Rapid identification of dairy mesophilic and thermophilic sporeforming bacteria using DNA high resolution melt analysis of variable 16S rDNA regions

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Due to their ubiquity in the environment and ability to survive heating processes, sporeforming bacteria are commonly found in foods. This can lead to product spoilage if spores are present in sufficient numbers and where storage conditions favour spore germination and growth. A rapid method to identify the major aerobic sporeforming groups in dairy products, including Bacillus licheniformis group, Bacillus subtilis group, Bacillus pumilus group, Bacillus megaterium, Bacillus cereus group, Geobacillus species and Anoxybacillus flavithermus was devised. This method involves real-time PCR and high resolution melt analysis (HRMA) of V3 (~70 bp) and V6 (~100 bp) variable regions in the 16S rDNA. Comparisons of HRMA curves from 194 isolates of the above listed sporeforming bacteria obtained from dairy products which were identified using partial 16S rDNA sequencing, allowed the establishment of criteria for differentiating them from each other and several non-sporeforming bacteria found in samples. A blinded validation trial on 28 bacterial isolates demonstrated complete accuracy in unambiguous identification of the 7 different aerobic sporeformers. The reliability of HRMA method was also verified using boiled extractions of crude DNA, thereby shortening the time needed for identification. The HRMA method described in this study provides a new and rapid approach to identify the dominant mesophilic and thermophilic aerobic sporeforming bacteria found in a wide variety of dairy products.

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1. Introduction

Aerobic sporeforming Bacillales members are of importance to the dairy industry as spores of these microorganisms, present in raw milk, can survive pasteurization and other processing events and ultimately become incorporated into final products (Cook and Sandeman, 2000; Coorevits et al., 2008; Crielly et al., 1994; Huck et al., 2007a). Also once present in a processing facility, spores from some thermophilic sporeformer organisms can germinate and multiply in biofilms in dairy processing equipment (Burgess et al., 2009, 2010).

In liquid-based dairy products, bacterial spores are rarely a health issue but may cause product spoilage under inadequate pasteurization/storage conditions, leading to product downgrades and losses in revenue. For dairy powders, thermophilic sporeforming bacilli can contaminate the final product and affect the value both in local and international markets (Burgess et al., 2010). In fresh raw and pasteurised milk and to an extent in cheeses, the predominant sporeforming species isolated using mesophilic incubation temperatures (i.e. ~30 °C) are Bacillus licheniformis, Bacillus subtilis, Bacillus pumilus, and Bacillus cereus (Cook and Sandeman, 2000; Coorevits et al., 2008; Cosentino et al., 1997; Crielly et al., 1994; Sutherland and Murdoch, 1994; Williams, 1958). In milk powders, sporeforming bacteria isolated using thermophilic incubation temperatures (i.e. ~55 °C) are Anoxybacillus flavithermus, Geobacillus species and B. licheniformis (Murphy et al., 1999; Reginensi et al., 2011; Ronimus et al., 2003; Scott et al., 2007).

Investigating and understanding the origins and microbial ecology of these sporeformers and developing strategies for controlling them require reliable rapid identification methods. Traditional culturing and biochemical test methods for identification of bacteria are widely being replaced by molecular methods, such as sequencing of variable regions in the 16S rDNA (Goto et al., 2000; Vardhan et al., 2011), 16S-23S ITS region (Xu and Cote, 2003) and the less conserved rpoB gene (Durak et al., 2006; Ki et al., 2009). Also a recently described
microarray-based genotyping method using 130 genomic markers has shown to discriminate 34 different strains from 6 *Bacillus* species and 4 species of *Geobacillus* genus isolated from a variety of food products (Caspers et al., 2011).

Real-time PCR has some advantages over sequencing and gene hybridization methods from the point of view of cost, time and data analysis (Fernández-No et al., 2010; Postollec et al., 2010; Rueckert et al., 2004; Rueckert et al., 2006). Also in the presence of other competing micro flora, PCR can sensitively detect species of specific interest in the absence or presence of an enrichment step (Postollec et al., 2011; Ranieri et al., 2012). A multiparametric TaqMan probe-based real-time PCR assay has recently been developed which discriminates 8 sporeforming genera and 6 *Bacillus* spp.; however this method is relatively low throughput, being able to examine 3 food samples at a time (Postollec et al., 2010, 2012). Fernández-No et al. (2010) reported a quantitative TaqMan-probe assay for *B. cereus*, *B. licheniformis* and *B. subtilis* directly from foods without pre-enrichment. This method however cannot differentiate between these three species. There remains a need for simple and cost-effective methods that allows species identification of important sporeforming bacteria from foods which has moderate throughput.

High-resolution melt analysis (HRMA) has emerged as a low-cost, single-step, closed-tube method for differentiating polymorphic PCR products (Vossen et al., 2009). In HRMA, the amplified fragments can be compared via both their melting temperature and changes in melt curve shape which are dependent upon G + C content, length, complementarity and sequence of the product. In recent years, HRMA has been used for rapid identification of clinical and biothreat bacterial species (Cheng et al., 2006; Slany et al., 2010; Wang et al., 2010; Won et al., 2010) but no applications of this technology in identifying foodborne bacteria species have been described. The objective of this study was to develop and validate a rapid, robust and inexpensive species identification method for the major 7 aerobic sporeforming bacteria isolated from a wide variety of dairy products. The single-tube method developed uses PCR coupled with HRMA and targets variable regions in the 165 rDNA without multiplexing or using hybridization probes. The utility of this approach is investigated and was shown to be able to distinguish representatives of *B. licheniformis* group, *B. subtilis* group, *B. pumilus* group, *Bacillus megaterium*, *B. cereus* group, *A. flavithermus* and the *Geobacillus* genus.

2. Materials and methods

2.1. Bacterial isolation

Raw and pasteurized fluid milk, cheddar cheese and milk powder samples were collected over a period of 5 months in 2010 from three different dairy processing plants in Victoria, Australia. Samples were maintained at −20 °C or below until used for microbiological analyses. In addition, commercial pasteurized milk, buttermilk and milk powders manufactured in Australia were also purchased at retail supermarkets in Brisbane, Australia, refrigerated and tested within 24 h of arrival in the laboratory.

Cheese samples were dispersed in 2% sodium citrate (90 ml to 10 g) using a stomacher while powder samples were dispersed in 0.1% (w/v) peptone water (90 ml to 10 g). Volumes of 20 ml each of raw fluid milk, buttermilk and dispersed cheeses and powder samples were heat treated to 80 °C for 10 min in a water bath and immediately cooled in an ice bath. Pasteurized fluid milk samples of the same volume were not heat treated, in accordance with that previously described (Huck et al., 2007b). Dilutions were made in 0.1% (w/v) peptone water and 1 ml of this was incorporated into nutrient agar (Oxoid, Basingstoke, England) containing 2% (w/v) starch (Sigma-Aldrich, Castle Hill, Australia) using the pour plate technique. Plates (once solidified) were overlayed with 3–5 ml of the same agar medium to reduce any colony spreading. Two sets of agar plates were prepared, with one set incubated at 30 °C and other at 55 °C for 24–48 h. Colonies with distinct morphologies were picked, streaked for purity using the same medium, grown in heart infusion (HI) broth (Oxoid, Basingstoke, England) and were then stored in 30% (v/v) glycerol at −80 °C.

2.2. DNA extraction

Total genomic DNA was extracted from 1.5 ml HI broth cultures using the DNeasy Blood and Tissue Lysis kit (Qiagen, Doncaster, Australia). DNA was eluted with 200 μl of elution buffer resulting in a DNA concentration of 20–80 ng/μl and then a 10-fold dilution was used as a DNA template for PCR and PCR coupled with HRMA. Additionally, crude genomic DNA was also isolated from bacterial cultures by resuspending one bacterial colony in 200 μl of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) followed by the addition of 5 μl of Proteinase K enzyme (20 mg/ml; Sigma-Aldrich, Castle Hill, Australia). Suspensions were incubated for 5 min at 22 °C and then for 15 min at 95 °C. Samples were centrifuged at 17,000 × g in a bench-top centrifuge (Eppendorf, South Pacific, North Ryde, Australia) for 10 min and the supernatant was diluted 10-fold and then used as DNA template for PCR coupled HRMA. Undiluted genomic DNA samples were stored at −20 °C (up to 12 months) while diluted samples were stored at 4 °C.

2.3. Amplification and sequencing of V1–V2 regions of 16S rDNA

PCR amplification of –1.4 kbp of the 16S rDNA was performed using primer pairs 16S_F (5′-AGAGTTTGATCCTGGCTC-3′) and 16S_R (5′-CGGAAACGTATTCAACC-3′), covering the 16S rDNA variable regions V1 to V9. Amplification was performed in a total volume of 10 μl containing 2 μl of a 10-fold dilution of the DNA template, 0.3 μM each primer and 5 μl Platinum SYBR®Green qPCRSuperMix-UDG (2×; Invitrogen Life Technologies, Mulgrave, Australia). The PCR cycling parameters were: 50 °C for 2 min; 95 °C for 2 min and then 40 cycles of 94 °C for 20 s, 45 °C for 30 s and 72 °C for 90 s. Partial sequencing of PCR products purified using the High-Pure PCR product purification kit (Roche Diagnostics, Castle Hill, Australia) was performed using Big Dye Terminator chemistry on ABI 3730x DNA analyser at the Australian Genome Research Facility (AGRF), Brisbane, Australia. Genus and species were identified by comparing –275 bp PCR fragment comprising V1–V2 regions (also called the HV region) of the 16S rDNA sequence to sequences in databases using the Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov/blast/) and also using the 16S ribosomal database (www.rdp.cme.msu.edu). This region as described in Goto et al. (2000) is considered a useful marker for grouping of species belonging to the order Bacillaceae including the genus *Bacillus*. However several *Bacillus* and related genera have groups of closely related species which share identical or highly similar 165 rDNA sequences. We carried out sequence comparison of the 16S rDNA HV region from type strains (available from GenBank) and found that equal to or greater than 98% identity was found for the strains listed in the following species clusters (*B. subtilis* subsp. *subtilis* 168 [AI009126] and *Bacillus mojavensis* IFOS15718 [AB021191], *Bacillus atrophaeus* JCM9070 [NR_024689], *Bacillus amylobacter* Y2 [CP003332] or *Bacillus vallismortis* DSM11031 [AB021198]; *B. licheniformis* ATCC 14580 [CP000002] and *Bacillus sonorensis* NVH 391-98 [CP000764]; *B. pumilus* SAFR-032 [NC_009848] and *Bacillus safensis* FO-036B [AF234854]; *B. cereus* Q1 [NC_011969] and *Bacillus thuringiensis* Bt407 [CP003889], *Bacillus mycoides* 273 [NR_036880], *Bacillus weihenstephanensis* DSM11821 [NR_024697], *Bacillus pseudomycoides* AF013121, *Bacillus anthracis* Sterne [AE017225] or *Bacillus cytoxicus* NIVH 391-98 [CP000764]; *Geobacillus thermoleovorans* CCB_US3UF5 [CP003125] and *Geobacillus stearothermophilus* BCRC 10285 [EU484358]). Therefore to take into account these close relationships, we identified isolates as members of “species groups” including *B. subtilis* group (contains either *B. subtilis*, *B. mojavensis*, *Bacillus atrophaeus*, *B. amylobacter* or *B. vallismortis*), *B. licheniformis*...
The assays were performed in a 10plex instrument with HRMA capability (Qiagen, Doncaster, Australia). Each bacterium was amplified by real-time PCR in a Rotor-Gene Q instrument containing EvaGreen dye (2×; Qiagen, Doncaster, Australia), for 20 s and 60°C for 20 s. A hold step of 50°C for 2 min followed cycling and then the HRMA was carried out over a temperature range of 65°C to 90°C with a machine HRMA setting of 0.1°C/2 s. Rotor-Gene Q series software version 1.7 (Qiagen, Doncaster, Australia) was used to analyse HRM data. Melting profiles were subjected to fluorescence normalisation to minimize inter- and intra-run variability. Analysis was performed separately for V3 and V6 regions. For the V3 region, curves were normalised using raw fluorescence data from 69–70°C (start) and 81–82°C (finish). For the V6 region, curves were normalised using raw fluorescence data from 77–78°C (start) and 86–87°C (finish). After normalisation, a difference graph was also generated for V3 and V6 curves by plotting the difference in fluorescence of a sample curve to a selected baseline or reference curve (obtained from a selected isolate representing that species/genus) at each temperature, using the software mentioned above. Differences in curves were measured in normalised fluorescence units (NFUs) from the y-axis. This type of analysis has been used previously (Stephens et al., 2008; Tong et al., 2009; Richardson et al., 2011). For the validation assay, isolates (n = 4) representing 7 different sporeforming bacteria were given random numerical codes (e.g., 1, 2, etc.) and then identified through HRMA.

### 3. Results

#### 3.1. Isolation and Identification of Aerobic Sporeforming Bacteria from a Variety of Dairy Products

A collection of 237 mesophilic and thermophilic bacterial isolates were assembled from Australian dairy product samples and raw milks sampled over a five month period (May–October 2010). All isolates of these bacteria were identified using V1–V2 regions containing ~275 bp 16S rDNA fragment, which of 196 were aerobic sporeforming bacteria and 41 represented non-sporeforming genera such as Microbacterium, Staphylocooccus, Streptococcus, Enterococcus, Enhydrobacter, Acinetobacter, and a non-sporeforming representative Microbacterium lactium. The sequence alignments were generated using ClustalW with sequences from the 16S ribosomal database. Positions identical to the first sequence are indicated by dots while gaps in sequences are indicated by dashes. The primer binding sequences for V3-F and V3-R and V6-F and V6-R are underlined.

### V3 region

- **B. licheniformis**: CTCGTTGTAAGGAAGACACACT
  - GAGAGTCCCT
  - G....

- **B. subtilis**: 
  - GAGAGTCCCT
  - G....

- **B. megaterium**: 
  - GAGAGTCCCT
  - G....

- **B. ceruis**: 
  - GAGAGTCCCT
  - G....

- **G. thermoleovorans**: 
  - GAGAGTCCCT
  - G....

- **A. flavigerinus**: 
  - GAGAGTCCCT
  - G....

- **M. lactium**: 
  - GAGAGTCCCT
  - G....

### V6 region

- **B. licheniformis**: ACCCTTACCAGGGCTTGGACACT
  - TCTGACACCG
  - GCTGCTGCTG

- **B. subtilis**: 
  - TCTGACACCG
  - GCTGCTGCTG

- **B. pumilus**: 
  - TCTGACACCG
  - GCTGCTGCTG

- **B. megaterium**: 
  - TCTGACACCG
  - GCTGCTGCTG

- **B. ceruis**: 
  - TCTGACACCG
  - GCTGCTGCTG

- **G. thermoleovorans**: 
  - TCTGACACCG
  - GCTGCTGCTG

- **A. flavigerinus**: 
  - TCTGACACCG
  - GCTGCTGCTG

- **M. lactium**: 
  - TCTGACACCG
  - GCTGCTGCTG

**Fig. 1.** Sequence alignments of ~70 bp of the V3 region and ~100 bp of the V6 region in the 16S rDNA for sporeforming bacteria representing Bacillus spp., Geobacillus thermoleovorans, A. flavigerinus, and a non-sporeforming representative Microbacterium lactium. The sequence alignments were generated using ClustalW with sequences from the 16S ribosomal database. Positions identical to the first sequence are indicated by dots while gaps in sequences are indicated by dashes. The primer binding sequences for V3-F and V3-R and V6-F and V6-R are underlined.
Kocuria, Brachybacterium, and Exiguobacterium (Table 1). Out of 135 sporeformers isolated at 30 °C, B. licheniformis group (n = 60) was the most common along with B. subtilis group (n = 25), B. pumilus group (n = 20), B. cereus group (n = 20) and B. megaterium (n = 8). Other bacilli identified but in very small numbers were Bacillus clausii (n =1) and Paenibacillus polymyxa (n = 1). Of 61 sporeformers isolated at 55 °C, Geobacillus spp. (n = 30), A. flavithermus (n = 19) and B. licheniformis (n = 12) were identified. All of these species have been reported as typical spore-formers in raw milks and dairy products worldwide with some species like B. licheniformis isolated at both 30 and 55 °C (Burgess et al., 2010; Cook and Sandeman, 2000; Criel et al., 1994; Huck et al., 2007a).

3.2. Selection of HRMA DNA targets for sporeformer identification

For developing a HRMA assay to identify bacterial species, PCR products should have the greatest sequence divergence between species but be conserved for strains within the same species. The 16S rDNA is an obvious target in this regard (Cilia et al., 1996) although some closely related species have shown to exhibit 98% or greater homology in the sequences of 16S rDNA gene. Comparisons of the 16S rDNA sequences from B. licheniformis, B. subtilis, B. pumilus, B. megaterium, B. cereus, Geobacillus and A. flavithermus available in databases revealed four variable regions within the 16S rDNA (V1, V2, V3 and V6) which appeared as suitable HRMA targets (data not shown). These regions show the greatest variability between the organisms examined here and as reported previously for other bacteria (Coenye and Vandamme, 2003). For PCR coupled HRMA, a ~275 bp fragment containing V1–V2 regions (Goto et al., 2000) and ~70 bp V3 and ~100 bp V6 regions in the 16S rDNA were selected.

We initially targeted the V1–V2 regions, however HRMA of a subset of Bacillus spp. isolates revealed that some isolates of the same species gave different HRM curves (data not shown), and so this region was not further investigated. Sequence analysis showed that the V3 and V6 regions varied between different sporeformers but had conserved flanking regions, making these ideal targets for HRMA (Fig. 1). However, the V6 region for 3 different Geobacillus spp. (G. steathermophilus, Geobacillus kaustophilus and G. thermoleovorans) was all identical while the primer binding sites flanking the V3 region varied significantly compared to those seen for the other Bacillus species. This meant both regions were unsuitable as HRMA targets to differentiate species within Geobacillus, although the genus itself could possibly be distinguished from other Bacillus species by targeting these regions. To maximise the resolving power of HRMA, shorter amplicons are preferred (Gundry et al., 2003), therefore for the regions V3 and V6, new primer sets were designed to minimize the size of the PCR products (Fig. 1).

3.3. HRMA of the V3 and V6 regions from seven sporeforming bacterial species

All the 194 aerobic sporeforming bacterial isolates identified by ~275 bp 16S rDNA sequencing (representing B. licheniformis group, B. subtilis group, B. pumilus group, B. megaterium, B. cereus group, Geobacillus and A. flavithermus) were analysed by HRMA. V3 and V6 melt curve profiles were found to be highly reproducible (i.e. from duplicate analysis of the same DNA template from the same isolate) for single isolates (examples shown in Fig. 2A and B) irrespective of the variations observed in the concentration and purity of DNA template. The V3 region melt curves for Geobacillus isolates however were not consistent and this was found to be due to this region not amplifying well for this bacterium, due to several primer mis-matches as shown in Fig. 1 (data not shown). The single isolates representing 5 different Bacillus species, A. flavithermus and Geobacillus were termed “reference controls” and were included (in duplicate) in each run of PCR coupled HRMA. It was found that V6 curves from A. flavithermus, Geobacillus spp., B. licheniformis group and B. subtilis group were well separated, while those from B. megaterium, B. cereus group and B. pumilus group curves were all close to each other (Fig. 2A). Fortuitously, V3 curves from B. megaterium, B. cereus group and B. pumilus group were well separated from each other (Fig. 2B). V3 curves from different B. licheniformis group isolates varied from each other, in some instances aligning with the B. subtilis group and B. pumilus group curves (Fig. 2C). In contrast, non-sporeformers (n = 41) were also analysed but all amplified inefficiently with a C of greater than 15 (Fig. 3) and could be readily differentiated from aerobic sporeformers using either or both V3 and V6 HRM curves. It was concluded that the ~70 bp V3 and ~100 bp V6 regions in the 16S rDNA (used in combination) are promising targets for HRMA-based rapid identification of at least six common

| Table 1 |
| Distribution of bacterial isolates obtained from different Australian dairy products. A total of 237 mesophilic and thermophilic isolates, representing sporeforming and non-sporeforming bacteria were obtained. |
| Bacterial isolate | Dairy products | Total no. of isolates | % of total |
| | Raw milk | Pasteurized milk | Milk powder | Butter milk | Cheese |
| Sporeforming bacteria | | | | |
| B. licheniformis group | 23 | 23 | 12 | 14 | 72 | 30.4 |
| Geobacillus spp. | 30 | | | | | 12.7 |
| B. subtilis group | 6 | 10 | 5 | 4 | 25 | 10.6 |
| B. pumilus group | 2 | 8 | 5 | 3 | 20 | 8.4 |
| B. cereus group | 5 | 13 | 1 | 1 | 20 | 8.4 |
| A. flavithermus | 1 | 12 | 2 | 4 | 19 | 8 |
| B. megaterium | 2 | 5 | 1 | 8 | 3.4 |
| B. clausii | 1 | | | | 1 | 0.4 |
| P. polymyxa | | 1 | | | 1 | 0.4 |
| Non-sporeforming bacteria | | | | |
| Microbacterium | 9 | 8 | 2 | 19 | 8 |
| Kocuria | 1 | 7 | | 8 | 3.4 |
| Staphylococcus | 1 | 3 | 1 | 5 | 2.1 |
| Streptococcus | 3 | | | 3 | 1.3 |
| Exiguobacterium | 2 | | | 2 | 0.8 |
| Enterococcus | 1 | | | 1 | 0.4 |
| Enhydrobacter | 1 | | | 1 | 0.4 |
| Acinetotobacter | 1 | | | 1 | 0.4 |
| Brachybacterium | 1 | | | 1 | 0.4 |
| Total | 237 | | | | 100 |

a Species groups contain several species which are described in the Materials and methods Section 2.3.

b The Geobacillus spp. includes several species which are described in Materials and methods Section 2.3.
dairy aerobic sporeforming bacterial species, and Geobacillus isolates could be identified at a genus level.

From the HRMA results from 194 sporeforming bacteria (P. polymyxa and B. clausii were not included due to their low abundance), we devised HRMA criteria and cut-off values which would allow grouping and therefore identification of dominant aerobic sporeforming bacteria using the two 16S rDNA regions (Fig. 4). Firstly, reactions must have a Ct of less or equal to 15 cycles (Fig. 3). Secondly, using difference graph analysis we set amplitudes ranging from between 5 and 13 NFU as cutoff values to group isolates as the same as reference curves (example is shown in Fig. 2D) similar to that which has been published previously (Stephens et al., 2008). Thirdly, identification using HRMA requires both analyses of the V3 and V6 regions. Bacterial isolates that did not generate curves which fit with these criteria and generated "atypical" melting curves (curves which did not fit within the criteria shown in Fig. 4) in either or both the regions of V3 and V6 were called

Fig. 2. HRMA graphs of the V3 and V6 regions. (A–E), reference controls considered as typical for each species are shown as solid lines and were run in duplicate: B. licheniformis group (pink), B. subtilis group (black), B. pumilus group (blue), B. cereus group (green), B. megaterium (red), Geobacillus spp. (orange) and A. flavithermus (aqua). (A & B), V3 and V6 HRM curves of 7 reference controls except for the Geobacillus spp. V3 region which did not amplify well. (C–E), dashed lines coloured the same show curves for isolates other than the reference controls. (C) Shows the heterogeneity of the V3 melting profiles of B. licheniformis group isolates (n = 5) which aligned with B. pumilus group and B. subtilis group reference control curves. (D), a typical difference graph of the V3 region for B. licheniformis group isolates (n = 10) and the curves from different isolates that fall within the ± 13 NFU of the baseline curve (reference control) are grouped as the same. (E), atypical isolates of B. subtilis group (n = 2) and B. pumilus group (n = 1) generating variation HRM curves in their V3 region.
variation’ and would need to be identified by a method other than HRMA. Out of the 194 sporeforming isolates which were analysed and used to generate the criteria shown in Fig. 4, only 3 isolates generated HRM curves which fell outside of the cut-off values and therefore were termed atypical. These included two B. subtilis group and one B. pumilus group isolates whose V3 HRM curves fell outside of the criteria set (Fig. 2E). The same result was obtained again for an independently prepared DNA template from these isolates. On comparison of their V3 region DNA sequences to the given sequences of our reference strains (Fig. 1), the B. pumilus isolate contained changes in 2 nucleotide bases (27G➔A and 40C➔T), while both isolates of B. subtilis group had one nucleotide change in them (25G➔A). As a result of such changes, V3 HRM curves for these isolates had lower melting temperatures and consequently did not align to the respective reference control curves (Fig. 2E).

3.4. Validation of HRMA

To determine the robustness of the HRMA method to correctly identify 7 dairy sporeforming bacteria, 4 representative isolates from each of the 7 sporeforming bacterial species/genera were randomised and de-identified. Following PCR and HRMA, V3 and V6 curves from each isolate were analysed and species/genera identification was based on the criteria shown in Fig. 4. The identities of the 28 de-identified sporeforming bacteria matched that previously obtained by 16S rDNA sequencing thereby confirming the method’s accuracy (trueness) under repeatability conditions according to the definitions in ISO 5725-1 (Anonymous, 1994).

4. Discussion

A new method for identifying common and predominant aerobic mesophilic and thermophilic sporeforming bacteria present in dairy products has been developed and validated. It is based on HRMA of 2 of the 9 variable regions in the 16S rDNA (Chakravorty et al., 2007). HRMA of PCR products using a double stranded DNA binding fluorescent dye was first performed in 2003 (Wittwer et al., 2003) and has since been used in clinical and biothreat bacterial species identification (Cheng et al., 2006; Wang et al., 2010) and bacterial genotyping (Fortini et al., 2007; Stephens et al., 2008; Tong et al., 2009). The ability to differentiate PCR products with single nucleotide differences makes it a powerful and rapid method for discerning whether or not sequences are the same. HRM curve shapes are largely determined by DNA length and G + C content and it has been shown that curves are highly reproducible (Richardson et al., 2011). We envisaged that this method would be useful for the identification of aerobic sporeformers for quality monitoring and isolate tracking purposes.

Fig. 3. Amplification curves of V3 and V6 regions from sporeforming and non-sporeforming bacteria. Sporeformers representing B. licheniformis group (n = 7; pink) and B. subtilis group (n = 2; black) amplify with Ct ≤ 15, while non-sporeformers like Microbacterium spp. (n = 2; purple) and Kocuria (n = 1; brown) amplified later in the PCR reaction.

Fig. 4. HRMA criteria for identification of 7 sporeforming bacteria: B. subtilis group, B. licheniformis group, B. pumilus group, B. megaterium, B. cereus group, A. flavithermus and Geobacillus genus, using regions V3 and V6 in the 16S rDNA. From difference graph analysis, HRMA curves of those isolates that fall outside the cutoff values (±5 to ±13 NFU) on comparison to the baseline curve were called ‘variation’ curves.
In studying a variety of dairy products obtained from processing plants and retail outlets, *B. licheniformis* group was the most common sporeformer isolated at 30 °C while *B. megaterium, B. subtilis* group, *B. cereus* group and *B. pumilus* group were isolated less frequently. This is in agreement with several studies conducted in Australia and around the world (Cook and Sandeman, 2000; Coorevits et al., 2008; Crielly et al., 1994; Sutherland and Murdoch 1994; Williams, 1958). For the sporeformers isolated at 55 °C and in particular the milk powders that we analysed, *Geobacillus spp., A. flavithermus* and *B. licheniformis* group were the most common organisms isolated. These are commonly found in milk powders as temperature parameters for processing milk powders are high thus allowing growth of thermophiles in evaporators and pre-heaters (Reginensi et al., 2011; Ruckert et al., 2004). A recent investigation of Uruguayan milk powders also found that the major contaminants were *A. flavithermus* and *B. licheniformis*, but not *Geobacillus* spp. (Reginensi et al., 2011).

Among the other major dairy sporeforming bacterial contaminants include species of *Paeunibacillus* which is a major psychrotolerant spoliage microbe for pasteurised fluid milk products and *Bacillus sporothermodurans* which has been found in ultra-high temperature treated milk products (Ranieri et al., 2012; Scheldeman et al., 2006). Due to this study being conducted on only early shelf life dairy products, psychrotrophic spoilage bacteria were not specifically targeted while *B. sporothermodurans* was not isolated from any products. Other bacilli such as *B. clausii* (*n* = 1) and *P. polymyxa* (*n* = 1) which upon examination by HRMA did not give curves which aligned to that of the 7 reference control sporeforming bacteria and were therefore unable to be identified using the current set of reference control species/genera (data not shown). Thus no false positive species/genera identification was made, allowing the current study to be specific for the targeted sporeformers only. In the future, other species could also potentially be incorporated as reference controls, allowing a greater number of sporeformers in dairy foods or other foods to be identified. Other than bacilli, we also isolated a range of non-sporeforming bacteria that have previously been described to survive pasteurization or enter foods as post-pasteurisation contaminants and hence are frequently isolated from dairy products (Coorevits et al., 2008; Fromm and Boor, 2004; Ranieri and Boor, 2009). Under the conditions described in this study, amplification was inefficient and HRM curves of non-sporeformers did not align with those obtained from the sporeforming bacteria reference controls (data not shown).

For species identification, the analysis of highly conserved small ribosomal subunit RNA is widely accepted (Cilia et al., 1996) but the presence of multiple gene copies and intragenomic heterogeneity among rDNA sequences could be an issue for HRMA. To limit the possibility of isolating particular dominant clonal strains, we isolated sporeformers from a diverse range of dairy products which were processed at different locations in Australia. Note that reference/type strains were not used in this study, however identification of all isolates was done using the well accepted method of Goto et al (2000) and they are from relevant dairy food sources. Previous findings have found that intragenomic heterogeneity can sometimes affect HRMA leading to an inability to identify bacteria (Cheng et al., 2006; Slany et al., 2010). In our study isolates of *B. subtilis* (*n* = 2) and *B. pumilus* (*n* = 1) groups could not be identified by our HRMA criteria (Fig. 2E) due to nucleotide changes seen in their V3 region DNA sequence when compared to the reference strains used. These isolates however were not identified as an incorrect species/genera, but instead gave a variation result, and therefore required identification by a method other than HRMA.

Through analysis of available genome sequences we observed that most intragenomic heterogeneity between multiple 16S rDNA copies in the same organism occurred in the V1–V2 variable regions, which probably explains why we observed different V1–V2 curves for isolates of the same species (data not shown). While V6 region curves of the *B. licheniformis* group isolates were very close, the V3 region curves in contrast showed considerable variation. For example, in Fig. 2C, the V3 region curve of *B. licheniformis* group isolate aligned closely with that of *B. pumilus* and was found to contain a single nucleotide variation (43T → C) causing the PCR product to melt at a higher temperature. Analysis of the *B. licheniformis* strain ATCC 14580 genome (GenBank accession number CP000002) also revealed considerable sequence heterogeneity between gene copies for the V3 region; out of seven 16S rDNA gene copies, three were identical and four copies had the same one nucleotide difference (43C/T). Variation between gene copies in the V6 region was slightly less; among seven 16S rDNA gene copies, five were identical and 2 copies had the same one nucleotide difference (28A/G). It is possible that the sequence heterogeneity of the PCR products allows heteroduplex formation in HRMA which subsequently creates differences within the melting amplicons; however this is yet to be confirmed.

Despite the high discriminatory power of HRMA, we found that the targeted 16S rDNA sequences will be unlikely to resolve the known species of *Geobacillus* and close relatives of the *B. cereus, B. subtilis, B. licheniformis* and *B. pumilus* groups. It should be noted that the HRMA method described here will always be unable to differentiate closely related species that share identical V3 and V6 region sequences as these target regions need to be different in order to generate different HRM curves. Species of *Geobacillus* including *G. stearothermophilus* and *G. thermoleovorans* are indistinguishable in their 16S rDNA sequences (Weng et al., 2009). Therefore other housekeeping genes such as *recA, rpoB, gyrB* and *parE* have been investigated to differentiate them (Tourova et al., 2010; Weng et al., 2009). Similarly the *B. cereus* group consists of six genetically related species, *B. cereus, B. anthracis, B. mycoides, P. polymyxa, B. pseudomyxodes* and *B. wiihestenophonainis* and *B. thuringiensis*.

Although physiologically different from each other, these organisms show a high degree of genetic homology within their 16S rDNA sequences (Ash et al., 1991; Guinebretière et al., 2008). Therefore, other less conserved housekeeping genes have been also investigated for differentiating *B. cereus* group isolates (Blackwood et al., 2004; Helgason et al., 2004; Tourasse et al., 2006). Likewise, *B. safensis* a recently characterised species from spacecraft surfaces was found indistinguishable from *B. pumilus* on the V3 and V6 regions analysed but can be differentiated by one a nucleotide difference in the HV region (data not shown), *gyrB* gene sequences, repetitive element primer-PCR fingerprinting and DNA–DNA hybridization (Satomi et al., 2006).

The randomised and blinded validation experiment using 28 aerobic sporeforming isolates confirmed the accuracy of this HRMA identification method. Overall, these results show that the HRMA method can be used as a first pass identification method for the most common sporeforming bacteria in dairy products and that this procedure could be extended for the identification of additional bacteria with the inclusion and testing of more reference controls. In other work we have screened 180 thermophilic bacterial isolates from milk powder production samples by HRMA and found that 136 isolates generated melt curves that aligned with the *B. licheniformis* group control, thereby allowing us to dramatically reduce the number of isolates that needed to be identified using other methods, such as 16S rDNA sequencing (Dhakal et al., 2013). The HRMA method described here is a closed-tube assay which costs approximately US $1.30 per identification. With 7 sporeformers included in the control panel, 21 unknown bacteria or DNA samples can be analysed in a single run using a 72 tube rotor well. The method requires isolation of a pure culture of the organism and allows differentiation of typical dairy food sporeformers both through specific primer selection (Fig. 1) and HRM curve analysis (Fig. 2). Excluding the time taken to culture the bacteria, the identification method can take only a few hours (2 h for genomic DNA extraction and less than 3 h for PCR coupled HRMA). We have also demonstrated that accurate HRMA results are produced when using crude DNA extracted from a suspended single colony (following treatment with proteinase K and/or boiling), thereby shortening the time for identification (data not shown).
In conclusion, our described method is envisaged to be useful for rapid identification of important sporeforming bacteria groups in dairy products and likely in other foods as well, with potential application for surveillance and source tracking with the goal of ultimately improving food quality.

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