Spore populations among bulk tank raw milk and dairy powders are significantly different

Rachel A. Miller, David J. Kent, Matthew J. Watterson, Kathryn J. Boor, Nicole H. Martin, and Martin Wiedmann¹

Milk Quality Improvement Program, Department of Food Science, Cornell University, Ithaca, NY 14853

ABSTRACT

To accommodate stringent spore limits mandated for the export of dairy powders, a more thorough understanding of the spore species present will be necessary to develop prospective strategies to identify and reduce sources (i.e., raw materials or in-plant) of contamination. We characterized 1,523 spore isolates obtained from bulk tank raw milk ($n = 33$ farms) and samples collected from 4 different dairy powder-processing plants producing acid whey, nonfat dry milk, sweet whey, or whey protein concentrate 80. The spores isolated comprised 12 genera, at least 44 species, and 216 *rpoB* allelic types. *Bacillus* and *Geobacillus* represented the most commonly isolated spore genera (approximately 68.9 and 12.1%, respectively, of all spore isolates). Whereas *Bacillus licheniformis* was isolated from samples collected from all plants and farms, *Geobacillus* spp. were isolated from samples from 3 out of 4 plants and just 1 out of 33 farms. We found significant differences between the spore population isolated from bulk tank raw milk and those isolated from dairy powder plant samples, except samples from the plant producing acid whey. A comparison of spore species isolated from raw materials and finished powders showed that although certain species, such as *B. licheniformis*, were found in both raw and finished product samples, other species, such as *Geobacillus* spp. and *Anoxybacillus* spp., were more frequently isolated from finished powders. Importantly, we found that 8 out of 12 genera were isolated from at least 2 different spore count methods, suggesting that some spore count methods may provide redundant information if used in parallel. Together, our results suggest that (1) *Bacillus* and *Geobacillus* are the predominant spore contaminants in a variety of dairy powders, implying that future research efforts

targeted at elucidating approaches to reduce levels of spores in dairy powders should focus on controlling levels of spore isolates from these genera; and (2) the spore populations isolated from bulk tank raw milk and some dairy powder products are significantly different, suggesting that targeting in-plant sources of contamination may be important for achieving low spore counts in the finished product. These data provide important insight regarding the diversity of spore populations isolated from dairy powders and bulk tank raw milk, and demonstrate that several spore genera are detected by multiple spore count methods.

Key words: spore populations, dairy powders, bulk tank raw milk

INTRODUCTION

Bacterial endospores (referred to here as spores) represent important quality and safety indicators for dairy powders (Burgess et al., 2010; Watterson et al., 2014; Hwang and Park, 2015). Their resistance to several environmental stresses, including high temperature, low pH, and low water activity, makes spores well suited to survive the various environments encountered during dairy powder production (Setlow, 2006; Burgess et al., 2010; Witthuhn et al., 2011).

Previous accounts of aerobic spore populations in dairy powders were restricted to nonfat dry milk (NDM) and whole milk powder (WMP). For these dairy powders, *Anoxybacillus flavithermus*, *Geobacillus stearothermophilus*, and *Bacillus licheniformis* represent the most frequently isolated organisms (Scott et al., 2007; Burgess et al., 2010; Yuan et al., 2012). However, *A. flavithermus* and *G. stearothermophilus* are rarely isolated from bulk tank (BT) raw milk, where the predominant spore species include *B. licheniformis*, *Bacillus pumilus*, and *Bacillus subtilis* (Coorevits et al., 2008; Burgess et al., 2010; Yuan et al., 2012). The disparity in spore populations isolated from dairy powders and BT raw milk is often attributed to in-plant sources of con-

Received June 10, 2015.

Accepted August 20, 2015.

¹Corresponding author: mw16@cornell.edu

tamination. Scott et al. (2007) isolated *A. flavithermus* and *Geobacillus* spp. from multiple sampling points in a WMP processing plant. Those authors noted that *A. flavithermus* was the predominant organism in pre-heating processing steps, but both *Geobacillus* spp. and *A. flavithermus* were isolated at subsequent processing steps (Scott et al., 2007). This demonstrates the dynamic nature of spore populations in dairy powders and highlights the need for an improved understanding of the spore species, which are associated with raw material and in-plant contamination.

Currently, no public reports exist of spore populations in dairy powders, such as whey protein concentrate 80 (**WPC-80**), sweet whey (**SW**), and acid whey (**AW**); this would be of significant economic value to dairy powder exporters due to customer requirements for powders to meet strict spore count limits. Therefore, it is important to identify the spores present in these dairy powders so that preventative approaches to limiting spore contamination in these products can be developed.

Previous studies have identified contamination originating from biofilms formed by spore-forming bacteria as an important source of bacterial spores in dairy powders (Parker et al., 2001; Scott et al., 2007; Burgess et al., 2010). In favorable environments, spores are capable of germinating into bacterial cells, which are then able to adhere to processing equipment and, subsequently, establish biofilms. Additionally, the heat tolerance of several spore species suggests that they are capable of surviving in the product throughout processing (te Giffel et al., 2002; Scheldeman et al., 2005; Burgess et al., 2010). For these reasons, mandating low spore levels in raw materials has been identified as an important approach to reducing spore loads in the finished product (Watterson et al., 2014). However, few studies have examined differences in spore populations isolated from raw materials and finished products (Scott et al., 2007; Watterson et al., 2014). An improved understanding of the processing steps at which spores contaminate dairy powders would provide relevant information that would be useful for developing enhanced control strategies.

Spore levels may be quantified using a variety of spore count methods (**SCM**), each requiring different temperature and time combinations [referred to as spore pasteurization (**SP**)] to eliminate vegetative bacterial cells, as well as different incubation temperatures and plating media to enumerate the resulting spore-forming bacteria (Ronimus et al., 2003; Bienvenue, 2014; Watterson et al., 2014). For example, the standard spore pasteurization (**SSP**) method requires a heat treatment of 80°C for 12 min, whereas the highly heat resistant (**HHR**) SCM uses a heat treatment of 100°C for 30 min (Frank and Yousef, 2004; Watterson

et al., 2014). Currently, it is unknown how these different SCM may influence the resulting spore count of the product tested or the composition of the spore populations recovered. Work by Watterson et al. (2014) suggests differences exist in the resulting spore counts obtained by HHR and SSP methods, but it is unknown how these methods affect the resulting spore species that are isolated. Because dairy powder customers may require that multiple SCM be performed to assess spore levels in a dairy powder, it is important to determine the spore species that may be detected or excluded by different SCM.

Therefore, the goals of this study were to (1) characterize the spore populations of a variety of dairy powder (AW, NDM, SW, and WPC-80) and BT raw milk sources; (2) determine differences in spore populations isolated from raw materials and finished products; and (3) determine which spore genera are typically detected by different SCM.

MATERIALS AND METHODS

Description of Spore Isolates Used in This Study

A total of 1,949 bacterial isolates, collected from BT raw milk and dairy powder samples that had been SP, were characterized in this study. Isolates were obtained from 2 previous studies examining spore counts in (1) BT raw milk from 33 different farms throughout New York State sampled every other month for 1 yr (i.e., 6 BT raw milk samples per farm, total of 198 samples; Miller et al., 2015); and (2) 4 different dairy powder processing plants sampled every other month for 1 yr (i.e., 6 sampling dates per plant, total of 147 samples from plants processing AW, NDM, SW, or WPC-80; Watterson et al., 2014). Samples collected in the 4 plants included (1) raw materials (e.g., raw milk, whey powders, liquid whey), (2) product collected at intermediate processing stages (work in process; **WIP**), and (3) finished product (see Table 1; Watterson et al., 2014). All samples had been SP by heating at 80°C for 12 min for SSP, or 100°C for 30 min for HHR SP (only performed for dairy powder plant samples), followed by incubation at 6°C for 10 d [psychrotolerant spore count (**PSC**)] to enumerate psychrotolerant spores, and at 32° and 55°C for 48 h to enumerate mesophilic [mesophilic spore count (**MSC**)] and thermophilic [thermophilic spore count (**TSC**)] spores, respectively (Frank and Yousef, 2004). An additional enrichment step, in which SP samples were incubated at either 6°C for 10 d, or at 32 or 55°C for 48 h, followed by plating on brain-heart infusion (**BHI**) agar and subsequent incubation at 6°C for 10 d, or at 32 or 55°C for 48 h, was done to detect spores present at levels below detection (present at

Table 1. Summary of all spore isolates collected from acid whey, nonfat dry milk, sweet whey, whey protein concentrate-80, and bulk tank raw milk sources

Source ¹	Raw material ²		Work in process ³		Finished ⁴		Total ⁵	
	No. of samples	No. of isolates	No. of samples	No. of isolates	No. of samples	No. of isolates	No. of samples	No. of isolates
SW	18	62	24	213	6	57	48	332
NDM	6	39	24	266	6	69	36	374
AW	6	3	12	25	6	6	24	34
WPC-80	9	13	24	131	6	44	39	188
BT Raw Milk	198	595	NA ⁶	NA	NA	NA	198	595

¹Description of the product sample from which a given spore isolate was collected [sweet whey (SW), nonfat dry milk (NDM), acid whey (AW), whey protein concentrate 80 (WPC-80), bulk tank (BT) raw milk].

²Samples included raw milk, whey by-products, WPC-80, lecithin, and lactose powder (Watterson et al., 2014).

³Samples included materials collected midprocess at intermediate steps in processing (Watterson et al., 2014).

⁴Samples included dairy powders (SW, NDM, AW, WPC-80) after packaging.

⁵Includes all spore isolates collected from all samples from a given source.

⁶NA = not applicable, only raw samples were collected for BT raw milk.

levels <10 cfu/mL). Morphologically distinct colonies obtained from PSC, MSC, and TSC analyses had been substreaked onto BHI plates, followed by incubation at 6, 32, or 55°C, depending on the original temperature of isolation. Isolated colonies from substreaked plates had been propagated in BHI broth (Difco; Franklin Lakes, NJ) overnight at either 32°C for isolates from PSC or MSC analyses, or at 55°C for isolates obtained from TSC analyses. Visibly turbid cultures had been preserved in 15% glycerol stocks maintained at −80°C before characterization.

Gene Sequencing

All spore isolates were characterized using PCR amplification of a segment of the *rpoB* gene (Durak et al., 2006; Ivy et al., 2012). Briefly, frozen aliquots of spore isolates were plated on BHI agar, followed by incubation at 32 or 55°C for 24 to 48 h. Bacterial lysates were prepared either by (1) heating colonies in a microwave on high for 2 min, or (2) by inoculating 100-μL aliquots of 0.05 M NaOH with colonies, followed by heating at 95°C for 15 min. The PCR reagents and thermocycling conditions used were as described by Durak et al. (2006). A second set of primers (refer to Table 2 for primers used in our study) was designed to characterize

isolates collected from dairy powder sources. Electrophoresis of PCR products was performed using a 1.5% agarose gel. The PCR products were electrophoresed at 120 V for 30 min. Sequencing reactions were performed as per manufacturer's instructions (Big Dye Sequencing Kit, Life Technologies Inc., Grand Island, NY), and were analyzed as described previously (Ivy et al., 2012).

rpoB AT Assignment, Sequence Alignment, and Species Identification

Spore isolates were assigned an *rpoB* allelic type (AT), as described previously (Huck et al. 2007; Ivy et al., 2012); *rpoB* AT represent unique 632 nucleotide sequences of the *rpoB* gene that differ from previous *rpoB* sequences in the *rpoB* database (MQIP, Cornell University, Ithaca, NY) by 1 or more nucleotides. For a spore isolate representing a new AT, a 700-nucleotide segment of the 16S rRNA gene was analyzed for species identification as previously described (Huck et al., 2007). Sequences were aligned and trimmed to their respective lengths (632 nucleotides for *rpoB* and 700 nucleotides for 16S rRNA) using Mesquite software (version 3.03, Mesquite software; <http://mesquiteproject.org>). All sequences were edited using Sequencher software 5.0 (Gene Codes Corporation, Ann Arbor, MI).

Table 2. Primers used in this study

Primer name	Primer description	Primer sequence (5'→3') ¹
RZrpoBRV3	Reverse primer for <i>rpoB</i>	TGNARYTTRTCRTCRACCATGTG
RZrpoBFV3	Forward primer for <i>rpoB</i>	AARYTNGGHCCDGARGAAAT
RZrpoBRV2	Reverse primer for <i>rpoB</i>	AARYTNGGHCCCTGAAGAAAT
RZrpoBFV2	Forward primer for <i>rpoB</i>	TGNARYTTRTCATCAACCATGTG
16S-P3SH	Reverse primer for 16S rRNA gene	CTACGGTTACCTTGTACGACTT
16S-PEU7	Forward primer for 16S rRNA gene	GCAAACAGGATTAGATACCC

¹IUPAC standard nomenclature; N = A, C, G, or T; R = A or G; Y = C or T; H = A, C, or T; D = A, G, or T.

A maximum-likelihood phylogenetic tree was generated as described previously, using the rapid maximum-likelihood algorithm (Ivy et al., 2012), to provide genus and species identification.

Final Spore Isolate Data Set

Of the 1,949 bacterial isolates characterized, a total of 1,523 spore isolates were included in the final data set (see Table 1). Isolates which were identified as non-spore-forming (e.g., *Staphylococcus* spp., *Carnobacterium* spp., *Planococcaceae* spp., *Streptococcus* spp., *Lactococcus* spp., and *Micrococcus* spp.) were excluded from all analyses ($n = 68$ isolates). Likewise, if multiple isolates with the same *rpoB* AT were isolated from the same sample and receiving the same SCM, only one isolate for the given AT, sample, or SCM was retained; this strategy eliminated 358 isolates from the final data set. Therefore, the final data set included unique isolates representing AT obtained by a given SCM (i.e., SP and subsequent incubation temperature) for each sample. All information pertaining to isolate source, isolation methods, and *rpoB* and 16S rRNA sequences are available online in the Food Microbe Tracker Database (<http://www.foodmicrobetracker.net>).

Statistical Analysis

All statistical analyses were performed using R statistical software (version 3.0.1; R-project, Vienna, Austria). Nonmetric dimensional scaling and analysis of similarities (ANOSIM) analyses were performed using the R package Vegan (Oksanen et al., 2015). Post hoc pairwise comparisons of ANOSIM statistics were performed using the Holm-Bonferroni multiple comparisons method included in the R package stats (version 3.0.1; R-project).

RESULTS AND DISCUSSION

To identify potential differences between populations of bacterial spores in both BT raw milk and dairy powder samples, we characterized and identified spores previously isolated from samples from (1) BT raw milk (from 33 different farms; Miller et al., 2015) and (2) 4 different powder plants (AW, NDM, SW, and WPC-80 processing plants; Watterson et al., 2014).

Dairy Powder and BT Raw Milk Samples Contain Diverse Populations of Bacterial Spores

Overall, we identified 12 different genera among 1,523 spores isolated from BT raw milk samples and samples collected from the 4 different powder plants (AW, NDM,

SW, and WPC-80 processing plants). *Bacillus* and *Geobacillus* were the predominant genera, representing 68.9 and 12.1%, respectively, of all spores collected. At least 44 different species were identified; the 3 most common species were *B. licheniformis* (36.5% of all isolates), *Bacillus cereus* group spp. (7.6% of all isolates), and *B. pumilus* (5.2% of all isolates; see Table 3). The *B. cereus* group includes spore isolates that may represent species *anthracis*, *cereus*, *cytotoxicus*, *mycoides*, *pseudomycoides*, *thuringiensis*, and *weihenstephanensis* (Dréan et al., 2015). The genetic relatedness of the species within the *B. cereus* group is high, therefore requiring more discriminatory analyses than single gene sequencing, as was used in our study, to distinguish between species within the *B. cereus* group (Kolstø et al., 2009; Zwick et al., 2012). Whereas the *rpoB* AT-based classification approach used here was unable to speciate *Geobacillus* and *Anoxybacillus* isolates, other studies have reported isolating *G. stearothermophilus* and *A. flavithermus* from dairy powders (Scott et al., 2007; Postollec et al., 2012). Consistent with our findings, *B. licheniformis* has been identified previously as the predominant spore species in BT raw milk (Scheldeman et al., 2005; Coorevits et al., 2008), and different dairy products (infant formula powder and whole milk powder) in different countries (Ronimus et al., 2003; Rückert et al., 2004; Yuan et al., 2012).

Characterization of a portion of the *rpoB* gene also allowed us to differentiate the 1,523 spore isolates into 216 different *rpoB* AT. The 3 most common AT were 1, 353, and 6, representing *B. licheniformis*, *Anoxybacillus* spp., and *B. licheniformis*, respectively. These data provide important baseline information on the most common spores found throughout the dairy continuum, from BT raw milk to finished dairy powder products.

Bacillus licheniformis and *Bacillus pumilus* Are the Predominant Spore Species in BT Raw Milk

The 595 spore isolates collected from BT raw milk from 33 different farms comprised 10 different genera and at least 36 different species. *Bacillus licheniformis* and *B. pumilus* were the 2 most frequently isolated spore species, accounting for a combined total of 56.5% of all isolates from BT raw milk (Table 3). We isolated *B. licheniformis* from BT raw milk from all 33 farms, highlighting the ubiquitous distribution of this spore species. Our results are consistent with other studies reporting that *B. licheniformis* and *B. pumilus* are frequently isolated from BT raw milk in Belgium (Coorevits et al., 2008) and the Midwestern United States (Buehner et al., 2014). Additionally, in agreement with other studies, we did not isolate *Anoxybacillus* spp. from any of the 198 milk samples tested (Coorevits et

al., 2008; Burgess et al., 2010). In contrast, we isolated *Geobacillus* spp. (1 isolate) from 1 BT raw milk sample. Whereas *Geobacillus* spp. are traditionally believed to be present in BT raw milk at very low levels, they are rarely isolated from BT raw milk (Scheldeman et al., 2005; Burgess et al., 2010).

Among 140 *rpoB* AT isolated from BT raw milk, *B. licheniformis* AT 1 was identified in raw milk collected from every farm, suggesting that this AT is ubiquitous among BT raw milk from farms throughout New York State. Other AT which were commonly isolated from BT raw milk included *B. licheniformis* AT 6 (isolated from 28 farms), *B. licheniformis* AT 169 (isolated from 14 farms), and *B. pumilus* AT 20 (isolated from 13 farms). On average, 11 different AT (range = 4–19 different AT; SD = 3.5 different AT) were isolated from BT raw milk collected from each farm. A total of 87 AT were isolated from only 1 farm; the majority ($n = 84$) of these AT were only isolated from a single BT raw milk sample (i.e., these AT were only isolated from 1 out of 198 total samples).

Although our data support the common presence of certain spore species (e.g., *B. licheniformis* and *B. pumilus*) and subtypes (e.g., AT 1), we hypothesized that BT raw milk from certain regions or specific farms may yield different spore populations. Farms belonged to 4 regions in New York State (Ithaca, Canton, Cobleskill, and Geneseo; see Materials and Methods; Miller et al., 2015). Despite being located in the same state, when farms were stratified by region the distribution of spore genera isolated from BT raw milk samples collected from farms within each region was significantly

different ($P = 0.045$; ANOSIM analysis). To further discriminate differences in spore populations we examined the spore populations from each region at the AT level. Aside from AT 1 (representing *B. licheniformis*), which was isolated from BT raw milk from all 33 farms, no other AT were isolated from BT raw milk from all farms (i.e., present in BT raw milk collected from all 33 farms). Few other studies have examined differences in the spore ecology of BT raw milk collected from farms in different geographical regions. By comparison, Coorevits et al. (2008), who sampled BT raw milk from farms in Belgium, reported that *B. licheniformis* and *B. pumilus* spores were present in all BT raw milk samples collected from 10 different farms sampled twice. A recent study by Buehner et al. (2014) also identified *B. licheniformis* as the predominant spore in BT raw milk from farms in South Dakota. Together, the results presented here and the findings of Buehner et al. (2014) and Coorevits et al. (2008) suggest that *B. licheniformis* and *B. pumilus* are the predominant spore species in BT raw milk, regardless of geographic location.

Bacillus and Geobacillus Are the Predominant Spore Genera Isolated from Dairy Powders

Among a total of 928 spore isolates obtained from raw materials, WIP, and dairy powder samples collected from 4 dairy powder plants over 1 yr, a total of 10 different genera were identified (see Table 3). The majority of the isolates collected from all 4 powder plant sources were *Bacillus* spp. (56.4%), *Geobacillus* spp. (19.8%), and *Anoxybacillus* spp. (17.3%).

Table 3. Spore genera and species isolated from samples from 4 different dairy powder processing plants and bulk tank raw milk from 33 farms

Genus and species	Spore isolates from each source, ¹ % (no. of isolates)					
	SW	NDM	AW	WPC-80	BT raw milk	Total
<i>Aeribacillus pallidus</i>	0.6 (2)	2.1 (8)	0.0 (0)	4.3 (8)	1.3 (8)	1.7 (26)
<i>Anoxybacillus</i> spp.	38.0 (126)	9.4 (35)	0.0 (0)	0.0 (0)	0.0 (0)	10.6 (161)
<i>Bacillus cereus</i> group	17.2 (57)	1.3 (5)	0.0 (0)	13.3 (25)	4.7 (28)	7.6 (115)
<i>Bacillus clausii</i>	0.3 (1)	2.1 (8)	5.9 (2)	0.5 (1)	5.2 (31)	2.8 (43)
<i>Bacillus licheniformis</i>	6.3 (21)	44.9 (168)	73.5 (25)	31.9 (60)	47.4 (282)	36.5 (556)
<i>Bacillus pumilus</i>	0.0 (0)	6.4 (24)	0.0 (0)	0.5 (1)	9.1 (54)	5.2 (79)
<i>Bacillus subtilis</i> s.l.	2.4 (8)	1.1 (4)	0.0 (0)	2.7 (5)	4.9 (29)	3.0 (46)
<i>Bacillus thermoamylovorans</i>	0.0 (0)	2.1 (8)	2.9 (1)	16.0 (30)	6.4 (38)	5.1 (77)
<i>Geobacillus</i> spp.	27.1 (90)	16.0 (60)	0.0 (0)	18.1 (34)	0.2 (1)	12.1 (185)
<i>Lysinibacillus</i> spp.	0.0 (0)	1.3 (5)	0.0 (0)	1.1 (2)	3.2 (19)	1.7 (26)
<i>Paenibacillus</i> spp.	0.9 (3)	3.5 (13)	2.9 (1)	0.5 (1)	4.2 (25)	2.8 (43)
<i>Ureibacillus</i> spp.	0.3 (1)	1.1 (4)	0.0 (0)	1.6 (3)	0.3 (2)	0.7 (10)
Other ²	0.9 (3)	1.1 (4)	5.9 (2)	0.0 (0)	2.4 (14)	1.5 (23)
Other <i>Bacillus</i> spp. ³	6.0 (20)	7.5 (28)	8.8 (3)	9.6 (18)	10.8 (64)	8.7 (133)
Total isolates, n	332	374	34	188	595	1,523

¹SW = sweet whey; NDM = nonfat dry milk; AW = acid whey; WPC-80 = whey protein concentrate 80; BT = bulk tank.

²Other includes *Brevibacillus* spp., *Oceanobacillus* spp., *Solibacillus* spp., *Sporosarcina* spp., and *Terribacillus* spp.

³Other *Bacillus* spp. includes species *aerophilus*, *coagulans*, *firmus*, *gibsonii*, *horneckiae*, *megaterium*, *muralis*, *nealsonii*, *neutrophilus/sojae*, *safensis*, *smithii*, and other *Bacillus* spp.

A total of 7 genera were identified among the 332 spore isolates from the SW processing plant (Table 3). The 2 most common genera were *Anoxybacillus* and *Bacillus*, which accounted for 38.0 and 32.2% of all spores, respectively; 27.1% of spore isolates from SW samples represented the genus *Geobacillus*. We also identified at least 20 species and 39 AT. The predominant spore species isolated from SW samples were *B. cereus* group spp. and *B. licheniformis*. Although we were unable to speciate the *Anoxybacillus* spore isolates, just 3 different AT were found in our study, with AT 353 representing the predominant AT for that genus, contributing 122 out of a total 126 *Anoxybacillus* spp. isolated from samples collected from the SW processing plant. Of the *Anoxybacillus* spp. isolated in our study (157 isolates from all sources), AT 353 was the predominant subtype among *Anoxybacillus* spp. in dairy powders from the northeastern United States. Similarly, among all *Geobacillus* spp. isolates, 4 AT were identified from the SW processing plant samples, but the majority of the isolates belonged to AT 439 (68 out of 90 *Geobacillus* spp. isolates from SW samples).

Among the 374 spores isolated from the NDM processing plant samples (see Table 3), 10 different genera were identified, with the majority belonging to *Bacillus* (65.5%), *Geobacillus* (16.0%), and *Anoxybacillus* (9.4%). We identified at least 28 species and 71 AT; the species *B. licheniformis* (44.9%) and *B. pumilus* (6.4%) represented the majority of spore isolates from this source, and just 1 and 2 AT of *Anoxybacillus* and *Geobacillus* spp., respectively, were isolated. Similar to BT raw milk, *Paenibacillus* spp., *Lysinibacillus* spp., and *Ureibacillus* spp. were also isolated. Silo raw milk samples were only obtained for the NDM processing plant. Among the silo raw milk spore isolates ($n = 41$), we identified 5 genera (*Bacillus*, *Lysinibacillus*, *Oceanobacillus*, *Paenibacillus*, and *Ureibacillus*). Not surprisingly, the majority of the silo raw milk spores isolated were *B. licheniformis* ($n = 21$ isolates).

Very few spore isolates ($n = 34$) were obtained from samples from the AW processing plant (see Table 3). For this plant, only 4 genera were isolated, representing 9 species and 14 AT. *Bacillus licheniformis* accounted for 73.5% of all spores isolated. In contrast to the SW and NDM samples, we did not isolate any *Anoxybacillus* or *Geobacillus* spp. from AW samples.

A total of 188 spores were isolated from the WPC-80 processing plant (Table 3). We identified 6 genera and at least 18 different species. The most common genera were *Bacillus* (74.5%) and *Geobacillus* (18.1%). Overall, *B. licheniformis* (31.9%), *B. thermoamylovorans* (16.0%), and *B. cereus* group spp. (13.3%) represented the most commonly isolated species for this source. Fifty-four different AT were identified; AT

1 (*B. licheniformis*), 380 (*B. cereus* Group spp.), and 439 (*Geobacillus* spp.) were the most common. Similar to AW, we did not isolate any *Anoxybacillus* spp. from this plant.

In previous studies (Rückert et al., 2004; Postollec et al., 2012; Yuan et al., 2012) that characterized spore species in dairy powders, the common genera and species identified included *B. licheniformis*, *Anoxybacillus* spp. (typically *A. flavithermus*), and *Geobacillus* spp. For example, Yuan et al. (2012) reported that *B. licheniformis* was the most frequently isolated spore species from dairy powders collected from 22 different dairy powder processing plants in China. Other studies (Rückert et al., 2004; Yuan et al., 2012) have reported that *Anoxybacillus* and *Bacillus* spores were widely distributed among WMP and NDM dairy powders. Interestingly, we did not isolate *Anoxybacillus* spp. from AW or WPC-80 samples. Therefore, at least in the current study, we did not find that *Anoxybacillus* spores are widely distributed among powder processing facilities. Further sampling of other AW and WPC-80 powder samples will be necessary to confirm whether this result is reflective of a plant- or product-specific phenomenon.

Overall, our results confirm that spores of *Bacillus* spp. and *Geobacillus* spp. are the most prominent among dairy powders produced in the northeastern United States. Whereas spores of *Bacillus* spp. seem to be ubiquitously distributed among all dairy powders, *Geobacillus* and *Anoxybacillus* spp. are not.

Spore Populations Isolated from Dairy Powder and BT Raw Milk Are Significantly Different

The large variety of species and AT identified among the spore isolates was further investigated to determine if the spore populations of BT raw milk and the 4 dairy powder plants were significantly different. Our initial analysis revealed that spore populations in BT raw milk samples were significantly different ($P = 0.001$; ANOSIM analysis of spore isolates from SSP samples only) from spore populations isolated from dairy powder plant samples. To further discriminate specific differences between sources, we performed nonmetric dimensional scaling and ANOSIM analyses using all spore isolates (i.e., those collected from both HHR and SSP dairy powder plant samples and SSP BT raw milk samples). These analyses showed that the spore population isolated from BT raw milk was significantly different from the spore populations isolated from NDM, WPC-80, and SW samples (Table 4), even though silo raw milk isolates were included in the NDM spore population. The SW samples contained spore populations that were significantly different from the spore populations in AW, WPC-80, and BT raw milk

Table 4. Significance of pairwise comparisons between spore populations isolated from acid whey, nonfat dry milk, sweet whey, or whey protein concentrate 80 dairy powders and BT raw milk samples

Sample source ^{1,2}	Sample source ^{1,2,3}			
	AW ^a	BT raw milk ^a	NDM ^b	SW ^b
BT raw milk ^a	0.35	—	—	—
NDM ^b	0.01	0.01	—	—
SW ^b	0.01	0.01	0.45	—
WPC-80 ^c	0.01	0.01	0.01	0.01

¹Sample source: AW = acid whey; BT = bulk tank; NDM = nonfat dry milk, SW = sweet whey; WPC-80 = whey protein concentrate 80.

²Superscripts (a,b) show grouping based on statistical differences observed between spore populations from each group; sample sources that share the same superscript do not show significant differences in spore populations.

³Holm-Bonferroni-corrected *P*-value for multiple pairwise comparisons of significant differences in the analysis of similarities (ANOSIM) statistic between different sample sources.

samples (Table 4), but not from the spore population in NDM samples. Consistent with our finding that a large number of *Anoxybacillus* spp. were isolated from NDM and SW samples, the spore populations isolated from these sources were significantly different from that of AW samples. Finally, WPC-80 samples contained spore populations that were significantly different from AW, BT raw milk, NDM, and SW samples (Table 4).

The finding that significant differences exist among the spore populations isolated from BT raw milk and the different dairy powder-processing plant samples examined in our study was not surprising given the large differences in both the processing environments and the raw and finished product samples obtained. However, the differences in the observed spore populations from particular sources were somewhat unexpected (e.g., BT raw milk and NDM). Several key explanations exist for the observed differences between spore populations from the various sources analyzed in our study.

The differences in the overall composition of each dairy powder product, and especially in the raw materials used in the manufacture of each dairy powder, could have affected the resulting spore populations. Interestingly, the spore population isolated from BT raw milk and NDM samples were significantly different, suggesting that, at least in this specific case, BT raw milk is likely not the only source of spores in the finished product. It is important to note that although the BT raw milk collected and analyzed in our study originated from dairy farms located in the same geographic regions as the powder processing facilities, information on the specific BT raw milk sources used by this NDM powder processing facility, as well as the raw milk used to produce the raw materials (e.g., acid whey) for the other powder processing facilities, was not available to us. To our knowledge, no studies have specifically examined spore populations in liquid whey from cheese processing, which represented the raw materials for 3

out of 4 plants included in our study; however, spore-forming bacteria such as *Clostridium tyrobutyricum* are known to be present in cheese, suggesting that spores are likely present in whey by-products as well (Klijn et al., 1995; Bassi et al., 2013). Overall, our finding that the spore populations isolated from dairy powder plant and BT raw milk samples were significantly different implicates the processing environment as an important source of spore contamination.

Differences in manufacturing processes and environments could also have contributed to the observed differences among the spore populations from each source. Whereas the overall processing of each dairy powder was similar, the specific processes differed. For example, some processes included UF or reverse osmosis steps, which may affect levels of spores in milk (Elwell and Barbano, 2006; Tomasula et al., 2011). Differences in heat treatments used during processing could also have influenced spore levels. For example, the temperatures encountered during the processing run could have effectively reduced some heat-sensitive spore species or could have selected for certain spore species. It is also possible that some or all processing plants could have either harbored biofilms of a particular spore species, or acquired them during the processing run. The length of the processing run could affect whether exponential growth (and sloughing off) of spore-forming bacteria within biofilms established during the processing run occurs. All of these factors are important, as bacterial biofilms are a documented source of spore contamination (Scott et al., 2007).

Importantly, our data provide evidence that the spore populations among different dairy powders are not homogenous. We hypothesized that the observed differences in spore populations could be attributed to (1) differences in processing parameters (i.e., time of processing run, temperatures), (2) differences in the incoming spore loads due to the properties of raw ma-

terials (e.g., pH, total acidity), (3) differences in spore species found within the processing facility (e.g., the presence of biofilms), or (4) a combination of these factors.

***Bacillus licheniformis* Is Found Consistently in Both Raw Materials and Finished Dairy Powders**

Previous studies had suggested that the spore populations of raw milk and finished dairy powders were different (Rückert et al., 2004; Scott et al., 2007). Two possible explanations for this phenomenon have been proposed. First, biofilms of spore-forming bacteria within the plant environment are able to contaminate the product during the processing run (Scott et al., 2007; Burgess et al., 2010). Second, spores are concentrated in the product through the removal of water during the drying process, allowing spores that were previously present at levels below detection to be concentrated to detectable levels (Watterson et al., 2014). Overall, our data support both in-plant and raw material derived sources of spore contamination in dairy powders.

The ubiquitous distribution of *B. licheniformis* spores among both BT raw milk and raw materials collected from all 4 dairy powder plants suggests that *B. licheniformis* contamination of dairy powders often originates from contaminated raw materials. In particular, *B. licheniformis* AT 1 was isolated from both raw and WIP or finished samples for all 4 dairy powder plants (Figure 1). Additionally, 5 out of 7 total *B. licheniformis* AT isolated from the dairy powder plant sources were also isolated from BT raw milk samples (representing 99.3% of *B. licheniformis* isolates from all 4 powder plant sources). Furthermore, for the NDM processing plant, we isolated the same subtypes of *B. licheniformis* from both silo raw milk and finished powder (AT 1, 6, 9, 169), supporting our conclusion that the presence of *B. licheniformis* spores are a result of raw material contamination for this plant.

Interestingly, *Anoxybacillus* spore subtypes were isolated from both raw and WIP or finished samples for the SW processing plant. Other studies have isolated *Anoxybacillus* spores from finished dairy powders, but not from raw materials such as raw milk (Ronimus et al., 2003; Scott et al., 2007; Yuan et al., 2012). Importantly, the SW processing plant included whey powder as a raw ingredient, suggesting that the presence of *Anoxybacillus* spores in the finished powder could have resulted from raw material contamination. However, for the NDM processing plant, *Anoxybacillus* spores were only isolated from WIP or finished samples, suggesting that for this plant the contamination was likely due to in-plant sources. In addition, the same *Anoxybacillus* spore subtypes were isolated during all

6 sample collections, therefore supporting the idea of an in-plant contamination source for the NDM processing plant. *Anoxybacillus* spores are frequently isolated from dairy powders produced throughout the world, but have yet to be isolated from raw milk (Rückert et al., 2004; Coorevits et al., 2008; Postollec et al., 2012). Taken together, our results suggest that *Anoxybacillus* spore contamination of dairy powders may not solely be attributed to in-plant sources if dairy powders are included as a raw material.

Similar to *Anoxybacillus*, *Geobacillus* spores have been primarily associated with in-plant sources of contamination due to both their demonstrated ability to form biofilms in powder-processing plants (Burgess et al., 2010) and their being isolated at high levels from powders but present at levels below detection in BT raw milk (Scott et al., 2007; Burgess et al., 2010). In our study, *Geobacillus* spp. were isolated from both raw and WIP or finished product samples from the SW and WPC-80 processing plants. However, for these sources certain *Geobacillus* spore subtypes (AT 439 and 444) were isolated from both raw materials (including WPC-80, raw whey retentate, liquid whey) and finished product (Figure 1), whereas other subtypes (AT 357, 420, and 442) were exclusively isolated from WIP or finished powders. This suggests that, for certain plants, contamination of dairy powder products with *Geobacillus* spores is attributable to both raw material and in-plant contamination. For the NDM processing plant, *Geobacillus* spores (n = 60 isolates) were found exclusively in WIP or finished powder samples. For the NDM processing plant, in-plant contamination is likely the dominant source of *Geobacillus* spores found in the final powder.

Altogether, we found evidence to support both in-plant sources of spore contamination and the concentration of spores from low levels in raw materials to higher levels in finished dairy powders. One limitation of the identification method used in our study is that isolates were selected for *rpoB* AT identification based on differences in colony morphology. Therefore, our data limited our analyses to presence or absence and do not allow for quantification of a given spore species from a sample. The presence of multiple subtypes of a given spore genus in both raw materials and finished products implicates raw materials as an important source of spore contamination for some, but not all spore genera.

The Majority of Spore Genera Were Isolated by Both HHR and SSP Methods

Several different SCM exist and may be used in parallel to quantify spore loads in dairy powders (Bi-

Genus and species	AT	Source				
		A	B	C	D	E
<i>Aeribacillus pallidus</i>	355	0	0	0	1	0
	356	2	0	0	0	1
	359	0	3	0	2	1
	376	0	5	0	3	3
	433	0	0	0	0	1
	435	0	0	0	0	1
	491	0	0	0	1	0
	494	0	0	0	1	0
	523	0	0	0	0	1
<i>Anoxybacillus</i> spp.	353	122	35	0	0	0
	440	3	0	0	0	0
	509	1	0	0	0	0
<i>Bacillus aerophilus</i>	69	0	0	0	0	8
	135	0	1	0	1	4
	172	0	1	0	1	1
	350	0	0	0	1	0
	374	0	0	0	0	1
	381	0	0	0	0	1
	437	1	0	0	0	0
	449	0	1	0	0	0
<i>Bacillus cereus</i> s.l.	61	0	1	0	0	0
	90	0	0	0	0	1
	92	4	0	0	1	0
	120	1	2	0	0	0
	125	26	1	0	2	0
	128	0	0	0	0	2
	129	0	0	0	0	3
	148	0	0	0	0	1
	154	0	1	0	3	0
	194	0	0	0	0	1
	308	0	0	0	0	1
	362	0	0	0	0	2
	363	0	0	0	0	1
	365	0	0	0	0	1
	366	0	0	0	0	1
	380	22	0	0	18	4
	409	0	0	0	0	2
	410	0	0	0	0	3
	412	0	0	0	0	3
	417	0	0	0	0	1
	424	0	0	0	1	1
<i>Bacillus clausii</i>	463	2	0	0	0	0
	481	2	0	0	0	0
	55	0	2	1	0	11
	287	1	3	0	1	17
	318	0	2	1	0	1
	377	0	1	0	0	1
	418	0	0	0	0	1
<i>Bacillus coagulans</i>	354	0	2	0	3	1
	358	0	6	0	0	0
	487	7	0	0	0	0
	504	0	1	0	0	0
	505	0	1	0	0	0
<i>Bacillus firmus</i>	121	1	0	0	0	0
<i>Bacillus gibsonii</i>	497	0	1	0	0	0

Genus and species	AT	Source				
		A	B	C	D	E
<i>Bacillus horneckiae</i>	223	0	0	0	0	6
	240	0	0	0	0	2
	434	0	0	0	0	2
<i>Bacillus licheniformis</i>	1	17	111	15	28	159
	6	2	26	7	11	70
	9	0	7	1	1	10
	31	1	16	1	6	12
	169	0	7	1	14	29
	215	1	0	0	0	0
<i>Bacillus megaterium</i>	398	0	0	0	0	1
	428	0	0	0	0	1
	476	0	1	0	0	0
	151	0	0	0	0	3
	467	2	0	0	0	0
<i>Bacillus muralis</i> <i>Bacillus nealsonii</i>	455	3	0	1	0	0
	431	0	0	0	0	1
	288	0	0	0	0	5
	368	0	2	0	0	1
	393	0	0	0	1	1
	427	0	0	0	0	1
	448	0	1	0	0	0
	458	0	0	0	2	0
	460	1	0	0	1	0
	484	0	1	0	0	0
<i>Bacillus</i> <i>neutrophilus/sojae</i> <i>Bacillus pumilus</i>	498	0	0	0	1	0
	510	0	0	1	0	0
	382	0	1	0	0	1
	20	0	7	0	0	15
	47	0	2	0	0	4
	62	0	1	0	0	5
	68	0	4	0	0	11
	72	0	0	0	0	2
	137	0	4	0	0	2
	144	0	5	0	0	4
<i>Bacillus safensis</i>	175	0	0	0	1	1
	337	0	0	0	0	9
	373	0	0	0	0	1
	485	0	1	0	0	0
	106	1	0	0	1	1
	124	0	0	0	1	0
	140	1	0	0	0	0
	235	0	0	0	0	2
	254	0	4	0	0	1
	370	0	0	0	0	1
<i>Bacillus smithii</i>	371	0	0	1	0	1
	378	0	0	0	0	4
	384	0	0	0	0	1
	415	0	0	0	0	1
	426	0	0	0	0	1
	436	0	0	0	0	1
	486	1	0	0	0	0
	446	1	0	0	0	0
	447	1	0	0	0	0
	461	0	0	0	1	0
	506	0	0	0	1	0

Figure 1. Stratification of *rpoB* allelic types (AT) collected from dairy powder processing samples (A = sweet whey, B = nonfat dry milk, C = acid whey, D = whey protein concentrate 80) and (E) bulk tank raw milk, by general source. Shaded cells represent spores isolated from raw materials (dark gray-shaded cells), work-in process or finished product (light gray-shaded cells), or from both raw and work-in-process or finished product samples (black-shaded cells). Numbers within cells represent the number of isolates of a specific AT isolated from a given source.

Genus and species	AT	Source				
		A	B	C	D	E
<i>Bacillus subtilis</i> s.l.	65	0	2	0	1	7
	66	6	0	0	1	7
	211	1	0	0	1	0
	231	1	0	0	0	2
	262	0	0	0	0	2
	330	0	2	0	0	0
	331	0	0	0	0	1
	360	0	0	0	0	1
	361	0	0	0	1	3
	372	0	0	0	0	2
	375	0	0	0	1	2
	405	0	0	0	0	2
<i>Bacillus thermoamylovorans</i>	352	0	4	1	10	18
	385	0	0	0	0	1
	386	0	1	0	0	1
	387	0	0	0	0	4
	397	0	0	0	2	6
	399	0	0	0	0	1
	400	0	0	0	6	4
	401	0	1	0	4	1
	402	0	0	0	1	1
	422	0	0	0	0	1
	483	0	1	0	0	0
	488	0	1	0	3	0
	489	0	0	0	1	0
	495	0	0	0	1	0
	499	0	0	0	1	0
	500	0	0	0	1	0
Other <i>Bacillus</i> spp.	71	0	0	0	0	1
	277	0	0	0	1	2
	396	0	0	0	0	1
	407	0	0	0	1	3
	414	0	1	0	0	1
	419	0	0	0	0	1
	459	0	0	0	1	0
	472	0	1	0	0	0
	477	0	2	0	0	0
	479	0	1	0	0	0
	514	0	0	0	0	1
	517	0	0	0	0	1
<i>Brevibacillus</i> spp.	430	0	0	0	0	1
	438	3	0	0	0	0
	518	0	0	0	0	1
	519	0	0	0	0	2
<i>Geobacillus</i> spp.	357	15	50	0	0	0
	420	0	0	0	2	1
	439	68	10	0	25	0
	441	1	0	0	0	0
	442	2	0	0	0	0
	444	4	0	0	7	0
<i>Lysinibacillus</i> spp.	294	0	1	0	0	1
	299	0	1	0	0	7
	303	0	3	0	2	3
	329	0	0	0	0	1
	364	0	0	0	0	1

Genus and species	AT	Source				
		A	B	C	D	E
<i>Lysinibacillus</i> spp.(cont.)	367	0	0	0	0	2
	383	0	0	0	0	1
	389	0	0	0	0	1
	404	0	0	0	0	1
	423	0	0	0	0	1
	268	0	0	0	0	1
<i>Oceanobacillus</i> spp.	392	0	1	0	0	2
	395	0	0	0	0	1
	406	0	0	0	0	1
	429	0	1	0	0	1
<i>Paenibacillus amylolyticus</i> s.l.	23	0	1	0	0	0
	93	0	0	0	0	1
	264	0	0	0	0	1
<i>Paenibacillus cookii</i>	502	0	1	0	0	0
	138	0	0	1	0	1
	332	0	0	0	0	2
<i>Paenibacillus glucanolyticus</i>	452	0	1	0	0	0
	159	1	0	0	0	0
	139	1	2	0	0	4
<i>Paenibacillus lactis</i>	425	0	0	0	0	1
	496	0	1	0	0	0
	451	0	1	0	0	0
<i>Paenibacillus lautus</i>	515	0	0	0	0	1
	343	0	0	0	0	1
	2	0	1	0	0	0
<i>Paenibacillus macerans</i>	50	0	1	0	0	0
	411	0	0	0	0	1
	67	0	0	0	0	1
<i>Paenibacillus peoriae</i>	179	0	0	0	0	2
	432	0	0	0	0	1
	379	0	0	0	0	2
Other <i>Paenibacillus</i> spp.	390	0	0	0	0	1
	450	0	1	0	0	0
	473	0	1	0	0	0
<i>Solibacillus</i> spp.	475	0	1	0	0	0
	490	0	0	0	1	0
	503	0	1	0	0	0
<i>Sporosarcina luteola</i>	507	1	0	0	0	0
	516	0	0	0	0	2
	520	0	0	0	0	1
<i>Terribacillus</i> spp.	521	0	0	0	0	1
	522	0	0	0	0	1
	126	0	0	0	0	1
<i>Ureibacillus</i> spp.	220	0	0	0	0	1
	471	0	0	1	0	0
	478	0	1	0	0	0
<i>Ureibacillus</i> spp.	456	0	1	1	0	0
	408	0	0	0	0	2
	351	0	3	0	1	1
<i>Ureibacillus</i> spp.	413	1	0	0	2	1
	468	0	1	0	0	0

Figure 1 (Continued). Stratification of *rpoB* allelic types (AT) collected from dairy powder processing samples (A = sweet whey, B = nonfat dry milk, C = acid whey, D = whey protein concentrate 80) and (E) bulk tank raw milk, by general source. Shaded cells represent spores isolated from raw materials (dark gray-shaded cells), work-in process or finished product (light gray-shaded cells), or from both raw and work-in-process or finished product samples (black-shaded cells). Numbers within cells represent the number of isolates of a specific AT isolated from a given source.

Table 5. Detection of spore genera using different spore pasteurization and spore count methods

Genus	Proportion of all isolates, ¹ %	No. of isolates obtained from each SP method ²				
		SSP			HHR	
		PSC ³	MSC ³	TSC ³	MSC	TSC
<i>Bacillus</i>	68.9	4	567	380	44	54
<i>Geobacillus</i>	12.1	—	—	85	—	100
<i>Anoxybacillus</i>	10.6	—	—	127	—	34
<i>Paenibacillus</i>	2.8	3	32	8	—	—
<i>Aeribacillus</i>	1.7	—	—	15	—	11
<i>Lysinibacillus</i>	1.7	—	26	—	—	—
<i>Ureibacillus</i>	0.7	—	—	8	—	2
<i>Oceanobacillus</i>	0.5	—	8	—	—	—
<i>Brevibacillus</i>	0.5	—	2	3	—	2
<i>Solibacillus</i>	0.3	1	3	—	—	—
<i>Sporosarcina</i>	0.1	—	2	—	—	—
<i>Terribacillus</i>	0.1	—	2	—	—	—

¹Proportion of isolates from all sources from a given genus, compared with all isolates (n = 1,523) analyzed.

²SP = spore pasteurization; SSP = standard spore pasteurization (80°C for 12 min); HHR = highly heat resistant (100°C for 30 min).

³PSC = psychrotolerant spore count (incubation of spore pasteurized samples at 6°C for 10 d); MSC = mesophilic spore count (incubation of spore pasteurized samples at 32°C for 48 h); TSC = thermophilic spore count (incubation of spore pasteurized samples at 55°C for 48 h).

envenue, 2014; Watterson et al., 2014). To assess the relationship between SCM and the spore genera that were detected, we stratified the spore isolates by SCM [consisting of either HHR or SSP heat treatments and subsequent incubation temperature (6, 32, or 55°C)] of the sample from which they had been isolated. In total, we analyzed 5 different SCM (we did not detect any spores from HHR pasteurized samples incubated at PSC temperatures). Overall, we found that 8 out of 12 spore genera were detected by at least 2 different SCM (Table 5).

Bacillus spore isolates were recovered from all 5 SCM. This result was not surprising given the broad range of growth temperatures reported among the *Bacillus* genera (De Vos et al., 2009). Importantly, this result suggests that *Bacillus* spores present in SP dairy powder samples will likely be detected by multiple SCM, although we were unable to determine from our present data set whether any differences exist in the quantity of spores that can be recovered by each method. Scheldeman et al. (2005) also found that *Bacillus* spores were detected from various environmental and BT raw milk samples receiving a HHR heat treatment. *Geobacillus* and *Anoxybacillus* spores were recovered from samples receiving either SSP or HHR SP but were restricted to SCM with incubation at 55°C.

No spore genera were exclusively isolated following HHR but not SSP heat treatments. Genera that were only isolated following SSP included *Paenibacillus*, *Lysinibacillus*, *Oceanobacillus*, *Solibacillus*, *Sporosarcina*, and *Terribacillus*. It should be noted that some of these isolates were recovered on very few occasions

(see Table 5; e.g., just 2 spore isolates were obtained for *Sporosarcina* and *Terribacillus* genera), and any conclusions drawn about their sensitivity to HHR pasteurization should be done with caution. Spore genera that were only isolated following sample incubation at 55°C (true thermophilic) include *Geobacillus*, *Anoxybacillus*, *Aeribacillus*, and *Ureibacillus*. Genera that were only isolated from MSC (incubation at 32°C) represented *Lysinibacillus*, *Oceanobacillus*, *Sporosarcina*, and *Terribacillus* spp. (Table 5). Importantly, this suggests that some spore species may not be detected by some SCM. For example, *Lysinibacillus* spores (n = 26 spore isolates) were only recovered from SSP (80°C for 12 min) with incubation at 32°C. Therefore, SCM using either HHR SP or incubation at 6 or 55°C would likely fail to detect *Lysinibacillus* spores. Overall, this finding suggests that SCM should be selected to enumerate the spore species of interest.

Other studies have reported isolating *Paenibacillus* spores from raw milk using multiple SCM (Coorevits et al., 2008). In contrast to our findings, Scheldeman et al. (2005) reported that *Paenibacillus* spp. were isolated from raw milk and environmental samples subjected to HHR SP. This result could be attributed to differences in species isolated or differences in levels of *Paenibacillus* spores present in the sample. The results of Scheldeman et al. (2005) largely confirm our finding that fewer spore genera are found in samples subjected to HHR pasteurization.

Whereas our analyses suggested that certain spores are capable of surviving various SP heat treatments and that the resulting bacteria were capable of growing

at multiple temperatures, further analyses will be beneficial in more definitively identifying which bacterial spores cannot be isolated from various SP methods. Importantly, our results highlight the potential for the redundant detection of the majority of spore-forming genera that were commonly isolated from BT raw milk and dairy powder samples.

CONCLUSIONS

Overall, we found that the spore populations isolated from a variety of dairy powder and BT raw milk samples were significantly different. A broad range of spore species were isolated from BT raw milk and the 4 dairy powder-processing plants. In general, *Bacillus* and *Geobacillus* represented the most frequently isolated spore genera. Very few spore subtypes were isolated solely from raw materials (i.e., not isolated from WIP or finished product), suggesting that spore contaminants found in BT raw milk are typically capable of persisting in the product throughout the processing run. Our results also suggest that several spore genera that are frequently isolated from dairy powders are commonly isolated using multiple SCM, suggesting that the simultaneous use of multiple methods may provide redundant information. Our study provides important data regarding the ecology of spores present in a variety of different dairy powders. Altogether, our results suggest that controlling spore counts in raw materials, as well as limiting in-plant contamination, are both important for producing dairy powders with low spore counts.

ACKNOWLEDGMENTS

Funding for this project was provided by the Dairy Research Institute (Rosemont, IL) and the New York State Milk Promotion Advisory Board (Albany, NY) through the New York State Department of Agriculture and Markets (Albany, NY). The authors thank the members of the Cornell University Milk Quality Improvement Program and the Cornell University Food Safety Laboratory (Cornell University, Ithaca, NY) for their invaluable assistance with sample processing and technical support. A special thank you to the Cornell Statistical Consulting Unit (Cornell University) for their advice with the statistical analyses performed. Finally, we thank the dairy farms and powder processing facilities that voluntarily participated in this study.

REFERENCES

Bassi, D., C. Fontana, S. Gazzola, E. Pietta, E. Puglisi, F. Cappa, and P. S. Cocconcelli. 2013. Draft genome sequence of *Clostridium*

- tyrobutyricum* strain UC7086, isolated from grana padano cheese with late-blowing defect. *Genome Anounc.* 1:e00164–13.
- Bienvenue, A. 2014. Spores from a global perspective. *Midwest Dairy Research Forum*. Accessed Apr. 3, 2015. http://northcentralcheese.org/wp-content/uploads/2014/10/Opp-for-Low-Spore-Milk-Powder_AB.pdf.
- Buehner, K. P., S. Anand, G. D. Djira, and A. Garcia. 2014. Prevalence of thermophilic bacteria and spores on 10 Midwest dairy farms. *J. Dairy Sci.* 97:6777–6784.
- Burgess, S. A., D. Lindsay, and S. H. Flint. 2010. Thermophilic bacilli and their importance in dairy processing. *Int. J. Food Microbiol.* 144:215–225.
- Coorevits, A., V. De Jonghe, J. Vandroemme, R. Reekmans, J. Heyrman, W. Messens, P. De Vos, and M. Heyndrickx. 2008. Comparative analysis of the diversity of aerobic sporeforming bacteria in raw milk from organic and conventional dairy farms. *Syst. Appl. Microbiol.* 31:126–140.
- De Vos, P., G. M. Garrity, D. Jones, N. R. Krieg, W. Ludwig, F. A. Rainey, K. Schleifer, and W. B. Whitman. 2009. *Bergey's Manual of Systematic Bacteriology*, Vol. 3. The Firmicutes. Springer, New York, NY.
- Dréan, P., C. M. McAuley, S. C. Moore, N. Fegan, and E. M. Fox. 2015. Characterization of the spore-forming *Bacillus cereus sensu lato* group and *Clostridium perfringens* bacteria isolated from the Australian dairy farm environment. *BMC Microbiol.* 15:38.
- Durak, Z. M., H. I. Fromm, J. R. Huck, R. N. Zadoks, and K. J. Boor. 2006. Development of molecular typing methods for *Bacillus* spp. and *Paenibacillus* spp. isolated from fluid milk products. *J. Food Sci.* 71:50–56.
- Elwell, M. W., and D. M. Barbano. 2006. Use of microfiltration to improve fluid milk quality. *J. Dairy Sci.* 89:E10–E20.
- Frank, J. F., and A. E. Yousef. 2004. Tests for groups of microorganisms. Pages 227–248 in *Standard Methods for the Examination of Dairy Products*. 17th ed. H. M. Wehr and J. F. Frank, ed. Am. Public Health Assoc., Washington, DC.
- Huck, J. R., B. H. Hammond, S. C. Murphy, N. H. Woodcock, and K. J. Boor. 2007. Tracking spore-forming bacterial contaminants in fluid milk-processing systems. *J. Dairy Sci.* 90:4872–4883.
- Hwang, J.-Y., and J.-H. Park. 2015. Characteristics of enterotoxin distribution, hemolysis, lecithinase, and starch hydrolysis of *Bacillus cereus* isolated from infant formulas and ready-to-eat foods. *J. Dairy Sci.* 98:1652–1660.
- Ivy, R. A., M. L. Ranieri, N. H. Martin, H. C. den Bakker, B. M. Xavier, M. Wiedmann, and K. J. Boor. 2012. Identification and characterization of psychrotolerant sporeformers associated with fluid milk production and processing. *Appl. Environ. Microbiol.* 78:1853–1864.
- Klijn, N., F. J. Nieuwenhof, J. D. Hoolwerf, C. B. van der Waals, and A. H. Weerkamp. 1995. Identification of *Clostridium tyrobutyricum* as the causative agent of late blowing cheese by species-specific PCR amplification. *Appl. Environ. Microbiol.* 61:2919–2924.
- Kolstø, A.-B., N. J. Tourasse, and O. A. Okstad. 2009. What sets *Bacillus anthracis* apart from other *Bacillus* species? *Annu. Rev. Microbiol.* 63:451–476.
- Miller, R. A., D. J. Kent, K. J. Boor, N. H. Martin, and M. Wiedmann. 2015. Different management practices are associated with mesophilic and thermophilic spore levels in bulk tank raw milk. *J. Dairy Sci.* 98:4338–4351.
- Oksanen, J., F. G. Blanchet, R. Kindt, P. Legendre, P. R. Minchin, B. O'Hara, G. L. Simpson, P. Solymos, M. Henry, H. Stevens, and H. Wagner. 2015. *Community Ecology Package*. R package version 2.2–1. Accessed Apr. 6, 2015. <http://cran.r-project.org/web/packages/vegan/index.html>.
- Parker, S. G., S. H. Flint, J. S. Palmer, and J. D. Brooks. 2001. Factors influencing attachment of thermophilic bacilli to stainless steel. *J. Appl. Microbiol.* 90:901–908.
- Postollec, F., A.-G. Mathot, M. Bernard, M.-L. Divanac'h, S. Pavan, and D. Sohier. 2012. Tracking spore-forming bacteria in food: From natural biodiversity to selection by processes. *Int. J. Food Microbiol.* 158:1–8.

- Ronimus, R. S., L. E. Parker, N. Turner, S. Poudel, A. Rückert, and H. W. Morgan. 2003. A RAPD-based comparison of thermophilic bacilli from milk powders. *Int. J. Food Microbiol.* 85:45–61.
- Rückert, A., R. S. Ronimus, and H. W. Morgan. 2004. A RAPD-based survey of thermophilic bacilli in milk powders from different countries. *Int. J. Food Microbiol.* 96:263–272.
- Scheldeman, P., A. Pil, L. Herman, P. De Vos, and M. Heyndrickx. 2005. Incidence and diversity of potentially highly heat-resistant spores isolated at dairy farms. *Appl. Environ. Microbiol.* 71:1480–1494.
- Scott, S. A., J. D. Brooks, J. Rakonjac, K. M. R. Walker, and S. H. Flint. 2007. The formation of thermophilic spores during the manufacture of whole milk powder. *Int. J. Dairy Technol.* 60:109–117.
- Setlow, P. 2006. Spores of *Bacillus subtilis*: Their resistance to and killing by radiation, heat and chemicals. *J. Appl. Microbiol.* 101:514–525.
- te Giffel, M. C., A. Wagendorp, A. Herrewegh, and F. Driehuis. 2002. Bacterial spores in silage and raw milk. *Antonie Van Leeuwenhoek* 81:625–630.
- Tomasula, P. M., S. Mukhopadhyay, N. Datta, A. Porto-Fett, J. E. Call, J. B. Luchansky, J. Renye, and M. Tunick. 2011. Pilot-scale crossflow-microfiltration and pasteurization to remove spores of *Bacillus anthracis* (Sterne) from milk. *J. Dairy Sci.* 94:4277–4291.
- Watterson, M. J., D. J. Kent, K. J. Boor, M. Wiedmann, and N. H. Martin. 2014. Evaluation of dairy powder products implicates thermophilic sporeformers as the primary organisms of interest. *J. Dairy Sci.* 97:2487–2497.
- Witthuhn, M., G. Lücking, Z. Aatamer, M. Ehling-Schulz, and J. Hinrichs. 2011. Thermal resistance of aerobic spore formers isolated from food products. *J. Dairy Technol.* 64:486–493.
- Yuan, D.-D., G.-C. Liu, D.-Y. Ren, D. Zhang, L. Zhao, C.-P. Kan, Y.-Z. Yang, W. Ma, Y. Li, and L.-B. Zhang. 2012. A survey on occurrence of thermophilic bacilli in commercial powders in China. *Food Contr.* 25:752–757.
- Zwick, M. E., S. J. Joseph, X. Didelot, P. E. Chen, K. A. Bishop-Lilly, A. C. Stewart, K. Willner, N. Nolan, S. Lentz, M. K. Thomason, S. Sozhamannan, A. J. Mateczun, L. Du, and T. D. Read. 2012. Genomic characterization of the *Bacillus cereus sensu lato* species: Backdrop to the evolution of *B. anthracis*. *Genome Res.* 22:1512–1524.