijerinckii*, *C. tyrobutyricum* and *C. butyricum* are frequently isolated from raw milk and cheeses [24]. *C. tyrobutyricum*, *C. butyricum*, *C. beijerinckii* and *C. sporogenes* are the major contributors for the gas production in cheese blocks, commonly known as late blowing of cheeses [25–27]. *C. butyricum* and *C. sporogenes* can be the major sources of gas defects in cheeses, and with high proteolysis occurring during the ripening of cheese, the increase in the pH and release of amino acids would favour the propagation of other *Clostridium* species, particularly *C. tyrobutyricum* [28]. Spores present in cheese mainly originate from the raw milk which becomes contaminated during various activities on a farm including the milking process and feeding animals with contaminated feed (especially silage) [6,28–30].

Spore-forming bacteria are not only involved in food spoilage but can also be a risk to food safety. Food poisoning caused by these bacteria is usually linked with mishandling or improper storage conditions of heat-treated foods. This event results in the germination of a spore followed by bacterial growth and food with high levels of bacteria or toxins produced by them. *B. cereus* is recognised as one of the most hazardous human pathogens because some strains can produce a heat-stable toxin such as the emetic toxin, cereulide. However, some strains of *Bacillus weihenstephanensis* have also been found to produce these heat-stable toxic molecules. In addition, some strains of *B. licheniformis*, *B. pumilus*, *B. circulans*, *B. subtilis*, *B. simplex*, *B. megatarium* and *B. amyloliquefaciens* have also been reported to produce heat-stable toxins that resemble physiological and chemical characteristics of cereulide and may be toxic to mammalian cells [31–40]. Common pathogenic *Clostridium* species with the ability to produce toxins include *C. difficile*, *C. perfringens*, and *C. botulinum*, with *C. perfringens* mostly contaminating raw-milk products [41]. Although rare, *C. butyricum* has also been reported to produce a Type E botulinum neurotoxin [42].

The pasteurization of raw-milk and dairy products is an important processing step for the dairy industry to control the growth of spoilage and pathogenic microorganisms; however, pasteurization can be ineffective against some of the bacterial spores [43]. Hence, it is important to understand how farm management and practices may increase or decrease the risk of raw-milk contamination.

Every year, dairy companies in New Zealand process about a billion litres of milk, most of which is exported globally as whole-milk powder (~1.52 million metric tonnes). Hence, ensuring the safety of the starting material is essential. If studies to explore mitigation strategies to control or reduce the entry of spores in the food chain are to be assessed, it is important to investigate the prevalence and diversity of spore-forming bacteria in raw milk. This will relate back to what is present in the dairy environment and how this can be managed.

Limited studies are available on the detection and identification of both aerobic and anaerobic spore-forming bacteria in raw milk; hence, our pilot study aimed to investigate the numbers and diversity of various spore-forming bacteria in raw milk collected from four different New Zealand dairy farms. Furthermore, important food-spoilage and pathogenic

spore-forming bacteria were examined for the presence and characterization of toxin types and their spoilage potential. To date, we believe that studies investigating the occurrence and diversity of both aerobic and anaerobic spore-forming bacteria in raw milk have never been undertaken in New Zealand.

2. Materials and Methods

2.1. Study Sites

A cross-sectional pilot study was carried out on four farms in the Manawatu region of New Zealand. All the farms had pasture grazing for animals along with feed supplements such as grains, molasses, corn, silage and palm-kernel extract. The farm size ranged between 350 and 600 ha, with the number of cows ranging between 400 and 500. The analysis was conducted on the basis of "composite raw milk" by collecting samples of bulk raw milk from the farm vats. The vats were maintained at a temperature between 3 and 4 °C, and raw-milk samples were transported to the lab in chilled conditions.

2.2. Processing of Raw-Milk Samples

Raw bulk-tank milk was collected from four bovine dairy farms in the Manawatu region, in December–January 2015 (summer) and July–September (winter–early spring). From each farm, 2.5 L of raw milk was collected in duplicate to make a total of five litres of raw milk. Sterile Schott Duran glass bottles were used for collecting milk, which were kept cold in an insulated box containing ice packs prior to and after raw-milk collection. Raw milk was collected from the farm vat, which was maintained at temperature between 3 and 4 °C, immediately after milking. Samples were then transported to the laboratory in chilled conditions and processed on the same day. For recording purposes and to maintain confidentiality of each farm, farms were assigned a number, with summer designated as 'S' and winter as 'W'. Duplicate milk samples from each farm were mixed thoroughly to obtain a "cumulative" milk sample, of which 200 mL was centrifuged (ThermoFisher Scientific, Osterode am Harz, Germany) at 3466× *g* for 1 h at 4 °C. The cream at the top and the aqueous supernatant were carefully removed, and the pellet was suspended in five mL of pre-warmed Butterfield's diluent (BD; Fort Richard, Auckland, New Zealand). To isolate spores, the respective suspensions were heated at 80 °C for 10 min in a water bath, with intermittent shaking to kill the vegetative cells.

2.3. Bacterial Isolates

Clostridium perfringens NCTC 8237 Type A, *Clostridium perfringens* NCTC 8533 Type B, *Clostridium perfringens* NCTC 8081 Type C, *Clostridium perfringens* NCTC 8504 Type D, *Clostridium perfringens* NCTC 8084 Type E, *Bacillus licheniformis* NCTC 6346, *Bacillus cereus* NCTC 11143 and *Bacillus pumilus* NCTC 8241 were used as positive control strains for different assays in this study. These bacteria were procured from environmental science and research (ESR), New Zealand.

2.4. Isolation of Mesophilic Spore-Forming Bacteria from Raw-Milk Samples

Aerobic and anaerobic spore-forming bacteria were isolated using methodology as described by [44]. Briefly, for aerobic spore formers, one mL aliquots from each of the heated suspensions, as mentioned above, was serially diluted in BD and plated directly on sheep blood agar (SBA; Fort Richard, Auckland, New Zealand) in duplicates. Plates were incubated aerobically at 35 °C for 24 h, and colonies were enumerated. This preparation was termed as "heated and directly plated" on the media (HD). For anaerobic spore formers, one mL aliquot of each of the heated suspensions was added to nine mL of pre-reduced supplemented cooked meat-glucose broth (CMG; Fort Richard, Auckland, New Zealand) and incubated in an anaerobic chamber (Don Whitley Scientific, Bingley, UK) set at 35 °C, for 48 h. This treatment was termed as "heated, and enriched" (HE). HE cultures were centrifuged at 10,000× *g* for 15 min, and pellets were suspended in two mL of BD. A one mL preparation from each of the enriched suspensions was then serially diluted in BD and

plated in duplicates on egg yolk agar (EYA, Fort Richard, Auckland, New Zealand). Plates were incubated anaerobically at 35 °C in an anaerobic chamber for 24–48 h. This methodology was developed in our previous study [44] to specifically isolate *Clostridium* species.

All the colonies from both HD and HE plates were further subcultured on SBA to obtain pure cultures. HD cultures were further inoculated in tryptic soy broth (TSB; Fort Richard, Auckland, New Zealand) for genomic analysis and culture bank storage, whereas HE cultures were inoculated in TSB and thioglycollate broth (Fort Richard, Auckland, New Zealand) for genomic analysis and culture bank storage, respectively.

The number of anaerobic spore formers was not counted as the samples were enriched in the CMG medium. However, aerobic spore formers were counted to deduce the number of aerobic spores present in raw-milk samples.

2.5. Extraction of DNA

A boiled cell lysate and extracted genomic DNA were used for two different experiments. 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 10,000× *g* for 5 min. For Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR, this supernatant was used as the template DNA, whereas for amplification and sequencing of the 16S rRNA gene and identification of toxin genes, genomic DNA was used which was isolated using the Roche kit (Roche diagnostics, Manheim, Germany).

2.6. ERIC PCR Fingerprinting to Identify Unique DNA Patterns in Isolates for Further Analysis

All the spore-forming bacteria isolated from milk samples were subjected to ERIC PCR to obtain and identify genetic fingerprints (band patterns or ERIC profile) associated with all the isolates. Based on the different fingerprints or ERIC profiles, representatives (isolates) of each of the unique fingerprint groups were selected and examined for spoilage activity, presence and absence of toxin genes, and 16s rRNA gene sequencing. This selection was made to reduce the number of isolates for further testing.

For ERIC PCR, "ERIC 2" primer was used as described by [45] with the protocol of [46]. A total of 50 µL of the PCR mixtures contained 1 × Amplitaq Gold DNA Polymerases (Applied Biosystems, Melbourne, Australia), 1 µM of primer 5′-AAG TAA GTG ACT GGG GTG AGC-3′ (Thermo Fisher Scientific, Auckland, New Zealand) and 5 µL of the boiled lysate. PCR was carried out in a PTC-100™ Thermal Cycler (MJ Research Inc., Waltham, MA, USA) by following the protocol of [46]. Amplified products were observed on a 1.5% agarose gel (Fisher Scientific, Loughborough, UK), stained with 10 mg L^{-1} of ethidium bromide (Bio-Rad Laboratories, North Harbour, New Zealand), at 150 V for 4 h. Gel DocTM XR+ (Bio-Rad Laboratories, North Harbour, New Zealand) was used to visualise gels, and images of band patterns (fingerprints) were acquired using Image LabTM software version 3.0 (Bio-Rad Laboratories, North Harbour, New Zealand). Bio-Rad Quanti-One software (v. 4.5.2) was used to compare the fingerprints.

2.7. Amplification and Sequencing of 16S rRNA Gene

To identify isolated spore formers, amplification of the 16S rRNA gene was carried out using primer sequences, as mentioned in the previous research paper [47]. Each 50 µL reaction mix contained 1 × Amplitaq Gold DNA Polymerases (Applied Biosystems, Melbourne, Australia), 1 µM each of forward and reverse primers, and 5 µL of pure genomic DNA. PTC-100™ Thermal Cycler (MJ Research Inc., MA, USA) was used to run PCR with the following conditions: 93 °C for 3 min, 30 cycles each of 92 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, followed by a final extension at 72 °C for 3 min. PCR product of 1500 bp was visualised on a 0.8% ultrapure agarose (GibcoBRL) gel that was stained with 10 mg L^{-1} ethidium bromide (Bio-Rad). Amplified products were first purified using a Qiagen PCR purification kit (Qiagen, Bio-strategy Ltd., Auckland, New Zealand), and the products were sent for sequencing to Massey Genome Service, Palmerston North, New Zealand.

The 16S rRNA gene sequences were matched to the closest 16S rRNA sequence of the type strains with the help of RDP release 11 tool [48].

2.8. Detection of Toxin Genes Using PCR

Presumable isolates of *B. licheniformis*, *B. pumilus*, *B. cereus*, *B. mycoides* and *C. perfringens*, identified using 16S rRNA gene amplification and sequencing, were screened for different toxin genes using PCR. The lists of primers used in the study are shown in Tables 1–3.

Table 1. Primers used in PCR to detect toxin genes in *B. licheniformis* and *B. pumilus* isolates.

Primers and Target Genes	Sequence (5′-3′)	Product Size bp	Reference
*Lic*A-F	GTGCCTGATGTAACGAATG	735	[49]
*Lic*A-R	CACTTCCTGCCATATACC		
*Lic*B-F	TGATCAGCCGGCCGTTGTCT	904	[49]
*Lic*B-R	GGCGAATTGTCCGATCATGTCC		
*Lic*C-F	GCCTATCTGCCGATTGAC	1195	[49]
*Lic*C-R	TATATGCATCCGGCACCA		
*ces*B-F	GACAAGAGAAATTTCTACGAGCAAGTACAAT	635	[50]
*ces*B-R	GCAGCCTTCCAATTACTCCTTCTGCCACAGT		

Table 2. Primers used in PCR to detect toxin genes in isolates from *B. cereus* group.

Primers and Target Genes	Sequence (5′-3′)	Product Size bp	Reference
*hbl*A-F	ATT AAT ACA GGG GAT GGA GAA ACT T	237	[51]
*hbl*A-R	TGA TCC TAA TAC TTC TAG ACG CTT		
*hbl*C-F	CCT ATC AAT ACT CTC GCA ACA CCA AT	386	[51]
*hbl*C-R	TTT TCT TGA TTC GTC ATA GCC ATT TCT		
*hbl*D-F	AGA TGC TAC AAG ACT TCA AAG GGA AAC TAT	436	[51]
*hbl*D-R	TGA TTA GCA CGA TCT GCT TTC ATA CTT		
*nhe*A-F	ATT ACA GGG TTA TTG GTT ACA GCA GT	475	[51]
*nhe*A-R	AAT CTT GCT CCA TAC TCT CTT GGA TGC T		
*nhe*B-F	GTG CAG CTG TAG GCG GT	328	[51]
*nhe*B-R	ATG TTT TTC CAG CTA TCT TTC GCA AT		
*nhe*C-F	GCG GAT ATT GTA AAG AAT CAA AAT GAG GT	557	[51]
*nhe*C-R	TTT CCA GCT ATC TTT CGC TGT ATG TAA AT		
*ent*FM-F	CAA AGA CTT CGT AAC AAA AGG TGG T	290	[51]
*ent*FM-R	TGT TTA CTC CGC CTT TTA CAA ACT T		
*cyt*K1-F	ATC GGG CAA AAT GCA AAA ACA CAT	800	[51]
*cty*K1-R	ACC CAG TTT GCA GTT CCG AAT GT		
*ces*B-F	GACAAGAGAAATTTCTACGAGCAAGTACAAT	635	[50]
*ces*B-R	GCAGCCTTCCAATTACTCCTTCTGCCACAGT		

Table 3. Primers used in PCR to detect toxin genes in *C. perfringens* isolates.

Primers and Target Genes	Sequence (5′-3′)	Product Size bp
CPA-F	GTTGATAGCGCAGGACATGTTAAG	402
CPA-R	CATGTAGTCATCTGTTCCAGCATC	
CPB-F	ACTATACAGACAGATCATTCAACC	236
CPB-R	TTAGGAGCAGTTAGAACTACAGAC	
CPE-F	ACTGCAACTACTACTCATACTGTG	541
CPE-R	CTGGTGCCTTAATAGAAAGACTCC	
CPI-F	GCGATGAAAAGCCTACACCACTAC	317
CPI-R	GGTATATCCTCCACGCATATAGTC	

B. licheniformis NCTC 6346, *B. cereus* NCTC 11143, *B. pumilus* NCTC 8241, *Clostridium perfringens* NCTC 8237 (Type A), *C. perfringens* NCTC 8533 (Type B), *C. perfringens* NCTC 8081 (Type C), *C. perfringens* NCTC 8504 (Type D) and *C. perfringens* NCTC 8084 (Type E) were used as positive controls, in respective PCRs.

Detection of toxin genes in *Bacillus* species was carried out using different sets of primers and PCR conditions for *B. licheniformis*, *B. pumilus* and *B. cereus*.

Standard PCR reactions to detect *Lic*A, *Lic*B and *Lic*C genes encoding lichenysin A, B and C protein, respectively, in *B. licheniformis* and *B. pumilus* were carried out as described in a previous study [49], with few modifications. Representatives (isolates) from each ERIC type was tested for the presence and absence of toxin genes. The PCR reaction mixtures contained, 2 × AmpliTaq Gold DNA Polymerases (Applied Biosystems, Melbourne, Australia), 1 mM of primers (Table 1), ~100 ng of DNA, and a volume adjusted to 50 µL with distilled water. PCR programs used in this study were different for detecting *Lic*A/C and *Lic*B genes, respectively. To detect genes *Lic*A and *Lic*C, the program consisted of initial denaturation at 94 °C for 2 min, followed by 30 cycles each of 30 s at 94 °C, 30 s at 56 °C and 50 s at 72 °C and ending with a final extension for 5 min at 72 °C. To detect *Lic*B genes, a different program was used, with initial denaturation at 94 °C for 2 min, followed by consecutive 30 cycles of 30 s at 94 °C, 30 s at 64 °C and 50 s at 72 °C, and a final extension for 5 min at 72 °C. Ten microliters of the amplified products were analysed using electrophoresis on a 1.0% agarose gel. The presence and absence of the genes were recorded.

Similarly, PCR was carried out to detect haemolysin (*hbl*), non-haemolytic enterotoxin (*nhe*), enterotoxin (*ent*) and cytotoxin (*cyt*K) genes in isolates from the *B. cereus* group, using primers shown in Table 2 and PCR protocol by [51]. PCR protocol as described by [50] was used to detect the emetic/cereulide toxin-producing gene, *ces*B in isolates of *B. licheniformis*, *B. pumilus* and *B. cereus* group, however with some modifications. Reaction mixture of 50 µL contained 2 × of AmpliTaq Gold DNA Polymerases (Applied Biosystems, Melbourne, Australia), 1 mM of primers, ~100 ng of DNA, and volume adjusted with distilled water. The PCR program consisted of initial denaturation at 95 °C for 5 min, followed by 30 cycles of 30 s at 95 °C, 35 s at 65 °C, and 45 s at 72 °C, and a final extension for 7 min at 72 °C.

To detect different toxin genes in *C. perfringens* isolates, PCR conditions and primers (Table 3) as described by [52] were used, with modifications. In the present study, standard PCRs were conducted with single primer sets rather than a multiplex, as described in the previous study. PCRs were conducted using an MJ research thermal cycler, and the mixtures contained 2 × of AmpliTaq Gold DNA Polymerases (Applied Biosystems, Melbourne, Australia), 100 pmol of primers, ~100 ng of DNA template, and up to 50 µL of distilled water. The following PCR program was used in this experiment: initial denaturation for 5 min at 94 °C, followed by 30 cycles consisting of 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C and a final extension for 2 min at 72 °C. Ten microliters of the amplified products were visualised on a 1.5% agarose gel, stained with ethidium bromide, and the presence and absence of genes were recorded.

2.9. Toxin Production by Clostridium Perfringens

Production of different toxin types by *C. perfringens* isolates collected in this study was caried out using the Multiscreen ELISA kit (Bio-X Diagnostics, Rochefort, Belgium), as per the manufacturer recommendations. Briefly, *C. perfringens* isolates were grown in TSB (Fort Richard, Auckland, New Zealand) for 6 h under anaerobic conditions at 35 °C and were used undiluted in the ELISA reaction. A 100 µL aliquot of respective cultures was added to each of the wells of ELISA plates, covered with a lid, and incubated at 21 °C ± 3 °C for 1 h. Following incubation and washing with sterile water, a peroxidase-labelled conjugate was added. After a further wash, the chromogen (tetramethylbenzidine) was added, which was catalysed in the presence of conjugate and was visualised using a spectrophotometer after the reaction had been stopped with phosphoric acid. For each test well, a corresponding negative control containing non-specific immobilised antibodies was used. Positive controls consisting of pure solutions of the target antigens (*C. perfringens*

surface protein) were simultaneously assessed during each ELISA reaction. Each experiment was conducted in triplicate and intensity of the colour change was observed and corresponded to a low (+), intermediate (++), and high (+++) level of toxin production.

2.10. Screening for Spoilage Potential

Selective media were used to examine the spoilage potential of aerobic and anaerobic spore-forming bacteria identified using 16S rRNA gene sequencing, as described by [53]. Briefly, colonies from each isolate were cultured on selective media to visualise enzymatic action (a clear zone around the bacterial growth), e.g., milk IDF agar (MA) for detection of proteolysis, and tributyrin agar (TA) for detection of lipolytic activity. All media were procured from Fort Richard, New Zealand. Each isolate was cultured on 3 plates of each of the selective media to determine the reproducibility of the spoilage activity. Depending on the clear zone, results are depicted as low- (small clear zones and medium size zones) to high- (big clear zones that will clear the whole plate) spoilage enzyme producers.

3. Results

3.1. Enumeration of Aerobic Spore-Forming Bacteria Present in Raw Milk

Aerobic and anaerobic mesophilic spore-forming bacteria were isolated and identified from raw-milk samples from the farms, except from Farm 3 during summer where bacterial growth was not detected on any growth medium. In total, 1680 aerobic and 920 anaerobic isolates were cultured. Of these isolates, representatives from different ERIC profile groups were chosen for further analyses. Enumeration of only aerobic spore-forming bacteria from HD samples was carried out, whereas due to enrichment the enumeration of anaerobic spore-forming bacteria in HE samples was not conducted, but the diversity of anaerobic spore-forming bacteria was investigated. In summer, a low count of aerobic spore formers was obtained from raw milk collected from Farm 2 (1.30 log CFU mL^{-1}), whereas no colonies were detected (or under the detectable range) from raw milk obtained from Farm 3. However, a higher count of spore-forming bacteria (2.4 log CFU mL^{-1} and 2.5 log CFU mL^{-1}) was obtained in summer from Farms 1 and 4, respectively. Milk samples from Farm 4 did not show any difference in the spore counts between summer and winter (2.53 log CFU mL^{-1} in summer and 2.50 log CFU mL^{-1} in winter). An increase in numbers was observed during all the winter sampling, where 2.51 log CFU mL^{-1}, 2.11 log CFU mL^{-1} and 2.38 log CFU mL^{-1} of spore-forming bacteria were detected in raw milk from Farms 1, 2 and 3, respectively. The level of detection of the methodology used in this study was 20 CFU mL^{-1}.

3.2. ERIC Profiles and 16S rRNA Sequencing

ERIC PCR was used to differentiate between isolates cultured from all raw-milk samples to select unique representatives for sequencing and other experiments. In comparison to type strains, the maximum identity for the 16S rRNA gene sequences found in this study ranged from 96 to 100% (Supplementary Table S1). Therefore, species were identified as the closest related taxonomically described species. It should be noted that isolates included in this table have been grouped based on different ERIC profiles obtained (irrespective of same genus and species from different farms and season), and a representative from each ERIC profile groups was selected for sequencing and future analysis. It was observed that *B. licheniformis* had the largest number of ERIC fingerprinting patterns as shown in the Supplementary Table S1.

3.3. Different Aerobic Spore-Forming Bacteria Present in Raw-Milk Samples

An increase in the diversity of aerobic spore formers was observed in winter as compared to summer (Figure 1). From the four farms, a total of 12 different mesophilic *Bacillus* spp., 7 different *Paenibacillus* spp. and 4 other aerobic spore-forming bacteria (identified using the 16S rRNA gene closest taxonomically described species) were isolated (Figure 1 and Supplementary Table S2). The common species of *Bacillus* isolated from

milk were those that matched with 16S rRNA sequences of *B. licheniformis* (maximum identity 100%) followed by that of *B. pumilus* (maximum identity 99–100%) and *B. clausii* (maximum identity 99–100%). Other *Bacillus* spp. included *B. mycoides*, *B. simplex*, *B. kochii*, *B. subtilis*, *B. circulans*, and *B. altitudinis* with maximum identity ranging between 98 and 99.6%. *B. cereus*-like sequence types with a maximum identity of 99% were also detected in the raw milk collected from Farm 3 in winter. Sequence types similar to *B. licheniformis* were isolated in both seasons and from all farms, except for from Farm 3, and showed the maximum ERIC types per farm (Supplementary Table S1). *Paenibacillus* isolates (maximum identity ranging between 97 and 100%) were detected from all farms during winter and from Farms 1 and 4 in summer. *Lysinibacillus fusiformis* was also detected from Farm 4 in both summer and winter (Supplementary Table S1).

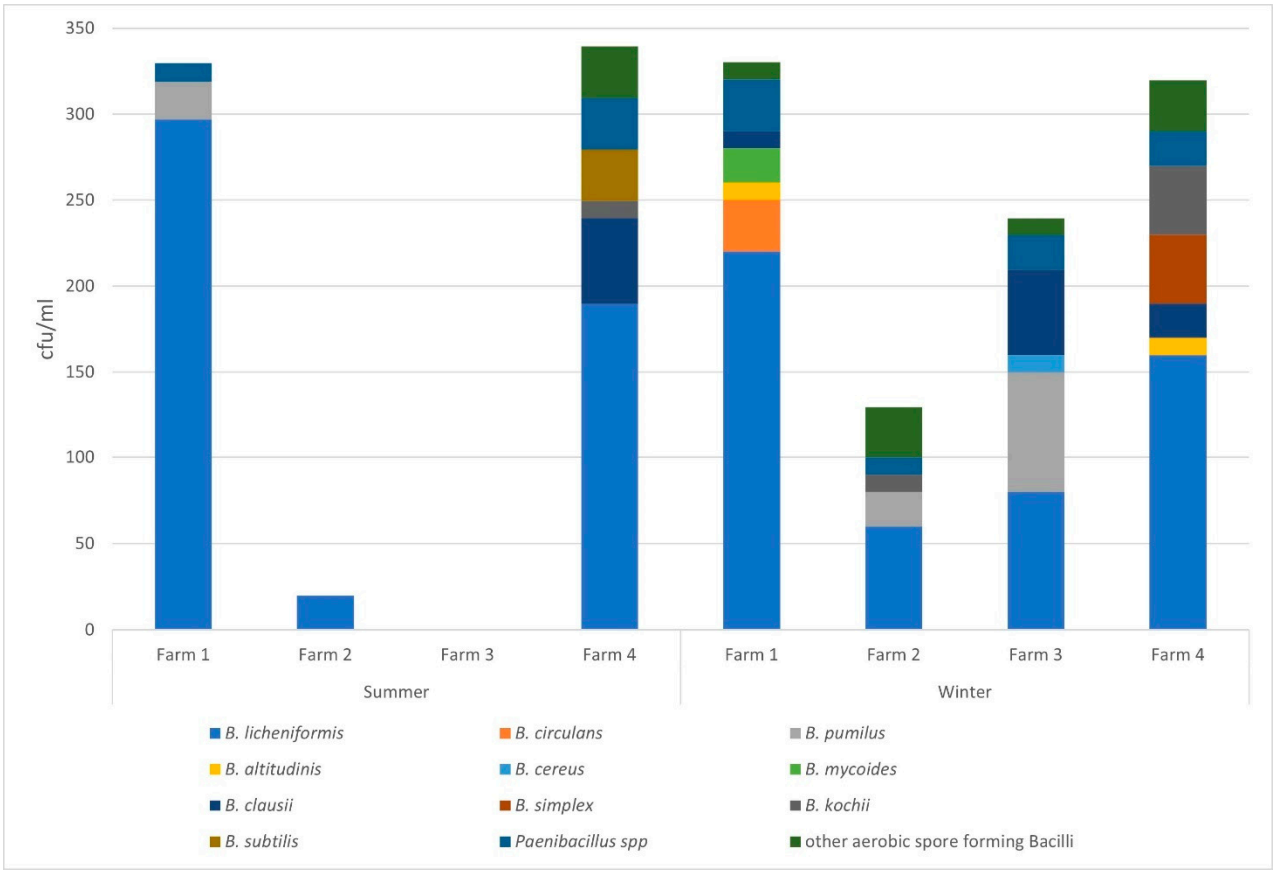

Figure 1. Number of aerobic spore-forming bacteria isolated from raw milk from each farm during summer and winter. Aerobic spore-forming bacteria were under a detectable range from the Farm 3 sample in summer.

3.4. Different Anaerobic Spore-Forming Bacteria Present in Raw Milk

In total, six different mesophilic *Clostridium* spp. (identified using the 16S rRNA gene closest taxonomically described species) were isolated from all farms. Sequence types similar to *C. cochlearium* (identity between 99.8 and 100%) and *C. sporogenes* (identity 100%) were isolated from Farm 1 during summer and winter, respectively. Interestingly, anaerobic spore-forming bacteria were not detected or under a detectable level from Farms 2 and 3 in summer, whereas *C. sulfidigenes* (identity between 98.9 and 99.3%) was isolated from Farm 2, and *C. perfringens* (identity 100%) from Farms 3 and 4, only in winter. *C. beijerinckii* (identity between 99 and 99.5%) and *C. sartagoforme* (identity between 98 and 99.0%) were isolated from raw milk collected from Farm 4 in summer. Overall, *Clostridium* spp. were found to be less diverse on the four farms compared to *Bacillus* spp.; however, this could be due to culture enrichment undertaken for *Clostridium* spp. isolation.

3.5. Detection of Toxin Genes in Bacillus spp. and C. perfringens

Representatives (isolates) from each unique ERIC profile of *Bacillus* spp. and *C. perfringens* were tested for the presence and absence of different toxin genes using standard PCR with primer pairs specific to the target genes. Results showed that none of the *B. licheniformis* and *B. pumilus* isolates harboured genes for cereulide/emetic toxins. Most of the *B. licheniformis* isolates were positive for lichenysin A, B and C coding genes (*Lic*A, *Lic*B and *Lic*C); however, some isolates were found to be negative for either of these genes (in Farm 1) or for all the three genes (1 isolate from Farm 4) (Table 4). All the tested isolates of *B. pumilus* were found to be negative for *Lic*A, *Lic*B and *Lic*C genes. Presumptive *B. cereus* and *B. mycoides* isolates did not harbour *ces*B genes for emetic toxin. However, they were found to harbour some hemolysin genes of the *hbl* operon; *B. cereus* isolate harboured *hbl*D gene, and *B. mycoides* isolates harboured *hbl*A and *hbl*B genes. *B. mycoides* isolates also harboured gene coding for non-haemolytic enterotoxin (NHE); however, it was positive for only *nhe*A and *nhe*B genes and not *nhe*C. The *B. cereus* isolate also harboured the enterotoxin gene, *ent*FM. None of the isolates of *B. cereus* and *B. mycoides* obtained in this study were positive for *cyt*K1 genes (Table 5).

Table 4. Presence and absence of toxin genes in *B. licheniformis* and *B. pumilus.*

Isolates	***Lic*A**	***Lic*B**	***Lic*C**	***ces*B**
Farm 1, Summer 1	−	−	+	−
Farm 1, Summer 5	+	+	+	−
Farm 1, Summer 7	+	−	+	−
Farm 1, Summer 8	+	−	+	−
Farm 1, Summer 9	−	+	+	−
Farm 1, Summer 11	+	−	+	−
Farm 1, Summer 12	+	−	−	−
Farm 1, Summer 13	+	−	−	−
Farm 1, Summer 2 (*B. pumilus*)	−	−	−	−
B. licheniformis +ve control	+	+	+	−
B. pumilus -ve control	−	−	−	−
Farm 1, Winter 1	+	+	+	−
Farm 1, Winter 3	+	+	+	−
Farm 1, Winter 4	+	+	+	−
Farm 1, Winter 8	+	+	+	−
Farm 1, Winter 9	+	+	+	−
Farm 1, Winter 19	+	+	+	−
Farm 1, Winter 25	+	+	+	−
Farm 1, Winter 26	+	+	+	−
Farm 1, Winter 27	+	+	+	−
B. licheniformis +ve control	+	+	+	−
Farm 2, Summer 1	+	+	+	−
Farm 2, Summer 2	+	+	+	−
Farm 2, Winter 2	+	+	+	−
Farm 2, Winter 9 (*B. pumilus*)	−	−	−	−
B. licheniformis +ve control	+	+	+	−
B. pumilus -ve control	−	−	−	−
Farm 3, Winter 1	+	+	+	−
Farm 3, Winter 9	+	+	+	−
Farm 3, Winter 10	+	+	+	−
Farm 3, Winter 3 (*B. pumilus*)	−	−	−	−
B. licheniformis +ve control	+	+	+	−
B. pumilus -ve control	−	−	−	−
Farm 4, Summer 1	+	+	+	−
Farm 4, Summer 5	+	+	+	−
Farm 4, Summer 29	−	−	−	−
B. licheniformis +ve control	+	+	+	−

Table 4. *Cont.*

Isolates	*Lic*A	*Lic*B	*Lic*C	*ces*B
Farm 4, Winter 1	+	+	+	−
Farm 4, Winter 4	+	+	+	−
Farm 4, Winter 5	+	+	+	−
Farm 4, Winter 15	+	+	+	−
Farm 4, Winter 31	+	+	+	−
B. licheniformis +ve control	+	+	+	−

*Lic*A—gene encoding Lichenysin A protein. *Lic*B—gene encoding Lichenysin B protein. *Lic*C—gene encoding Lichenysin C protein. *ces*B—gene encoding for emetic toxin. (+) Presence of the toxin gene; (−) absence of the toxin gene.

Table 5. Presence and absence of toxin genes in *B. mycoides* and *B. cereus*.

	Isolates			
Toxin Genes	***B. cereus* NCTC 11143 (Positive Control)**	**Farm 1 Winter 11 (*B. mycoides*)**	**Farm 1 Winter 12 (*B. mycoides*)**	**Farm 3 Winter 17 (*B. cereus*)**
*hbl*A	+	+	+	−
*hbl*C	+	+	+	−
*hbl*D	+	−	−	+
*nhe*A	+	+	+	−
*nhe*B	+	+	+	−
*nhe*C	+	−	−	−
*ent*FM	+	−	−	+
*cyt*K1	+	−	−	−
*ces*B	+	−	−	−

*hbl*A: gene encoding hemolysin A protein; *hbl*C: gene encoding hemolysin protein C; *hbl*D: gene encoding hemolysin D protein; *nhe*A: gene encoding non-hemolytic enterotoxin protein A; *nheB:* gene encoding non-hemolytic enterotoxin protein B; *nhe*C: gene encoding non-hemolytic enterotoxin protein C; *ent*FM: gene encoding enterotoxin FM protein; *cyt*K1: gene encoding cytotoxin K1 protein; *ces*B: gene encoding emetic toxin protein; (+) presence of the toxin gene; and (−) absence of the toxin gene.

In this study, *C. perfringens* were isolated only during winter and were found to be positive for *cpa*, *cpb* and *etx* (alpha, beta, and epsilon) genes. All the 27 isolates of *C. perfringens* harboured *cpa* genes, seven also harboured *etx* genes; three isolates harboured *cpb* genes; and three isolates harboured *cpa*, *cpb*, as well as *etx* genes. None of the isolates were found to harbour genes for iota. Isolates possessing only alpha genes, i.e., Type A *C. perfringens* were found to be most prevalent (20 isolates), followed by Type D (positive for both alpha and epsilon toxins; 4 isolates). ELISA results showed the production of alpha, beta and epsilon toxins; however, there was one isolate which was positive for epsilon genes but did not produce the protein (Table 6). Type B *C. perfringens* were also isolated (3 isolates) from Farm 4's raw milk. To the best of our knowledge, this is the first study that detected Type B *C. perfringens* in raw milk.

Table 6. Presence of different toxin genes and production of respective proteins in *C. perfringens* strains isolated from raw-milk samples.

Isolate	**PCR**				**ELISA**			**Toxinotype**
	cpa	***cpb***	***etx***	***iap* and *ibp***	**Alpha**	**Beta**	**Epsilon**	
Farm 3, Winter 1	+	−	+	−	++	−	++	D
Farm 3, Winter 2	+	−	+	−	++	−	++	D
Farm 3, Winter 3	+	−	−	−	++	−		A
Farm 3, Winter 4	+	−	+	−	++	−		D[#]
Farm 3, Winter 5	+	−	+	−	++	−	++	D
Farm 3, Winter 6	+	−	−	−	++	−		A
Farm 3, Winter 7	+	−	−	−	++	−		A

Table 6. *Cont.*

Isolate	PCR				ELISA			Toxinotype
	cpa	*cpb*	*etx*	*iap* and *ibp*	Alpha	Beta	Epsilon	
Farm 3, Winter 8	+	−	−	−	++	−		A
Farm 3, Winter 9	+	−	−	−	++	−		A
Farm 3, Winter 10	+	−	−	−	++	−		A
Farm 3, Winter 11	+	−	−	−	++	−		A
Farm 3, Winter 12	+	−	−	−	++	−	−	A
Farm 3, Winter 13	+	−	−	−	++	−	−	A
Farm 3, Winter 14	+	−	−	−	++	−	−	A
Farm 3, Winter 15	+	−	−	−	++	−	−	A
Farm 4, Winter 1	+	−	−	−	++	−		A
Farm 4, Winter 2	+	−	−	−	++	−		A
Farm 4, Winter 3	+	−	−	−	++	−		A
Farm 4, Winter 4	+	−	−	−	++	−		A
Farm 4, Winter 5	+	−	−	−	++	−		A
Farm 4, Winter 6	+	−	−	−	++	−		A
Farm 4, Winter 7	+	−	−	−	++	−		A
Farm 4, Winter 8	+	−	−	−	++	−		A
Farm 4, Winter 9	+	−	−	−	++	−		A
Farm 4, Winter 10	+	+	+	−	++	++	++	B
Farm 4, Winter 11	+	+	+	−	++	++	++	B
Farm 4, Winter 12	+	+	+	−	++	++	++	B

(+) Presence of toxin genes; (−) absence of toxin genes; (++; intermediate) intensity of colour change with the production of the protein and an indication of toxin production; different alphabets indicate toxin types of *C. perfringens*; and D# gene for epsilon protein was present, but protein was not produced. This isolate was typed on the basis of the genes present.

3.6. Spoilage Activity

Representatives (isolates) of aerobic spore-forming bacteria, from each unique ERIC group and identified using 16s rRNA gene sequencing (n = 72) were selected and examined qualitatively for their spoilage potential. Most of the isolates identified were either proteolytic or lipolytic, or both, from lower to higher potential (colour code depicts small clearing to high clearing zones; Table 7). Of the 72 isolates, 56 (77.7%) produced proteases, 49 (68%) produced lipases, 46 (63.8%) produced both enzymes and 12 (16.6%) did not produce proteases or lipases. *B. licheniformis* isolates were predominantly producing both the spoilage enzymes, followed by *B. mycoides*. Other aerobic spore formers were also found to produce spoilage enzymes, such as *Paenibacillus* and *Lysinibacillus* species. Anaerobic spore-forming bacteria isolated in this study were not subjected to spoilage testing, except *C. beijerinckii*. All the isolates of this species were found to produce proteases (personal communication).

Table 7. Spoilage activity of different aerobic spore-forming bacteria isolated from raw milk.

		Spoilage Activity		
Farm, Season	Isolate Number	Proteolytic	Lipolytic	Bacteria
Farm 1, Summer	1			*B. licheniformis*
	2			*B. pumilus*
	4			*P. marinisediminis*
	5			*B. licheniformis*
	7			*B. licheniformis*
	8			*B. licheniformis*
	9			*B. licheniformis*
	11			*B. licheniformis*
	12			*B. licheniformis*
	13			*B. licheniformis*
Farm 1, Winter	1			*B. licheniformis*
	2			*P. xylanexedens*

Table 7. *Cont.*

		Spoilage Activity		
Farm, Season	**Isolate Number**	**Proteolytic**	**Lipolytic**	**Bacteria**
Farm 1, Winter	3			*B. licheniformis*
	4			*B. licheniformis*
	6			*B. clausii*
	8			*B. licheniformis*
	9			*B. licheniformis*
	10			*P. peoriae*
	12			*B. mycoides*
	14			*B. altitudinis*
	17			*B. circulans*
	19			*B. licheniformis*
	24			*S. silvestris*
	25			*B. licheniformis*
	26			*B. licheniformis*
	27			*B. licheniformis*
	28			*B. circulans*
Farm 2, Summer	1			*B. licheniformis*
	2			*B. licheniformis*
Farm 2, Winter	1			*B. pumilus*
	2			*B. licheniformis*
	8			*B. kochii*
	9			*B. pumilus*
	10			*S. silvestris*
	11			*B. rigui*
	12	NT	NT	*S. luteola*
	13			*P. polymyxa*
Farm 3, Winter	1			*B. licheniformis*
	2			*B. clausii*
	3			*B. pumilus*
	6			*B. pumilus*
	8			*Ornithinibacillus*
	9			*B. licheniformis*
	10			*B. licheniformis*
	11			*B. licheniformis*
	12			*B. licheniformis*
	13			*B. pumilus*
	17			*B. cereus*
	22			*B. clausii*
	23			*P. amylolyticus*
Farm 4, Summer	1			*B. licheniformis*
	5			*B. licheniformis*
	6			*B. subtilis*
	17			*B. clausii*
	22			*P. timonensis*
	25			*L. fusiformis*
	27			*B. kochii*
	29			*B. licheniformis*
Farm 4, Winter	1			*B. licheniformis*
	3			*B. altitudinis*
	4			*B. licheniformis*
	5			*B. licheniformis*
	9			*B. simplex*
	15			*B. licheniformis*
	22			*B. kochii*
	24			*L. fusiformis*
	25			*P. cookii*

Table 7. *Cont.*

Farm, Season	Isolate Number	Spoilage Activity		Bacteria
		Proteolytic	Lipolytic	
Farm 4, Winter	26			*B. clausii*
	29			*B. simplex*
	30			*B. pocheonensis*
	31			*B. licheniformis*
	32			*P. lactis*

The colour gradient depicts the level of spoilage activity by different aerobic spore-forming bacteria. high level of spoilage activity (wide clearing zone); intermediate level of spoilage activity (low to medium size clearing zone); no spoilage activity detected; and NT not tested.

4. Discussion

Spoilage and pathogenic spore-forming bacteria responsible for the safety and quality of milk products are found to be present in the farm environment [54–57]. These bacteria circulate in the farm environment and enter the raw milk via milking of dirty teats and udders [5,6,29]. In this pilot study, raw milk was collected from four farms of the Manawatu region, over two seasons. The results showed low aerobic spore counts in raw milk (between 1.3 and 2.5 log CFU mL^{-1}), compared with other findings by [14,58], where Martin and other researchers reported a higher spore counts in the raw milk within the range of 3.5–5.1 log CFU mL^{-1}. The lower spore counts found in this study could be due to appropriate farm-management systems and cleaning regimes of the animals followed by the farmers. According to a study by [59], the aerobic spore counts of raw milk during the winter was higher (average 2.06 log CFU mL^{-1}) than in summer (1.08 log CFU mL^{-1}) in the state of South Dakota, United States. Similar results were obtained in the present study, where relatively higher spore counts were obtained in the winter as compared to summer. This could be due to higher rainfall in New Zealand during winter that may increase the contamination of udders with soil harbouring spores which can further contaminate the raw milk during milking. Also, a high number of spores can be associated with adverse temperature conditions during winter that can favour the sporulation of vegetative forms of spore-forming bacteria [59]. A similar number of aerobic spore-forming bacteria in both seasons from Farm 1 and 4 was obtained; however, raw milk from these farms had more diverse spore-forming bacteria in winter. Interestingly, spore-forming bacteria, both aerobic and anaerobic, were not detected from Farm 3 raw-milk samples, which indicates a very low level of spores present in the milk and signifies good farm-management practices to keep the spore-levels low. This may also indicate that the level of detection of the methodology used in this study may not be sufficiently sensitive to detect extremely low numbers of spores (level of detection is 20 CFU mL^{-1}). Considering only the aerobic spore-forming bacterial counts in this study, it can be expected that the raw milk from these farms is of better microbiological quality, which when pasteurised may have better average shelf life. This indicates good farm management is being followed in these farms.

Although, the spore counts were less, a diverse range of aerobic spore-forming bacteria were identified and isolated in this study. Of these, some spore formers had the potential to spoil milk and produce toxins. It should be noted that to obtain a wider knowledge of the presence of these spore-forming bacteria in raw milk, more samples should be collected from all over New Zealand. However, despite a lower number of samples and geographical and methodology differences, diversity of aerobic *Bacillus* species on the four farms was found to be similar to that seen globally [53,60–62]. *B. licheniformis* was the most common spore-forming bacteria (showing high sequence similarity to the type strain ATCC 14580) isolated from raw milk collected from all the dairy farms included in this study, irrespective of seasons. Some of these isolates were able to grow at a higher temperature of 55 °C (personal communication), also described by [63]. Reports elsewhere have shown *B. licheniformis* to be one of the most prevalent *Bacillus* species in raw milk and along the dairy processing chain [62,64,65]. Although, *B. licheniformis* is not considered an

important human pathogen, this bacterium can cause spoilage of milk and dairy products, affecting the organoleptic and functional properties of milk and dairy products [17,66]. In the present study, all the isolates of *B. licheniformis* tested were found to produce proteolytic and lipolytic enzymes, indicating their potential to spoil milk. However, some studies have identified *B. licheniformis* to be foodborne pathogens capable of producing diarrhoeal enterotoxin [33] in the reconstituted infant milk formula [67]. In the present study, none of the *B. licheniformis* isolates harboured genes for diarrhoeal or emetic toxins. The majority of the isolates harboured genes for lichenysin protein, which can be responsible for foodborne illness [33,34]. However, the presence of toxin genes in these isolates does not suggest the production of the toxin protein. The production of these compounds depends on multiple factors including lichenysin synthetase, transcription rate, as well as environmental conditions [68,69]. Future studies need to be carried out to prove the production of lichenysin by these isolates. Following *B. licheniformis*, another species found to be dominant in the raw-milk samples was *B. pumilus,* which has also been previously detected in raw milk and dairy products [17,53]. These bacteria can be pathogenic as well as associated with spoilage of dairy through the production of lipase enzymes [17,35]. However, in the present study, none of the *B. pumilus* isolates harboured genes for toxins, but as reported in other studies [17,66], produced lipase enzymes indicating their potential to produce off-flavours in milk. It has been reported that the most common aerobic mesophilic spore-forming species found in raw milk are *B. licheniformis*, *B. pumilus*, and *B. subtilis* [70–72], whereas the most common psychrotolerant species is *B. cereus* [70]. In this study, *B. licheniformis* was isolated from all the raw-milk samples, from both seasons, similar to what was reported by [59]. *B. pumilus* was isolated from one farm in summer and two farms in winter, whereas *B. subtilis* was isolated from only one farm in summer. *B. cereus*, an important foodborne pathogen, has been detected in a dairy farm environment, as well as in milk [73,74]. This bacterium (according to 16s rRNA sequencing, with 99% similarity) was detected in only one occasion from one raw-milk sample in this study. However, whole genome sequencing should be conducted to verify if this isolate was *B. cereus* and not other species from the *B. cereus* group [75], and this is in the scope of future research of this study. Alongside producing toxins, *B. cereus* is also known to spoil milk and milk products, causing bitterness and off-flavours [4]. In this study, the presumptive *B. cereus* isolate was found to produce proteases but not lipases which indicates that spoilage activity can be strain dependent.

This isolate did not harbour genes for the cereulide toxin (*ces A/B*) but confirmed the presence of *hblD* gene encoding of one of the lytic proteins of diarrhoeal toxin hemolysin BL (HBL). Borge and co-researchers reported that most of the dairy-product-related *B. cereus* strains in their study possessed *nhe* and *hbl* genes and were rarely cereulide producers [76,77]. Similarly, another study reported a low prevalence of emetic *B. cereus* (1.5%; out of 5668) from milk and products [78]. Moreover, for complete activity of diarrhoeal toxin, the presence of two other genes *hbl*A and *hbl*C along with *hbl*D are required [79,80], which were missing in the *B. cereus* isolated in the present study. The isolate was positive for the *ent*FM gene, which encodes a putative cell wall peptidase and is one of the pore-forming enterotoxins secreted by toxigenic *B. cereus* [81]. These peptidases are responsible for virulence, adhesion of the bacteria to the gut and biofilm formation [82]. Even though the strain isolated in this study is positive for the *ent*FM gene, it does not confirm the production of this toxin. It has also been reported that the prevalence of *ent*FM toxin genes varies among different *B. cereus* strains [83,84]. *B. cereus* strains harbouring *ent*FM genes have also been isolated from powdered infant formula, cheeses and milk powder [77,85–87]. The present study also isolated psychrotolerant *B. mycoides* that are able to grow at 5 °C and have been previously identified from soil, whole-milk powder and powdered infant-formula milk [88–91]. Although *B. mycoides* is not a known pathogen, a human case report has demonstrated its association with a rare bloodstream infection [92]. In the present study, two isolates that were identified as *B. mycoides* were screened for the presence of toxin genes (Table 2). None of the isolates harboured *ent*FM, *cyt*K1 and *ces*B genes; however, they were found to harbour *hbl*A, *hbl*C, *nhe*A and *nhe*B genes encoding some components of the

diarrhoeal toxins, hemolysin BL (Hbl) and non-hemolytic enterotoxin (Nhe), respectively. As mentioned above, for complete activity of the diarrhoeal toxins, the presence of all the three genes' coding for respective proteins are required [79,80,93]. Genes *hbl*D and *nhe*C of the respective operons were absent in *B. mycoides* isolates, indicating no toxin production by these isolates. Limited knowledge is available of the presence of genes encoding these toxins and their production by *B. mycoides*; hence, further studies need to be carried out to screen more *B. mycoides* for different toxin production. In terms of their spoilage potential, *B. mycoides* isolated in this study were found to be highly proteolytic and lipolytic, indicating potential of these isolates to spoil milk and dairy products. These results were, contrary to what was reported by [66], where none of the *B. mycoides* isolates were proteolytic or lipolytic in nature. This suggests that spoilage activity may be strain or isolate dependent, which will require further investigation.

Other than B. cereus, *B. licheniformis* and *B. pumilus*, *Paenibacillus* species can spoil raw, as well as processed, milk. These are psychrotolerant spore-forming bacteria that can grow at refrigeration temperatures and can negatively impact the quality of dairy products [54,94]. These bacteria have been detected in the present study and some species such as *P. polymyxa* from Farm 2 and *P. amylolyticus* from Farm 3 during winter were found to possess mild proteolytic and lipolytic characteristics, whereas *P. lactis* isolated from Farm 4 in winter showed strong proteolytic and lipolytic activities. Although in low numbers, these spore-forming bacteria have been isolated from raw milk and milk products elsewhere and have been associated with spoilage [66,95–98]. *Lysinibacillus fusiformis* is another spore-forming bacterium which has been isolated from raw milk in a previous study [99]. In the present study, two isolates of *L. fusiformis* were isolated from Farm 4 raw-milk samples, one from summer and one from winter. Both the isolates showed proteolytic activity; however, the winter isolate produced both protease and lipase enzymes, similar to what was reported earlier [17].

It should be noted that spoilage potential or toxin production may differ from strain to strain of the bacteria; hence, for future research all the isolated colonies/strains should be included to determine their spoilage potential and toxin production. Moreover, a larger number of isolates should be collected to deduce prevalence of toxigenic spore-forming bacteria. *Clostridium* species are abundant in soil and its presence in feed, especially in silage, is a common source of raw-milk contamination, where spores from silage can be transferred via faecal and soil contamination of the udder and eventually contaminating milk during milking [6,100]. A group from Northern Italy investigated raw milk for the presence of *Clostridium* and found that *C. tyrobutyricum* was abundant in spring, *C. butyricum* and *C. sporogenes* in winter and *C. beijerinckii* abundant in summer [24]. Similarly, in the present study, *C. sporogenes* were isolated in winter (Farm 1) and *C. beijerinckii* in summer (Farm 4), and isolates of *C. beijerinckii* were found to produce protease enzyme (personal communication). These bacteria, occur in raw milk and have been found to be associated with butyric acid fermentation and have potential to cause late blowing defects [101,102]. However, *C. tyrobutyricum*, considered as the principle causative agent of late blowing in cheeses [28,103], and *C. butyricum*, associated with butyric acid fermentation and also late blowing in cheeses, were not detected in any of the raw-milk samples investigated in the present study. None of the anaerobic spore-forming bacteria were detected (or were under detectable numbers) from Farms 2 and 3 in summer, indicating a very low level of spores present in the milk which did not germinate even after enrichment for 2 days.

C. perfringens is considered significantly important from a food safety perspective due to the ability of some strains to induce illness in humans [104]. Some strains have also been recognised as the causative agents of mastitis in ruminants [105].

Raw milk and dairy products have been implicated as a source of *C. perfringens* for a very long time [106–109]. In the present study, *C. perfringens* were isolated in winter from raw-milk samples collected from Farms 3 and 4. *C. perfringens* have been detected in Australian dairy farms, specifically from milk filters, faeces and soil [41]. *C. perfringens* can be of five Types, A, B, C, D and E, depending on the four major toxins, namely alpha (CPA), beta (CPB), epsilon (ETX) and iota (ITX) toxin types produced by the isolates [110]. The

alpha toxin coded by *cpa* gene is the major pathogenicity factor and is linked to gas gangrene in humans and necrotising enteritis in poultry, whereas beta toxin coded by *cpb* is the second major toxin that is responsible for necrotic enteritis in cattle, and lambs [111–113]. The epsilon toxin coded by *etx*, is linked with enterotoxaemia in sheep and goats, however, less frequently in cattle, and the iota toxin, coded by *iap* and *ibp*, was found to be associated with enterotoxaemia in calves, lambs and rabbits [112,114]. In the present study, mainly *C. perfringens* Type A was detected (20/27 isolates tested), on the basis of the presence of the *cpa* gene and the production of CPA toxin in these isolates. This is the most frequently isolated type in human, animal, or environmental samples, which indicates that these isolates may have entered the raw milk (in our study) from the farm environment, possibly including by animals [100,115,116]. Four out of twenty-seven isolates tested were designated to be *C. perfringens* Type D, as they produced both CPA and epsilon toxins (*cpa* and *etx* positive), which is known to cause enterotoxaemia in small ruminants. Studies have shown *C. perfringens* Type A to be the predominant type in milk, with one study showing the presence of Type D along with Type A in caprine milk [117], whereas [118] reported Type A and C to be more prevalent in bovine milk. The present study showed the presence of Type B *C. perfringens* in raw milk (3/27 isolates) that carried *cpa*, *cpb* and *etx* genes and also produced respective proteins. *C. perfringens* Type B are mostly associated with necrotic enteritis in cattle and lambs, and not much knowledge is available on its presence in raw milk. To the best of our knowledge, this will be the first study to report the presence of Type B *C. perfringens* in raw milk. In this study, none of the tested *C. perfringens* isolates carried genes for the iota toxin found in Types C and/or E. Since, the isolation method included enrichment, the number of isolates of each toxinotypes cannot depict the true prevalence, and there is a possibility that some isolates are clones.

This study also detected and isolated two other *Clostridium* species, *C. sulfidigenes* and *C. sartagoforme,* in raw-milk samples from Farm 2 and 4, respectively. *C. sulfidegenes* are proteolytic in nature [119], whereas *C. sartagoforme* have been found to produce acetic acid, lactic acid and butyric acid as part of their metabolism [120]. This indicates their potential to spoil milk and milk products; however, only limited knowledge is available on their presence in milk and dairy products and their possible role in milk spoilage [121].

The data presented in this study indicate the presence of different spore-forming bacteria which include pathogenic and spoilage-associated species in raw milk. Spore-forming bacteria of *Bacillus* and related genera, and of *Clostridium* spp., are key contaminants of the dairy food chain. They can be present in the farm environment from where spores can be transferred to raw milk through dirty and contaminated udders. There is always a possibility that if proper farming practices are not carried out, spores may enter from the environment into raw milk and can cause safety and quality challenges. Processing milk to reduce spore contamination in dairy plants is expensive, time consuming and labour intensive. Therefore, it is imperative to reduce the risk through the implementation of good on-farm management procedures. The use of high-quality silage, appropriate cleaning of milking equipment, stringent udder and teat cleaning prior to milking are gold standards of a good farming practice. Importantly, the reduction or elimination of spore-forming bacteria can result in better shelf life of pasteurised milk and higher quality of products. Considering the number of aerobic spore-forming bacteria in this study, the quality of the raw milk from different farms appeared to be of good microbiological quality.

Single intervention or processing may not be helpful in destroying all spore types. Hence, the diversity of the spore-forming bacteria described in this work, could aid in forming the basis for elaborative and effective preventive measures to reduce the contamination of raw, as well as processed, milk products. More effective interventions can be designed by understanding and determining the characteristics of different spore-forming bacteria present in raw bulk-tank milk.

5. Conclusions

This pilot study reported relatively low levels of contamination of raw milk with spore-forming bacteria. However, the diversity of spore formers obtained in this study was high. Some isolates were capable of producing spoilage enzymes and also harboured genes for different toxins. The results obtained in this study will help in designing effective interventions to inactivate different ranges of spore-forming bacteria to obtain good quality raw milk and products.

6. Future Directions

Future studies will be carried out to determine seasonal variation in number of spore-forming bacteria, as well as their diversity in a bigger sample size and from more farms, and will also develop and test intervention strategies based on the outcomes of this study to reduce contamination of milk.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/dairy4040045/s1, Table S1: Diversity of aerobic spore-forming bacteria present in raw milk obtained from four farms over two seasons; Table S2. Presence of different aerobic spore forming bacteria in raw milk from each farm over two seasons

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