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Development of a multiplex real-time PCR assay for the identification and quantification of group-specific *Bacillus* spp. and the genus *Paenibacillus*

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ABSTRACT

Spoilage microorganisms can occur at many points throughout food production systems. *Bacillus* spp. and *Paenibacillus* spp. are important aerobic spoilage bacteria in various sectors of the food industry. In this study, we developed a rapid detection and quantification technique for *Bacillus* group-specific and the genus *Paenibacillus* by using multiplex quantitative PCR (mqPCR). The 1st was the *Bacillus cereus* group containing *B. cereus* and *B. weihenstephanensis*; the 2nd was the *B. subtilis* group containing *B. subtilis*, *B. licheniformis*, *B. safensis*, and *B. pumilus*; the 3rd was the *B. simplex* group containing *B. megaterium* and *B. simplex*; and the 4th was the genus *Paenibacillus*. Depending on the assays, the detection limit was 10 copy numbers. In addition, mqPCR assays were validated by spiking potato salad and milk samples with four strains; *B. weihenstephanensis*, *B. licheniformis*, *B. megaterium*, and *P. lautus*. The detection dynamic range for potato salad was 10^5 CFU/mL–10^1 CFU/mL with *B. weihenstephanensis* and *B. licheniformis*, and 10^5 CFU/mL–10^2 CFU/mL with *B. megaterium* and *P. lautus*, while, for milk, all strains were 10^5 CFU/mL–10^2 CFU/mL. We also stored these food matrices spiked with four bacterial suspensions (approximately 10^3 CFU/mL) at various temperatures. Results showed that *B. weihenstephanensis* and *B. licheniformis* were able to grow in potato salad, whereas, the populations of *B. weihenstephanensis*, *B. licheniformis*, and *P. lautus* increased in milk. Consequently, the mqPCR assays developed here in facilitated the differentiation, quantification, and confirmation of the presence of the psychrophilic and psychrotolerant *Bacillus* group and *Paenibacillus* spp.

1. Introduction

Microbiological spoilage is a significant cause of food loss despite developments in food preservation techniques (Gram et al., 2002; Samapundo et al., 2014; Trmčić et al., 2015). Nearly one-third of all food products worldwide are estimated to be lost postharvest, and much of this loss can be attributed to microbial spoilage (Gustavsson et al., 2011). Among the food spoilage microorganisms, the psychrotolerant endospore forming bacteria, *Bacillus* spp. and *Paenibacillus* spp. have potential to survive conventional pasteurization regimens such as heat pasteurization and can grow during refrigerated temperature storage, resulting in off-flavors and curdling in the final products (Ivy et al., 2012).

Heat treatments are commonly applied as pasteurization and commercial sterilization techniques, which can be applied for the inactivation of vegetative microorganisms; however, they are not completely effective against microbial spores. Spore-forming microorganisms are more heat resistant and are not inactivated by pasteurization. Consequently spore-forming bacteria limit the shelf life of pasteurized foods with a neutral or mildly acidic pH. Thus, to avoid the germination and outgrowth of surviving spores, pasteurized foods are stored at refrigeration temperature. Furthermore, additional hurdle technologies such as low pH, the addition of antimicrobial compounds, low water activity (Samapundo et al., 2014), and advanced packaging techniques are often combined with chilled storage. These techniques including modified gas composition, vacuum, and active modified atmosphere can extend the shelf life but not prevent spoilage by either aerobic or anaerobic spore-forming bacteria (Helmond et al., 2017).

Because the introduction of these microorganisms can occur at many points throughout food production systems, including in raw materials both before and after treatment, and in the processing plant environment, controlling and reducing the presence of spore-forming microorganisms is a considerable challenge (Durak et al., 2006; Pereira and Sant'Ana, 2018). Taken together, these phenomena have raised challenges for the food industry in the prevention of spoilage without loss of food quality (Helmond et al., 2017).

Psychrotolerant spore-formers may have multiple potential entry points through raw material preparation and the final products (Huck et al., 2007), and are capable of not only surviving heat treatments commonly used in the food processing lines but also germinating in

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foods that are held under refrigeration temperatures (Ivy et al., 2012). Psychrotolerant *Bacillus* spp. and *Paenibacillus* spp. are important aerobic spoilage bacteria in various sectors of the food industry. A number of studies have reported the detection of spoilage *Bacillus* spp. on non-dairy food, including bread, liquid eggs, seafood, and sous vide products, further illustrating the importance of spore-forming bacilli in food-processing system (Cabo et al., 2009; Coton et al., 2011; Fangio et al., 2010; Jan et al., 2011; Sorokulova et al., 2003). *Paenibacillus* spp. have been recognized as spoilage microorganisms of pasteurized milk, dairy products; unpasteurized, chilled ready-to-eat meals; pasteurized vegetable purees; and new starchy foods. These include *Paenibacillus odorifer*, *P. graminis*, *P. peoriae*, *P. amyloluticus*, *P. lautus*, *P. pabuli*, *P. terrae*, *P. macquariensis,* and *P. polymyxa,* (Durak et al., 2006; Guinebretiere et al., 2001; Huck et al., 2007; Helmond et al., 2017; Ivy et al., 2012; Trmčić et al., 2015).

The rapid identification and enumeration of spoilage microorganisms are of prominent importance to the food industry and related agencies, and classical microbiological methods require multiple culturing steps for the isolation and identification of microbes, which may not provide results quickly enough for the implementation of appropriate interventions. Furthermore, these methods are not always appropriate since some spoilage microorganisms fail to grow in culture media.

Moreover, nucleic acid-based methods, such as quantitative PCR (qPCR)-based identification and quantification, provide a suitable alternative because they are comparatively easy, rapid, and have a high level of sensitivity. Several publications on pathogens in food have been proposed and validated the use of qPCR for a wide range and variety of microorganisms, with an emphasis on the main foodborne pathogens responsible for important medical and economic outbreaks including *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Staphylococcus aureus*, among others (Rodŕiguez-Lázaro et al., 2013).

To improve the ability to detect contamination in the food industry, rapid identification and quantification techniques are needed for the microbial populations. Currently, multiplex qPCR (mqPCR) assays have been a focus of development (Ercolini et al., 2007; Forghani et al., 2016; Pal et al., 2010; Toplak et al., 2012), allowing the simultaneous quantification of more than one target sequence in a single reaction by using different sets of primer and probe pairs. For instance, using TaqMan chemistry, several sequence-specific probes can be labeled with different fluorescence dyes and multiple targets can be coamplified and quantified within a single reaction (Chapela et al., 2015). Therefore, mqPCR detection provides several advantages, including a reduction of sample consumption and analysis time, as well as being cost effective. Thus, laboratory efforts are maintained at a minimum (Janse et al., 2010).

Accordingly, the aim of this study was to develop new primer and probe pairs for the rapid identification and quantification of *Bacillus* group-specific spp. and the genus *Paenibacillus* by using single PCR and mqPCR. In this study, four individual target primers and probes were evaluated using TaqMan chemistry. The 1st was the *Bacillus cereus* group, named *B. cereus sensu lato* containing *B. cereus sensu stricto*, *B. anthracis*, *B. thuringiensis*, *B. weihenstephanensis*, *B. mycoides*, *B. pseudomycoides*, *B. cytotoxicus*, *B. toyonensis*. *B. cereus* and *B. wiedmannii*; the *B. cereus* group contains closely related pathogens of mammals and insects (Qi et al., 2001). *B. weihenstephanensis* has been characterized as a psychrotolerant subgroup of *B. cereus* (Lechner et al., 1998; Stenfors and Granum, 2001) because of their ability to grow between 4 and 7 °C, but not at 43 °C (Huck et al., 2007). The 2nd *B. subtilis* group contained *B. subtilis*, *B. amyloliquefaciens. B. licheniformis*, *B. pumilus. B. safensis*, and *B. xiamenensis*, which have been characterized as psychrotolerant, further highlighting the spoilage potential of species present in refrigerated products (Guinebretiere et al., 2001; Shehata et al., 1971). In particular, *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, *B. pumilus*, and *B. safensis* were commonly found as dairy-associated spore-formers isolated from raw milk (Huck et al., 2007), dairy cattle feed (Huck et al., 2008). Thus, this 2nd target group was focused on these species. The 3rd was the *B. simplex* group containing *B. megaterium* and *B. simplex*, which have been reported to be capable of producing a heat-stable toxin without vacuolating activity, revealing a similar physical characteristic to the *B cereus* emetic toxin cereulide (Taylor et al., 2005). The 4th group was the genus *Paenibacillus*, and we tested species including; *P. lautus, P. amylolyticus, P. macquaeriensis, P. odorifer*, *P. pabili, P. terrae, P. polymyxa, P. pini, P. thiaminolyticus,* and two of *Paenibacillus* spp. The purposes of the present study were to develop an mqPCR assay for the simultaneous identification and quantification of *Bacillus* group-specific spp. and *Paenibacillus* spp. and to evaluate the performance of this method in food and beverage samples. The specificity and sensitivity of the novel designed primer and probe pairs were evaluated for target and nontarget species together with *in silico* analysis. *In silico* analysis was applied to ascertain not only sequence mismatches of target loci within taxa that are and are not closely related strains but also polymorphism conformation for different strains of the same species from the vast amounts of biological information available.

2. Materials and methods

2.1. Primer and probe design

The target genes for the development of specific primer and probe pairs for the qPCR and mqPCR assays included 16S rRNA, *rpoB*, *spo0A*, *motB*. The 16S rRNA gene sequences from 250 bacterial species were obtained from the GenBank nucleotide database at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/genbank/), the National Institute of Technology and Evaluation Biological Resource Center (NBRC) (http://www.nbrc.nite.go.jp/e/), and the RIKEN BRC through the National BioResource Project of NEXT/AMED, Japan (http://dna.brc.riken.jp/ja/). The *rpoB* gene sequences from 212 bacterial species, 82 *spo0A* gene sequences, and 201 *motB* gene sequences were obtained from GenBank. The retrieved sequences were aligned using the multiple alignment tool Clustal X (Thompson et al., 1997). Based on the alignment, specific probes and primers were manually selected using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi (Untregasser et al., 2007). Consequently, primers and probe sets successfully obtained and are listed in Table 1.

The qPCR was performed using TaqMan chemistry. According to the results of the alignment analysis, the primer and probe pairs for the 1st group and 2nd group were designed based on the sequence of the *rpoB* gene from *B. cereus* (JCM 20266) and *B. subtilis* (NBRC 13719^T), respectively. The 3rd and 4th group were based on the sequence of the 16S rRNA gene from *B. simplex* (JCM 12307^T) and *P. odorifer* (JCM 13339), respectively. All four TaqMan probes were labeled with the 6-carboxyfluorescein (FAM) reporter at the 5′ end and tetramethly-6-carboxyrhodamine (TAMRA) at the 3′ end. In addition, to evaluate the mqPCR assay, the group-specific probe for *B. subtilis* was also labeled at the 5′ end with the 6-carboxyrhodamine (VIC) reporter and at the 3′ end with nonfluorescent quencher minor groove binder (MGB).

2.2. Bacterial strains and genomic DNA extraction

A total of 55 strains, including *Bacillus*, *Paenibacillus*, and *Clostridium* strains and other close and distant genera, listed in Table 2, were used for the primer and probe specificity studies. The strains were obtained from various culture collections (Table 2) and maintained in the laboratory according to the reference information. The microbial genomic DNA was extracted using an Ultra clean DNA isolation kit (MO BIO Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions. Then, genomic DNA concentrations were determined using a spectrophotometer (μQuant, BioTek Instruments, Inc., VT, USA) and diluted to the appropriate concentration prior to use.

Table 1
Primers and probe sets used for multiplex qPCR assays.

Target group	Species[a]	Target gene	Primer and probe[b]	Oligonucleotide Sequences (5′-3′)[c]	T_m (°C)	Length	GC%	Amplicon length (bp)
1st *Bacillus cereus* group	*B. cereus*	*rpo*B	BG1-rpo2_F	GCACTTATGGGAGCGAACAT	56.3	20	50	284
	B. weihenstephanensis							
	B. anthracis[d]		BG1-rpo2_R	CTTACGATTGGACGTTGGTTG	56.5	21	47.62	
	B. thuringiensis$_d$							
	B. pseudomycoides$_d$		BG1-rpo2_P	FAM-GARCGCGTWGAAGCACGTGAAGTTTG-TAMRA	64.4	26	53.85	
	B. cytotoxicus$_d$							
2nd *Bacillus subtilis* group	*B. subtilis*	*rpo*B	BG2-rpo2_F	AACATGCARCGTCAGGCHGT	61	20	54.17	260
	B. licheniformis							
	B. safensis		BG2-rpo2_R	ACTTACGATCGGACGCTG	56	18	55.56	
	B. pumilus							
	B. amyloliquefaciens$_d$		BG2-rpo2_P	VIC-CAGCYTGCTGAARTTTGTCCGYTC-MGB	62	24	52.08	
	B. atrophaeus$_d$							
	B. glycinifermentans$_d$							
3rd *Bacillus simplex* group	*B. simplex*	16S rRNA	BG3–3_F	GGAATCTTCCGCAATGGAC	55.7	19	52.63	143
	B. megaterium		BG3–3_R	TGGCTTTCTGGTTAGGTACC	55.8	20	55	
	B. flexus$_d$		BG3–3_P	FAM-TCAAGGTACVAGCAGTTACTCTBG-TAMRA	58.4	24	47.22	
Paenibacillus spp. group		16S rRNA	P-82_F	TGGTAGTCCACGCCGTAAAC	60.1	20	55	207
			P-82_R	AGAGGGATGTCAAGACCTGGT	60.2	20	52.4	
			P-82_P	FAM-GTTAGGGGTTTCGATACCCTTGGTGC-TAMRA	63.9	26	53.85	

[a] The species included the strains by *in silico* analysis based on the individual primer/probe set.
[b] Primers and probes were designed based on the *B. cereus* JCM 20266T rpo gene, *B. subtilis* NBRS 13179T (ATCC 6051) rpo gene, *B. simplex* JCM 12307T (ATCC 49097) 16S rRNA gene, and *Paenibacillua odorifer* JCM 13339 16S rRNA gene. F, forward primer; R, reverse primer; P, probe.
[c] Primer design was carried out using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgo), and probes were designed manually.
[d] The species are assumed to be possible detection by sequence alignment together with the target strains as shown in supplementary materials.

2.3. Amplification conditions

Four primer and probe sets were used to study the qPCR and mqPCR assays. For the qPCR assay, the reactions were carried out in a 20-μL volume containing 10 μL 2× TaqMan Fast Advanced Master Mix (Life Technologies, Foster City, CA), 0.3 μM each individual primer, 0.25 μM each probe, and 2 μL microbial genomic DNA template for the specificity experiment (adjusted to a concentration of 2 ng to 3 ng/μL) in a QuantStudio 3 system (Life Technologies). For the mqPCR assays consisted of 0.15 μM each primer for BG1-rpo2, BG3–3, and P-82; 0.6 μM each primer for BG2-rpo2; and a probe concentration identical to be qPCR assay. The following one-step amplification protocol was identical in all the qPCR and mqPCR assays: 95 °C for 20 s, followed by 45 cycles of 95 °C for 1 s and 58 °C for 20 s. All of the reactions were run in duplicate and positive and nontemplate control reactions were included in each assays.

2.4. Specificity assays

The specificity of the primer and probe pairs was tested against genomic DNA in a volume of 2 μL (adjusted to a concentration of 2 ng/μL to 3 ng/μL) isolated from pure cultures of target organisms and nontarget organisms (Table 2). All reaction results obtained with a C_T value < 38 were scored as positive with all samples for the amplification of individual test strains: otherwise the C_T value was considered negative.

Furthermore, we also checked all of the primers and probes "*in silico*" with the region of target genes 16S rRNA genes and the *rpoB* genes of taxa that are and are not closely related, also ascertaining polymorphism within the target loci for multiple different species of the same strains. In general, a total of > 3 sequence mismatches located in the probe has a major influence on the efficiency of the TaqMan assay.

2.5. Standard curve preparation

For all qPCR and mqPCR assays, standard curves were prepared with DNA extracted from a pure bacterial culture of *B. subtilis* (NBRC 13719^T), *B. simplex* (JCM 12307^T), or *P. odorifer* (JCM 13339). The genomic DNA of *B. cereus* (JCM 20266) was purchased the RIKEN BRC. The genomic DNA of the individual standard strains *B. cereus*, *B. subtilis*, *B. simplex*, and *P. odorifer* was quantified using a spectrophotometer (μQuant, BioTek Instruments Inc., Winooski, VT, USA) and diluted to the appropriate concentration prior to use. Consequently, individual genomic DNA concentration were prepared a 10-fold serial dilution series in sterile distilled water as follows: *B. cereus* (9.6 ng to 0.96 fg/μL), *B. subtilis* (8.7 ng to 0.87 fg/μL), *B. simplex* (9.7 ng to 0.97 fg/μL), and *P. oforifer* (10.1 ng to 1.01 fg/μL). For each qPCR and mqPCR assay, the experiment was repeated three times on separate plates with triplicate qPCR per experiment (n = 3) to determine reproducibility. Nontemplate control reactions were included in each assay. The individual DNA concentration were converted to the equivalent genomic DNA copy number using the Bacteria *tuf* gene quantitative PCR kit according to the manufacturer's instructions (Takara Bio, Shiga, Japan).

2.6. Multiplex qPCR assay

The three tests for the mqPCR assays were performed using combinations of primer pairs as follows: BG1-rpo2 (1st *B. cereus* group) and BG2-rpo2 (2nd *B. subtilis* group), BG2-rpo2 and BG3–3 (3rd *B. simplex* group), BG2-rpo2 and P-82 (*Paenibacillus* spp.). All the probes, BG1-rpo2_P, BG2-rpo2_P, BG3–3_P, and P-82_P, were labeled with 6-carboxyfluorescein (FAM) at the 5′ end and with tetramethly-6-carboxyrhodamine (TAMRA) at the 3′ end. In addition, probe BG2-rpo2_P was labeled with the 6-carboxyrhodamine (VIC) reporter at the 5'end and with the nonfluorescent quencher minor globe binder (MGB) at the 3′ end. The amplification conditions are mentioned above, and the results were analyzed with the QuantStudio Design and Analysis software v1.2 (Life Technologies). From each amplification plot, a C_T value was calculated representing the cycle number at which the reporter dye fluorescence was detectable above an arbitrary threshold. The C_T values for each samples were determined by using the mean of triplicate samples.

2.7. Analysis of artificially contaminated food and beverages

The mqPCR assays were further evaluated *via* the identification and quantification of *B. weihenstephanensis*, *B. licheniformis*, *B. megaterium*, and *P. lautus* strains by spiking commercial potato salad and milk stored

Table 2
Bacterial strains used for assessment of primer specificity and the C_T values obtained in single and multiplex qPCR.

Species	Sources[a]	Single plex or multiplex qPCR	C_T values obtained in singleplex qPCR with[b]:					C_T values obtained in multiplex qPCR with[b]:					
		Primer probe pair[c]	BG1-rpo2	BG2-rpo2		BG3-3	P-82	BG1-rpo2/BG2-rpo2		BG2-rpo2/BG3-3		BG2-rpo2/P-82	
		Dye	FAM-TAMRA	FAM-TAMRA	VIC-MGB	FAM-TAMRA	FAM-TAMRA	FAM-TAMRA/VIC-MGB		VIC-MGB/FAM-TAMRA		VIC-MGB/FAM-TAMRA	
Bacillus subtilis	NBRC 13719^T (ATCC 6051)		–	19.99 ± 0.21	19.20 ± 0.25	–	–	–	18.35 ± 0.15	18.39 ± 0.03	–	19.0 ± 0.07	–
Bacillus coagurans	ATCC 80078		–	–	–	–	–	–	–	–	–	–	–
Bacilus licheniformis	NBRC 12200^T (ATCC 14580)		–	18.14 ± 0.21	18.89 ± 0.13	–	–	–	19.04 ± 0.08	18.72 ± 0.01	–	18.57 ± 0.17	–
Bacillus megaterium	JCM 2506 T (ATCC 14581)		–	–	–	19.07 ± 0.22	–	–	–	–	20.43 ± 0.23	–	–
Bacillus weihenstephanensis	NBRC 101238		19.46 ± 0.33	–	–	–	–	19.31 ± 0.04	–	–	–	–	–
Bacillus safensis	NBRC 100820		–	24.25 ± 0.09	24.75 ± 0.24	27.74 ± 0.69	–	–	23.41 ± 0.02	24.44 ± 0.06	27.51 ± 0.33	25.41 ± 0.20	–
Bacillus sporothermodurans	NBRC 108793		–	–	–	–	–	–	–	–	–	–	–
Bacilus pumilus	JCM 2508^T (ATCC 7061)		–	23.35 ± 0.25	24.93 ± 0.06	–	–	–	19.91 ± 0.12	24.53 ± 0.08	–	26.07 ± 0.12	–
Bacillus simplex	JCM 12307^T (ATCC 49097)		–	–	–	18.66 ± 0.09	–	–	–	–	19.05 ± 0.08	–	–
Bacillus infantis	JCM 13438^T		–	–	–	–	–	–	–	–	–	–	–
Bacillus cereus	JCM 20266		20.77 ± 0.12	–	–	–	–	20.77 ± 0.13	–	–	–	–	–
Bacillus cereus	NBRC 15305^T		20.02 ± 0.2	–	–	–	–	20.15 ± 0.11	–	–	–	–	–
Paenibacillus lautus	JCM 9073^T (ATCC 43898)		–	–	–	–	19.60 ± 0.098	–	–	–	–	–	20.60 ± 0.17
Paenibacillus amylolyticus	JCM 9906^T (ATCC 9995)		–	–	–	–	16.27 ± 0.12	–	–	–	–	–	16.50 ± 0.11
Paenibacillus macquaeriensis subsp *defensor*	JCM 14954^T (DSM 23149)		–	–	–	–	15.53 ± 0.41	–	–	–	–	–	16.00 ± 0.81
Paenibacillus odorifer	JCM 13339		–	–	–	–	15.49 ± 0.18	–	–	–	–	–	15.73 ± 0.13
Paenibacillus pabuli	JCM 9074^T (ATCC 43899)		–	–	–	–	15.80 ± 0.034	–	–	–	–	–	16.84 ± 0.016
Paenibacillus terrae	JCM 11466^T		–	–	–	–	15.77 ± 0.23	–	–	–	–	–	16.20 ± 0.91
Paenibacillus polymyxa	JCM 2507^T (ATCC 842)		–	–	–	–	17.19 ± 0.05	–	–	–	–	–	16.64 ± 0.43
Paenibacillus pini	JCM 16418^T		–	–	–	–	16.98 ± 0.87	–	–	–	–	–	17.22 ± 0.02
Paenibacillus thiaminolyticus	JCM 7540		–	–	–	–	17.40 ± 0.13	–	–	–	–	–	17.77 ± 0.23
Paenibacillus sp.	NBRC 13157		–	–	–	–	16.82 ± 0.22	–	–	–	–	–	16.77 ± 0.06
Paenibacillus sp.	NBRC 13637		–	–	–	–	16.48 ± 0.25	–	–	–	–	–	16.93 ± 0.23
Clostridium acetobutylicum	NBRC 13948^T (ATCC 824)		–	–	–	–	37.0 ± 0.18	–	–	–	–	–	36.55 ± 0.44
Clostridium clariflavum	NBRC 101661^T (DSM 19732)		–	–	–	–	–	–	–	–	–	–	–
Clostridium thermocellum	NBRC 103400^T (ATCC 27405)		–	–	–	–	–	–	–	–	–	–	–
Clostridium kluyveri	NBRC 12016^T (DSM 555)		–	–	–	–	–	–	–	–	–	–	–

(*continued on next page*)

Table 2 (*continued*)

Species	Sources[a]	Single plex or multiplex qPCR	C_T values obtained in singleplex qPCR with[b]:					C_T values obtained in multiplex qPCR with[b]:					
		Primer probe pair[c]	BG1-rpo2	BG2-rpo2		BG3-3	P-82	BG1-rpo2/BG2-rpo2		BG2-rpo2/BG3-3		BG2-rpo2/P-82	
		Dye	FAM-TAMRA	FAM-TAMRA	VIC-MGB	FAM-TAMRA	FAM-TAMRA	FAM-TAMRA/VIC-MGB		VIC-MGB/FAM-TAMRA		VIC-MGB/FAM-TAMRA	
Clostridium difficile	JCM 1296^T		–	–	–	–	–	–	–	–	–	–	–
Hathewaya histolyticum	JCM 1403^T		–	–	–	–	–	–	–	–	–	–	–
Clostridium perfringens	JCM 1290^T		–	–	–	–	–	–	–	–	–	–	–
Clostridium pasteurianum	JCM 1408^T		–	–	–	–	–	–	–	–	–	–	–
Clostridium symbiosum	JCM 1297^T		–	–	–	–	–	–	–	–	–	–	–
Clostridium sporogenes	NBRC 14293		–	–	–	–	–	–	–	–	–	–	–
Geobacillus stearothermophilus	NBRC 12550^T (ATCC 12980)		–	–	–	–	–	–	–	–	–	–	–
Geobacillus caldoxylosilycus	NBRC 107762^T (ATCC 700356)		–	–	–	–	–	–	–	–	–	–	–
Geobacillus jurassicus	NBRC 107824^T (DSM 15726)		–	–	–	–	–	–	–	–	–	–	–
Geobacillus kaustophilus	NBRC 102445^T (ATCC 8005)		–	–	–	–	–	–	–	–	–	–	–
Geobacillus thermoglucosidasius	NBRC 107763^T (ATCC 43742)		–	–	–	–	–	–	–	–	–	–	–
Geobacillus toebii	NBRC 107807^T (DSM 14590)		–	–	–	–	–	–	–	–	–	–	–
Geobacillus zalhae	NBRC 101842^T (DSM 18318)		–	–	–	–	–	–	–	–	–	–	–
Oceanobacillus sojae	NBRC 105379^T (ATCC 105379)		–	–	–	–	–	–	–	–	–	–	–
Sporosarcina globispora	JCM 10046^T (ATCC 23301)		–	–	–	–	–	–	–	–	–	–	–
Sporosarcina psychrophila	JCM 9075^T (IFO 15381)		–	–	–	–	–	–	–	–	–	–	–
Lactobacillus casei	JCM 1134^T		–	–	–	–	–	–	–	–	–	–	–
Lactobacillus lactic supsp. *Lactis*	JCM 5805^T		–	–	–	–	–	–	–	–	–	–	–
Lysinibacillus boronitolerans	JCM 21713^T		–	–	–	–	–	–	–	–	–	–	–
Brevibacillus brevis	JCM 6285		–	–	–	–	–	–	–	–	–	–	–
Brevibacillus brevis	NBRC 100599		–	–	–	–	–	–	–	–	–	–	–
Enterococcus faecalis	NBRC 100481		–	–	–	–	–	–	–	–	–	–	–
Staphylococcus aureus	NBRC 100910^T (ATCC 12600)		–	–	–	–	–	–	–	–	–	–	–
Pseudomonas putida	JCM 6156		–	–	–	–	–	–	–	–	–	–	–
Escherichia coli	NBRC 102203^T (ATCC 11775)		–	–	–	37.1 ± 1.6	–	–	–	–	–	–	–
Ammonifex spp.	NBRC 100904		–	–	–	–	–	–	–	–	–	–	–
Thermus thermophilus	NBRC 101084^T		–	–	–	–	–	–	–	–	–	–	–

(*continued on next page*)

Table 2 (*continued*)

Species	Sources[a]	Single plex or multiplex qPCR	C_T values obtained in singleplex qPCR with[b]:						C_T values obtained in multiplex qPCR with[b]:					
		Primer probe pair[c]	BG1-rpo2	BG2-rpo2		BG3-3	P-82		BG1-rpo2/BG2-rpo2		BG2-rpo2/BG3-3		BG2-rpo2/P-82	
		Dye	FAM-TAMRA	FAM-TAMRA	VIC-MGB	FAM-TAMRA	FAM-TAMRA	FAM-TAMRA/VIC-MGB		VIC-MGB/FAM-TAMRA		VIC-MGB/FAM-TAMRA		VIC-MGB/FAM-TAMRA
Deferibacter desulfuricans	NBRC 101012^T		–	–	–	–	–	–	–	–	–	–	–	–

[a] NBRC, NITE Biological Resource Center (Kisarazu, Chiba, Japan); JCM, the RIKEN BRC through the National BioResource Project of the NEXT/AMED (Tsukuba, Ibaraki, Japan): T, type strain. The references in parentheses indicate the corresponding reference number in an alternative collection,

[b] C_T values are the mean values ± standard deviations of duplicates. —, no detection above the threshold before cycle 38.

[c] Primer and probe information are detailed in Table 1.

at room temperature with these strains. The strains were cultured in trypticase soy bean broth up to optical densities of 0.3–0.4 (approximately 10^7–10^8 CFU/mL) for strains *B. weihenstephanensis* and *B. licheniformis* and 0.6–0.7 (approximately 10^7 CFU/nL) for *B. megaterium* and *P. lautus* at 600 nm. The vegetative cells harvested by centrifugation at 7800 ×*g* for 10 min at 4 °C. The resultant pellets were resuspended in phosphate buffered saline and washed using repeated centrifugation. Subsequently, individual cell suspensions were serially diluted to approximately 10^5–10^1 CFU/mL, and 50 μL of individual cell suspensions were spiked into 2 mL of milk and 350–360 mg of potato salad. Immediately after spiking, microbial genomic DNA was extracted using a NucleoSpin Food kit (MACHEREY-NAGEL, GmnH & Co. KG, Düren, Germany) according to the manufacturer's instructions with minor modifications. Briefly, the milk sample was centrifuged at 16,000 ×*g* for 15 min at 4 °C, following which the pellet was resuspended in 550 μL *CF* solution (a lysis buffer) and 10 μL of proteinase K solution was added and incubated at 65 °C for 30 min (Provided in NucleoSpin Food kit). Following lysis treatment, pellets were crushed using 40–400 μL glass beads (NucleoSpin Bead Tubes Type B) from the NucleoSpin Microbial DNA Kit and agitated on a swing mill using TurboMix Vortex attachment (Scientific Industries, Inc. Bohemia, NY, U.S.A.) for 15 min at maximum speed. Thereafter, genomic DNA was extracted using a NucleoSpin Food kit according to the manufacturer's instructions. Potato salad samples were resuspended in 550 μL *CF* solution, following which 10 μL of proteinase K solution was added to the samples and incubated at 65 °C for 30 min without a centrifugation step. Subsequently, the samples were crushed using glass beads as mentioned previously. Aliquots (5 μL) of microbial genomic DNA templates were used for mqPCR assays and the cycle threshold values were plotted. All samples and measurements were performed in duplicate, and all experiments were repeated twice to evaluate the reproducibility of the mqPCR assay.

Furthermore, strain cell suspensions (approximately 10^3 CFU/mL) were spiked into 350–360 mg of potato salad and 1.8 mL of milk, then the samples incubated for 10 days at 7 °C, 15 °C, and 22 °C; thereafter, microbial genomic DNA was extracted using the above mentioned method. Genomic DNA from artificially contaminated samples and non-inoculum potato salads were subjected to mqPCR assays and the cycle threshold values were plotted.

3. Results and discussion

3.1. Primer and probe design

Among the total of 22 primer and probe pairs designed and tested for the targeted 16S rRNA gene and *rpoB* gene, consequently, the primer and probe sets were successfully applied for the group-specific identification of the 1st *B. cereus* group, the 2nd *B. subtilis* group, 3rd *B. simplex* group, and genus *Paenibacillus* by qPCR and mqPCR (Table 1). Identification of the genus *Paenibacillus* resulted in poor specificity with false-positives for closely related species. In addition, in this study, the primer and probe designs were not successful based on the nucleotide sequence alignment of the *spoA* genes and *motB* genes due to the poorly conserved sequence within the individual group-specific intrasequences.

For the identification of endospore-forming *Firmicutes*, several functional molecular markers have been evaluated and proposed, including the *spoA* gene (Bueche et al., 2013), *motB* gene (Oliwa-Stasiak et al., 2011), *rpoB* gene (Ivy et al., 2012; Huck et al., 2007; Qi et al., 2001), and 16S rRNA gene (Fernández-No et al., 2011) as well as 16S−23S rRNA ITS region (Saikaly et al., 2007). The *spo0A* gene is a major regulator for the initiation of sporulation (Errington, 2003), and the *motB* gene a encoding a flagella motor protein is classified as an outer membrane protein (OmpA), which encodes a hypothetical protein that is used to differentiate *B. cereus* group species (Oliwa-Stasiak et al., 2011).

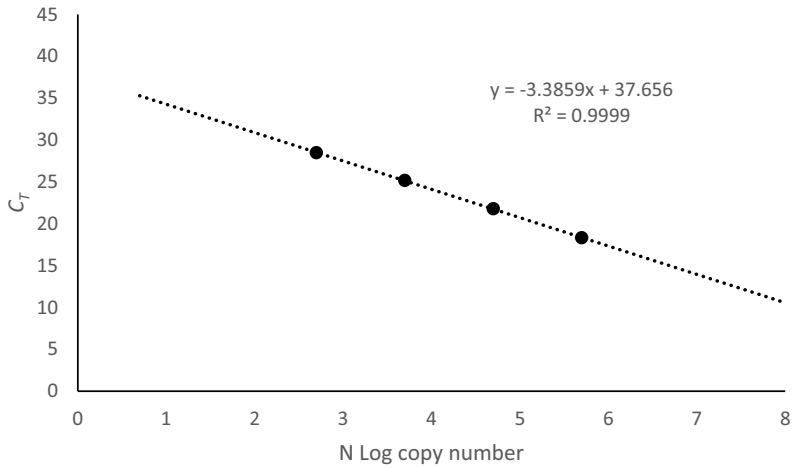

Fig. 1. Standard curve based on the TUF gene copy number quantitative PCR assay.

The early studies highlighted the difficulties of identifying a single set of PCR-based approaches allowing for discrimination and characterization of broad group of spoilage sporeformers, later studies illustrated that a combined *rpoB* and 16S rRNA gene based subtyping analysis not only provides a powerful tool for sensitive subtyping discrimination of *Bacillus* and *Paenibacillus* spp. but also may trace how these organisms contribute to spoilage through survival of heat treatment and the establishment of bacterial persistence in fluid milk processing systems (Durak et al., 2006; Huck et al., 2007).

3.2. Measurement of gene copy number of 16S rRNA gene based on the tuf gene

Here, we estimated microbial copy numbers of 16S rRNA gene to correct from individual genomic DNA using the bacterial *tuf* gene quantitative PCR kit, which was designed for rapid enumeration of bacterial genomic DNA derived from a wide range of bacterial species. The *tuf* gene, which encodes peptide elongation factor, is highly conserved, and has a low copy number (one to two) (Filler and Furano, 1981; Lathe III and Bork, 2001; Ludwig et al., 1990). This can be allowed to minimize the enumeration effect of bacterial counts using different target genes among multiple group-specific detection and quantification assays (Tanaka et al., 2010). The copy number of 16S rRNA gene in bacteria ranged from 1 to 15, with an average of 4.2 copies per genome. Moreover, an alternative core house-keeping gene, such as the *rpoB* gene, which has present at least one copy in all bacteria because of its essential role in cellular metabolism, could allow accurate measurement and avoid loss of phylogenetic resolution and biases in due to the presence of intragenomic heterogeneity (Case et al., 2007).

Multiple copies of the 16S rRNA gene are present in a given bacterium, *B. cereus* (range 10 to 16, mean value 13.6), *B. subtilis* (range 0 to 10, mean value 9.5), *B. simplex* (range 12 to 14, mean value 13), and *P. odorifer* (10 for all three species in DB, found at [https://rrndb.umms.med.umich.edu/]), and these intragenic copies can differ in sequence, leading to the identification of multiple ribotypes for a single bacterium (Case et al., 2007). Thus, it has bias for accurate determination of the cell number using the 16S rRNA gene in qPCR assay.

Our results generated, a linear correlation between *tuf* gene copy number/μL (5-log-copy number to 2-log-copy number/μL) and C_T value (18.32 ± 0.035, 21.78 ± 0.036, 25.16 ± 0.16, 28.48 ± 0.27). The mathematical equation that relates the copy number/μL *versus* the C_T value is as follows: y = −3.3859 (± 0.20) x + 37.656 (± 1.02) with an R^2 value of 0.9999 (± 0.0047) (Fig. 1). Based on the standard curve generated, the individual concentrated microbial template DNA average C_T value were as follows: *B. cereus* (15.73 ± 0.46, 9.6 ng/μL), *B. subtilis* (15.77 ± 0.26, 8.7 ng/μL), *B. simplex* (13.71 ± 0.37, 9.7 ng/μL), and *P. odorifer* (13.39 ± 0.24, 10.1 ng/μL). Consequently, the corresponding copy number for the concentrated DNA amounts of individual target species was estimated. Furthermore, individual standard curves were generated, and amplification parameters for both the qPCR and the mqPCR assays were summarized in Table 3. For the qPCR assays, the corresponding copy number for the concentrated DNA amounts of individual target species was estimated as follows: *B. cereus* (6.47 log and 1.47 log), *B. subtilis* (6.88 and 0.88 log, 6.69 log and 1.69 log), *B. simplex* (7.07 log and 1.07 log), and *P. odorifer* (7.16 log and 1.16 log) with qPCR assay (Table 3). Meanwhile, the simultaneous group–specific detection for mqPCR assays were generated between the *B. cereus* (6.06 log and 1.06 log) and *B. subtilis* (6.40 log and 1.40 log) group, the *B, subtilis* (6.33 log and 1.33 log) and *B. simplex* (6.47 log and 0.47 log) group, and the *B. subtilis* (6.37 log and 1.37 log) group and *Paenibacillus* spp. (6.29 log and 0.29 log) (Table 3). Overall, the detection range targeting *rpoB* and 16S rRNA was broader, covering a > 6-log-unit dynamic range detection with both the qPCR and the mqPCR assays.

3.3. Amplification parameters for the qPCR and mqPCR assays

For individual qPCR assays, targeting *B. cereus* and *B. subtilis*, the detection ranges encompassing BG1-rpo2 and BG2-rpo2, were slightly different between the dye used;6.47 to 1.47 log and 6.88 to 0.88 (FAM-TAMRA), and 6.69 to 1.69log (VIC-MGB), respectively, with obtained with a linear (both, R^2 > 0.997) 6 and 7-log-unit dynamic range detection. Meanwhile, the detection dynamic ranges encompassing BG3–3 and P-82, and targeting *B. simplex* and *P. odorifer*, were 7.07 to 1.07 log and 7.16 to 1.16 log, respectively, obtained with a linear (R^2 > 0.998, R^2 > 0.999) 7-log-unit dynamic range detection.

For individual mqPCR assay, the detection dynamic range of DNA showed a 6-log-unit target first, for *B. cereus* and second, for *B. subtilis*, with a linear values of R^2 > 0.98 and R^2 > 0.99, respectively. For the second, *B. subtilis* and the third, *B. simplex*, the group assay obtained indicated 6.33 to 1.33 log and 6.47 to 0.47log, respectively, with a linear (R^2 > 0.985 and > 0.998) 6 and 7-log-unit dynamic range detection. For the second, *B. subtilis* and the genus *Paenibacillus*, the group assay showed 6.37 to 1.37 log and 6.29 to 0.29 log, respectively, with a linear (R^2 > 0.98 and > 0.998) 6 and 7-log-unit dynamic range detection. A wide dynamic range detection is one of the key features of qPCR assay. The dynamic range cover should extend to 5 $\log_{10}$ to 6 $\log_{10}$ concentrations (Bustin et al., 2009). An R^2 value of 0.998 means that fit explains 99.8% of the total variation in the data about the average, thus, > 0.980 provides good confidence in

Table 3
Quantitative amplification parameters for the singleplex and multiplex PCR assays.

Parameter[a]	Singleplex PCR						Multiplex PCR[b, c]			
Taeget group	1st *B. cereus* group	2nd *B. subtilis* group	2nd *B. subtilis* group		3rd *B. simplex* group	*Paenibacillus* group	1st *B. cereus* group 2nd *B. subtilis* group	2nd *B. subtilis* group 3rd *B. simplex* group	2nd *B. subtilis* group *Paenibacillus* group
Target gene	*rpoB*	*rpoB*	*rpoB*		16S rRNA	16S rRNA	*rpoB*/rpoB	*rpoB*/16S rRNA	*rpoB*/16S rRNA
Dye	FAM-TAMRA	FAM-TAMRA	VIC-MGB		FAM-TAMRA	FAM-TAMRA	FAM-TAMRA VIC-MGB	VIC-MGB FAM-TAMRA	VIC-MGB FAM-TAMRA
Slope	−3.46 ± 0.15	−3.86 ± 0.04	−3.81 ± 0.07		−3.52 ± 0.05	−3.44 ± 0.025	−3.60 ± 0.08 −3.78 ± 0.01	−2.94 ± 0.10 −3.33 ± 0.08	−3.02 ± 0.13 −3.35 ± 0.06
y intercept	41.28 ± 0.70	39.50 ± 0.09	39.75 ± 0.26		36.22 ± 0.13	38.06 ± 0.042	40.40 ± 0.41 39.76 ± 0.13	35.09 ± 0.28 37.25 ± 0.15	35.67 ± 0.61 35.06 ± 0.24
R^2	0.998 ± 0.002	0.998 ± 0.0006	0.998 ± 0.002		0.998 ± 0.0009	0.999 ± 0.0001	0.999 ± 0.0004 0.997 ± 0.003	0.985 ± 0.001 0.998 ± 0.0004	0.98 ± 0.010 0.998 ± 0.0007
Efficiency	94.67 ± 5.49	81.46 ± 1.15	82.95 ± 2.06		92.41 ± 1.93	95.24 ± 0.97	89.54 ± 2.79 83.79 ± 0.42	119.27 ± 5.96 99.75 ± 3.65	110.3 ± 5.66 98.83 ± 2.57
Dynamic range of copy number[d] (log)	6.47–1.47	6.88–0.88	6.69–1.69		7.07–1.07	7.16–1.16	6.06–1.06 6.40–1.40	6.33–1.33 6.47–0.47	6.37–1.37 6.29–0.29

[a] The value for each parameter is the mean ± standard deviation, which was quantified individually in triplicate on the same plate ($n = 3$).
[b] The values of each parameter are shown in the upper detection group corresponding to the left column and the lower detection group shown in the right column.
[c] The upper and lower data correspond to the upper and lower primer and probe pairs with individual columns.
[d] The values of estimated copy number were determined from average values in triplicate on the same plate ($n = 3$) using TUF gene quantification bacterial kit.

correlating C_T and target copy number (Bustin and Huggett, 2017). These findings revealed no significant inhibition in the coamplification of the two targets in the same tubes.

The hallmarks of an optimized qPCR assay are; high amplification efficiency, a linear standard curve, consistency across replicate experiments, no primer dimer, and a wide dynamic range (Bustin and Huggett, 2017). The linear relationship makes the C_T value a reliable way to estimate the gene copy numbers per reaction. The slope from individual mqPCR assays ranged from −3.60 to −3.78 for the 1st and 2nd *Bacillus* group, −2.94 to −3.33 for the 2nd and 3rd *Bacillus group,* and −3.02 to −3.35 for 2nd *Bacillus* and genus *Paenibacillus* group. PCR efficiency was determined using the slope of a dilution curve based on the following equation: $E = 10^{-1/slope} - 1$, with $E = 1.0$ considered ideal. Therefore, a PCR efficiency of 100% is achieved when the slope is close to a theoretical value of −3.32 (Higuchi et al., 1993). A PCR reaction with lower efficiency will have lower sensitivity (Hilscher et al., 2005). In the present study, the amplification efficiencies of each target in the different mqPCR were between 83% and 119%, suggesting that the coamplification of the two targets in the same tubes was satisfactory, and revealing the mqPCR detection systems as sensitive and capable of delivering good linearly for the standard curves, as well as high PCR efficiency.

Optimization of a mqPCR assay poses several technical difficulties, including poor sensitivity or specificity and/or preferential amplification of certain specific targets (Polz and Cavanaugh, 1998). The presence of more than one primer pair in a mqPCR assay increases the chance of hybridization that can lead to spurious amplification reactions primarily because of primer dimer formation (Brownie et al., 1997). These nonspecific products may be amplified more efficiency than the desired target, thereby consuming reaction components and producing impaired annealing and extension rates. Thus, optimization should minimize and reduce such nonspecific interactions. Here this was achieved *via* the utilization of primers with nearly identical optimum annealing temperatures that did not display significant homology either internally or to one another.

The optimal amplicon length should be < 150 bp. In general, shorter amplicons are amplified more efficiently than longer amplicons and are more tolerant to sub-optimal reaction conditions (Chapela et al., 2015). Meanwhile, in practice, for many bacterial PCR amplicons, a longer amplicons can perform better than a shorter ones, since they have AT rich contents. Sometimes secondary structures simply cannot be avoided (Bustin and Huggett, 2017).

Additionally, statistical analysis revealed no significant differences ($P > 0.05$) among slopes, intercepts, R^2 values, and efficiencies, suggesting similar amplification performance between individual qPCR and mqPCR assays.

3.4. Specificity of the qPCR and mqPCR assays

The specificity of the primer and probe pairs in the present study was verified by qPCR and mqPCR using > 4 ng of genomic DNA from 55 strains, including closely related and unrelated strains (Table 2). When testing the specificity of the newly developed qPCR assays, the majority of nontarget species showed negative C_T values (C_T values > 38 were considered negative), with the exception of several species in which a fluorescence signal was detected but only at higher C_T values ($C_T \geq 36$) compared those of the target strains ($16 \leq C_T \leq 18$). The obtained results showed that the developed qPCR and mqPCR assays had higher specificity for the BG1–rpo2 and BG2-rpo2 primer and probe sets, demonstrating for all negative C_T values that a minimum of 4 ng of DNA is required to generate a positive signal for some nontarget strains. The 3rd *Bacillus* group-specific-primer and probe pair targeting the 16S rRNA gene BG3–3 generated a fluorescence signal to identify *B. simplex* and *B. megaterium* species. The BG3–3, successfully identified specifically the *B. simplex and B. megaterium*—except for one nontarget strain, *B. safensis* belonging to the 2nd *B. subtilis* group species, which elicited positive signals in the qPCR (27.74 ± 0.69) and mqPCR (27.51 ± 0.33)

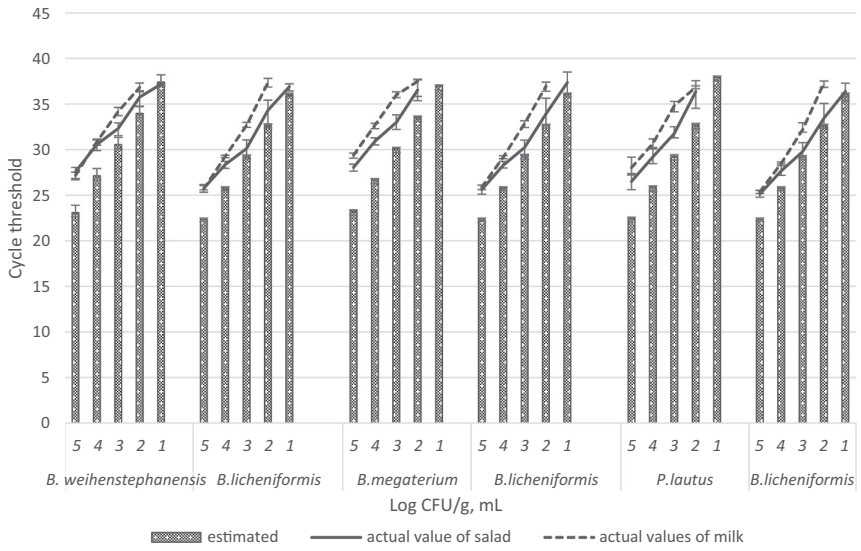

Fig. 2. The individual group-specific mqPCR assays of potato salad and milk samples artificially spiked with 10-fold dilutions of *B. weistephanenesis*, *B. licheniformis*, *B. megaterium,* and *P. lautus* microbial cells. Error bars represent duplicate samples and individuals quantified in triplicate on the same plate.

analyses, additionally, nontarget species belonging to nonrelated taxa for the slightly detectable strains *Escherichia coli,* with values of 37.1 ± 1.6 in the qPCR assay. The primer and probe pair P-82, targeting the 16S rRNA gene, generated a fluorescence signal with all the *Paenibacillus* spp. in the current experiment at lower C_T values ($15 \leq C_T \leq 19$) with both qPCR and mqPCR assays within the target organisms. However, P-82 resulted in weakly fluorescent C_T values within *Clostridium* spp. at C_T values ($36 \leq C_T \leq 38$), belonging to closely related taxa in the qPCR and mqPCR assays. These results suggested that probe P-82_P provided a mis-hybridization sequence region that should represent a specifically selected amplification area of 16S rRNA gene within these strains. Furthermore, the secondary and repetitive structure of the probe and primer sequences could lead to misbinding consequences resulting in amplification at high C_T values.

For the evaluation of these novel mqPCR assays, contradictory results were obtained, in which the sensitivity of the detection strain *B. pumilus* provided more sensitive C_T values (19.91 ± 0.12) with the mqPCR assay (1st and 2nd *Bacillus* group-specific detection assay) than at C_T (24.93 ± 0.06) with the single-plex qPCR assay. In contrast, for another two mqPCR assays (2nd and 3rd *Bacillus* group-specific detection assay, 2nd *Bacillus* and *Paenibacillus* spp. detection assay), the amplification results for strain *B. pumilus* provided C_T values of 24.53 ± 0.08 and 26.07 ± 0.12, respectively, which were similar to the values obtained with the single-plex qPCR assay. There results revealed the presence of sensitivity discrepancies occurred between the qPCR and mqPCR assays despite the use of DNA extracted from a pure culture of the strain *B. pumilus*. The reason for these sensitivity discrepancies among the qPCR and mqPCR assays remains unclear, but they could have been consequences of the complex interactions primers.

3.5. Primer and probe sequence specificity “in silico” analysis

The individual primer and probe sets were evaluated for target and nontarget species with “*in silico*” analyses (Table 4 to Table 7). The primer and probe pairs of BG1-rpo2 showed that the identified regions satisfied the individual group-specific detection (Table 4). The 1st *Bacillus* primer and probe pair BG1-rpo2_P had no mismatches within *B. cereus*, *B. anthracis*, *B. thuringensis*, *B. pseudomycoides*, and *B. cytotoxicus*.

Table 8
Quantitative amplification parameters of spiking experiment for mqPCR assays.

Parameter[a]	Potato salad[b]			Milk[b,c]		
Taeget group[c]	*B. weihenstephanensis* *B. licheniformis*	*B. licheniformis* *B. megaterium*	*B. licheniformis* *Paenibacillus lautus*	*B. weihenstephanensis* *B. licheniformis*	*B. licheniformis* *B. megaterium*	*B. licheniformis* *Paenibacillus lautus*
Target gene	*rpoB*/rpoB	*rpoB*/16S rRNA	*rpoB*/16S rRNA	rpoB/rpoB	rpoB/16S rRNA	rpoB/16S rRNA
Dye	FAM-TAMRA VIC-MGB	VIC-MGB FAM-TAMRA	VIC-MGB FAM-TAMRA	FAM-TAMRA VIC-MGB	VIC-MGB FAM-TAMRA	VIC-MGB FAM-TAMRA
Slope	−2.71 ± 0.29 −2.80 ± 0.09	−2.79 ± 0.83 −2.73 ± 0.25	−2.82 ± 0.21 −3.22 ± 0.75	−3.29 ± 0.28 −3.54 ± 0.25	−3.64 ± 1.30 −2.83 ± 0.44	−3.89 ± 0.11 −3.32 ± 0.31
y intercept	41.84 ± 1.38 41.0 ± 0.57	41.05 ± 0.91 42.48 ± 1.15	40.69 ± 0.97 43.89 ± 3.20	44.83 ± 1.30 45.33 ± 1.02	45.92 ± 0.65 45.40 ± 1.73	46.56 ± 0.54 46.22 ± 1.90
R^2	0.988 ± 0.014 0.959 ± 0.03	0.919 ± 0.04 0.949 ± 0.03	0.945 ± 0.01 0.977 ± 0.03	0.994 ± 0.008 0.998 ± 0.002	0.995 ± 0.008 0.983 ± 0.31	0.98 ± 0.010 0.985 ± 0.01
Efficiency	134.93 ± 1.98 127.85 ± 6.3	129.05 ± 11.9 134.25 ± 1.96	126.92 ± 15.1 109.39 ± 0.30	102.0 ± 13.0 91.86 ± 9.55	88.02 ± 4.20 117.7 ± 22.3	80.56 ± 3.29 100.99 ± 13.98
Dynamic range of copy number[d] (log)	5.32–1.32 5.54–1.54	5.54–1.54 5.27–2.27	5.54–1.54 5.51–2.51	5.32–2.32 5.54–2.54	5.54–2.54 5.27–2.27	5.54–2.54 5.51–2.51

[a] The value for each parameter is the mean ± standard deviation, which was quantified individually in triplicate on the same plate ($n = 3$).
[b] The values of each parameter are shown in the upper detection group corresponding to the left column and the lower detection group shown in the right column.
[c] The upper and lower data correspond to the upper and lower primer and probe pairs with individual columns.
[d] The values of estimated copy number were determined from average values in triplicate on the same plate ($n = 3$) using TUF gene quantification bacterial kit.

Table 9
Quantification of copy numbers spiked in potato salad and milk for mqPCR assays.

Strain	Quantity of DNA copy numbers spiked in potato salad and milk (CFU/mL)	Estimated C_T values yield from spiked copy numbers based on the calculation[a]	Actual C_T values yield from mqPCR assays[b]	Estimated DNA copy numbers from actual C_T values[b] (CFU/mL)
B. weihenstephanensis	$2.1 \times 10^5 \pm 0.3$	23.09 ± 0.80	27.56 ± 0.73	$1.4 \times 10^4 \pm 1.0 \times 10^4$
			27.17 ± 0.47	$1.4 \times 10^4 \pm 1.9 \times 10^4$
	$2.1 \times 10^4 \pm 0.3$	27.12 ± 0.80	30.57 ± 0.66	$1.9 \times 10^3 \pm 1.4 \times 10^3$
			30.84 ± 0.35	$1.0 \times 10^3 \pm 1.3 \times 10^3$
	$2.1 \times 10^3 \pm 0.3$	30.55 ± 0.80	32.32 ± 0.78	$6.2 \times 10^2 \pm 4.9 \times 10^2$
			34.18 ± 0.45	$9.4 \times 10^1 \pm 1.3 \times 10^2$
	$2.1 \times 10^2 \pm 0.3$	33.98 ± 0.80	35.75 ± 1.04	$6.6 \times 10^1 \pm 6.2 \times 10^1$
			36.82 ± 0.48	$1.4 \times 10^1 \pm 2.0 \times 10^1$
	$2.1 \times 10^1 \pm 0.3$	37.40 ± 0.80	37.18 ± 0.10	$2.6 \times 10^1 \pm 1.3 \times 10^1$
			–	–
B. licheniformis[c]	$3.5 \times 10^5 \pm 0.3$	22.33 ± 0.23	25.75 ± 0.42	$3.5 \times 10^4 \pm 1.3 \times 10^4$
			25.69 ± 0.36	$2.3 \times 10^4 \pm 4.6 \times 10^4$
	$3.5 \times 10^4 \pm 0.3$	25.76 ± 0.23	28.37 ± 0.42	$6.1 \times 10^3 \pm 2.3 \times 10^3$
			29.26 ± 0.14	$1.7 \times 10^3 \pm 3.7 \times 10^3$
	$3.5 \times 10^3 \pm 0.3$	29.19 ± 0.23	30.12 ± 0.94	$1.9 \times 10^3 \pm 1.0 \times 10^3$
			32.67 ± 0.32	$1.6 \times 10^2 \pm 4.2 \times 10^2$
	$3.5 \times 10^2 \pm 0.3$	32.61 ± 0.23	34.31 ± 1.48	$1.1 \times 10^2 \pm 8.6 \times 10^1$
			37.35 ± 0.47	$2.4 \times 10^1 \pm 2.0 \times 10^1$
	$3.5 \times 10^1 \pm 0.3$	36.04 ± 0.23	36.91 ± 0.46	$1.9 \times 10^1 \pm 7.0 \times 10^0$
			–	–
B. megaterium	$1.9 \times 10^5 \pm 0.3$	23.24 ± 0.40	28.05 ± 0.25	$7.5 \times 10^3 \pm 5.2 \times 10^3$
			29.40 ± 0.33	$3.0 \times 10^3 \pm 3.8 \times 10^3$
	$1.9 \times 10^4 \pm 0.3$	26.67 ± 0.40	30.90 ± 0.39	$1.1 \times 10^3 \pm 7.5 \times 10^2$
			32.72 ± 0.41	$3.3 \times 10^2 \pm 4.3 \times 10^2$
	$1.9 \times 10^3 \pm 0.3$	30.10 ± 0.40	32.32 ± 0.83	$4.3 \times 10^2 \pm 3.9 \times 10^2$
			36.25 ± 0.83	$3.0 \times 10^1 \pm 8.0 \times 10^1$
	$1.9 \times 10^2 \pm 0.3$	33.52 ± 0.40	36.53 ± 1.19	$2.7 \times 10^2 \pm 3.2 \times 10^2$
			36.94 ± 1.10	$1.9 \times 10^1 \pm 4.0 \times 10^1$
	$1.9 \times 10^1 \pm 0.3$	36.95 ± 0.40	–	–
			–	–
P. lautus	$3.3 \times 10^5 \pm 0.3$	22.42 ± 0.41	26.51 ± 0.90	$2.2 \times 10^4 \pm 1.2 \times 10^4$
			28.02 ± 1.15	$7.7 \times 10^3 \pm 1.7 \times 10^3$
	$3.3 \times 10^4 \pm 0.3$	25.85 ± 0.41	29.21 ± 1.02	$4.3 \times 10^3 \pm 2.4 \times 10^3$
			30.68 ± 0.59	$1.3 \times 10^3 \pm 1.9 \times 10^3$
	$3.3 \times 10^3 \pm 0.3$	29.27 ± 0.41	31.77 ± 0.73	$9.0 \times 10^2 \pm 4.2 \times 10^2$
			34.83 ± 0.67	$7.8 \times 10^1 \pm 1.2 \times 10^2$
	$3.3 \times 10^2 \pm 0.3$	32.70 ± 0.41	36.36 ± 1.19	$5.4 \times 10^1 \pm 3.4 \times 10^1$
			36.85 ± 0.15	$2.0 \times 10^1 \pm 2.2 \times 10^1$
	$3.3 \times 10^1 \pm 0.3$	36.13 ± 0.41	–	–
			–	–

[a] The data calculated based on the *tuf* bacterial gene quantification kit as shown in Fig. 1.
[b] The data of upper and lower column indicated data from potato salad and milk samples, respectivel; −indicated no detection.
[c] The data yield from specific mqPCR assay for the 1st *B. cereus* and 2nd *B. subtilis* group.

Additionally, both the forward and reverse primers provided no mismatches for the identification of *B. cereus*, *B. anthracis*, and *B. thuringensis, B. pseudomycoides,* and *B. cytotoxicus*. One mismatch and two mismatches with the forward and reverse primers for *B. pseudomycoides*, and two mismatches with the forward primer for *B. cytotoxicus* (Table 4). These findings implied that BG1-rpo2 was able to amplify the pathogenic species *B. anthracis*, *B. thuringensis*, *B. pseudomycoides*, and *B. cytotoxicus*, together with *B. cereus* and *B. weihenstephanensis*. As shown in previous studies, *B. anthracis*, *B. thuringiensis*, *B. cereus*, *B. weihenstephanensis*, *B. mycoides*, and *B. pseudomycoides* were members of the *B. cereus* group (Helgason et al., 2000), and later using TaqMan probe qPCR assay was established for detection and quantification of *Bacillus cereus* group species (Oliwa-Stasiak et al., 2011).

The probe BG2-rpo2_P also contained no mismatches with *B. amyloliquefaciens* or *B. atrophaeus* subsp. *globigii.* Moreover, the forward primer BG2-rpo2_F had no mismatches with *B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens*, *B. atrophaeus,* and *B. pumilus*. The reverse primer of BG2-rpo2_R had one mismatch with these species (Table 5). These results indicated that this probe and primer set can be used for the identification of these species.

The probe BG3-3_P also showed no mismatches with *B. flexus* together with the target species *B. simplex* and *B. megaterium*. These findings demonstrated that BG3–3 could amplify *B. flexus* along with *B. simplex* and *B. megaterium* strains with no mismatches with either the forward or reverse primer (Table 6). Thus, the results suggest that BG3–3 enables the discrimination of *B. simples*, *B. megaterium*, and *B. flexus* stains from other *Bacillus* species. In addition, "*In silico*" analysis provided the polymorphism information for *B. megaterium* within loci corresponding to the probe BG3–3_P.

The probe P-82_P had no mismatches within all aligned *Paenibacillus* spp. and one to two mismatches with the reverse primer in certain *Paenibacillus* species (Table 7). In fact, the probe P-82_P provided false positive results within certain *Clostridium* spp. (*C. acetobutylicum*) (Table 2) despite exhibiting > 5 mismatches but without continuous sequences (Table 7).

3.6. mqPCR amplification parameters in artificially contaminated potato salad and milk

The spiking experiments were performed to evaluate the detection limits of the mqPCR method as well as to determine the adequate differentiation of group-specific target species from food matrices and beverages. The results are visualized in Fig. 2, and quantitative parameters for mqPCR assays were summarized in Table 8.

For potato salad, the linear dynamic range of identification and quantification was 10^5–10^1 CFU/mL for *B. weihenstephanensis* and *B.*

Table 10
C_T values derived from the mqPCR assays of spiked potato salad and milk that were stored for 10 days at different temperatures.

Sample	Stored condition	Target species (Target group with mqPCR assays)	C_T values (Mean ± SD)[a]
	Temperature (°C)		
Potato salad	15	*Bacillus weihenstephanensis*	22.35 ± 1.69
	22	*Bacillus weihenstephanensis*	18.16 ± 2.74
		Bacilus licheniformis (1st *Bacillus* group × 2nd *Bacillus* group)	22.67 ± 1.37
		Bacilus licheniformis (2nd *Bacillus* group × 3rd *Bacillus* group)	22.44 ± 1.36
		Bacilus licheniformis (2nd *Bacillus* group × *Paenibacillus* group)	23.37 ± 0.62
Milk	7	*Bacillus weihenstephanensis*	28.81 ± 4.55
	15	*Bacillus weihenstephanensis*	25.34 ± 0.85
		Paenibacillus lautus	27.16 ± 0.84
	22	*Bacillus weihenstephanensis*	23.45 ± 0.50
		Bacilus licheniformis (1st *Bacillus* group ×2nd *Bacillus* group)	24.63 ± 0.33
		Bacilus licheniformis (2nd *Bacillus* group × 3rd *Bacillus* group)	24.47 ± 0.36
		Bacilus licheniformis (2nd *Bacillus* group × *Paenibacillus* group)	24.16 ± 0.50
		Paenibacillus lautus	23.54 ± 0.87

[a] C_T values were observed to be < 30, which indicated that the individual strain populations increased in potato salad and milk at respective temperatures.

licheniformis. The R^2 value was obtained 0.988 (± 0.014) only for *B. weihenstephanensis* and was < 0.980 for *B. licheniformis*, *B. megaterium*, and *P. lautus*. The amplification efficiencies for individual group–-specific exceed 110%; this could be caused by saturation of the qPCR, impairing discrimination between C_T values especially at higher dilutions. The C_T values showed negative results with these group–specific detections in non–inoculum potato salad.

For the milk sample, the dynamic range of the detection and quantification was 10^5–10^2 CFU/mL. The R^2 value was obtained > 0.980 for all group–specific assays. The amplification efficiencies for the individual group–specific detection were 1.02 (± 0.13), 1.17 (± 0.22), and 1.00 (± 0.13) for *B. weihenstephanensis*, *B. megaterium*, and *P. lautus*, respectively. Meanwhile, the values for *B. licheniformis* were between 0.80 (± 0.03) and 0.91 (± 0.09) depending on the mqPCR assays. C_T values were negative for the group–specific detections in non-inoculum milk. The detection and quantification limits of the mqPCR spiked in food and beverage samples obtained of 10^2 CFU/mL, these were comparable with other qPCR and mqPCR assays (Leblanc-Maridor et al., 2011; Toplak et al., 2012).

We assessed the accuracy of assays by comparing the actual C_T value yield from mqPCR assays with the estimated C_T values of the gene copy numbers of inoculated cells (Table 9). Overall, spiked genomic DNA copy numbers were estimated in observed 10–fold lower in potato salad and milk samples than in pure culture. The appropriate DNA extraction and purification method capable of maximum DNA recovery and inhibitor removal is a critical point for increasing the sensitivity of the qPCR assay.

3.7. mqPCR assay with spiking and storage of potato salad and milk at different temperatures

To evaluate the identities and quantify three species of the genus *Bacillus* and *Paenibacillus*, four suspensions of bacterial strain were spiked into potato salad and milk and stored for 10 days at different temperatures (7 °C, 15 °C, and 22 °C). The samples were subjected to mqPCR assays and analyzed further. The results are presented in Table 10. For potato salad, none of all the inoculum strains were observed to grow at 7 °C. Whereas, *B. weihenstephanensis* in particular, showed a higher growth than that of the other strains, which increased at 15 °C and 22 °C, C_T values were between 22 and 18 at 15 °C and 22 °C, respectively. The growth of another strain *B. licheniformis* increased at 22 °C, and the C_T values were between 22 and 23. In milk the growth of *B. weihenstephanensis* increased at all storage temperatures, with the C_T values as 28, 25, and 23 at 7 °C, 15 °C, and 22 °C, respectively. The growth of *B. licheniformis* increased at 22 °C, and the C_T values were 24. Additionally, *P. lautus* strain was able to grow at 15 °C and 22 °C, with C_T values between 27 and 23. Based on the spiking experiments, C_T values < 25, indicated that the strain populations had > 5 log gene copy numbers/mL and also indicated that the strains were able to grow in potato salad and milk at respective temperatures.

Our results showed that *B. weihenstephanensis* was able to grow in milk and potato salad at a broad range of temperatures (except at refrigeration temperatures for potato salad). This suggested that *B. weihenstephanensis*, in particular, is resistant to low pH and tolerant of broad temperature conditions. The observations revealed that potato salad offered the required growth conditions, thereby leading to a clear increase in populations at 15 °C and 22 °C. The pH and A_W values of potato salad were < 5 and > 0.976, respectively. *B. weihenstephanensis* was identified as a psychrotolerant species that can grow in skim milk broth at 6 °C (Ivy et al., 2012), whereas other studies have also demonstrated growth at ≤7 °C (Stenfors and Granum, 2001). Another *Bacillus* species, *B. licheniformis*, has been characterized as a psychrotolerant species with the potential to spoil refrigerated products, but no growth was observed in this study at 7 °C and 15 °C on both the substrates. Storage conditions at 20 °C–25 °C favored the growth of *B. licheniformis*, *B. subtilis*, and *B. cereus*, whereas *Paenibacillus* spp. are present at 10 °C in high concentrations (Guinebretiere et al., 2001). *P. lautus* was able to grow more at 22 °C than at 15 °C in milk but not in salad under any temperature condition, which may provide more optimal growth conditions required. Approaches for the simultaneously tracking and monitoring of spoilage microorganisms could be establish, which could facilitate the identification of entry points or site of contamination in the food industry, thereby improving the safety management and hygiene control.

4. Conclusions

qPCR and mqPCR assays were developed for the determination and quantification of three *Bacillus* group-specific species and *Paenibacillus* spp. using newly designed primer and probe pairs based on the *rpoB* gene and 16S rRNA genes. Sensitivity was evaluated by testing a total of 55 strains, including related and unrelated taxa. In particular, individual mqPCR assays between the *B. cereus* group and *B. subtilis* group and between the *B. subtilis* group and *B. simplex* group were highly species-specific, enabling the identification and quantification of these organisms. The method described herein provides a rapid, specific, reliable and accurate identification strategy for signature sequences for the detection, identification, and confirmation of the presence of psychrophilic and psychrotolerant spoilage microorganisms for food risk assessment and food control applications.

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References

Brownie, J., Shawcross, S., Theaker, J., Whitcombe, D., Ferrie, R., Newton, C., Little, S., 1997. The elimination of primer-dimer accumulation in PCR. Nucleic Acids Res. 25, 3235–3241.

Bueche, M., Wunderlin, T., Roussel-Delif, L., Junier, T., Sauvain, L., Jeanneret, N., Junier, P., 2013. Quantification of endospore-forming *Firmicutes* by quantitative PCR with the functional gene *spo0A*. Appl. Environ. Microbiol. 79, 5302–5312.

Bustin, S., Huggett, J., 2017. qPCR primer design revisited. Biomolecular Detection and Quantification 14, 19–28.

Bustin, S., Benes, V., Garson, J., Hellemans, J., et al., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611–622.

Cabo, L.M., Torres, B., Herrera, J.J., Bernárdez, M., Pastoriza, L., 2009. Application of nisin and pediocin against resistance and germination of *Bacillus* spores in sous vide products. J. Food Prot. 72, 515–523.

Case, J.R., Boucher, Y., Dahllöf, I., Holmström, C., Doolittle, F.W., Kjelleberg, S., 2007. Use of 16S rRNA and *rpoB* genes as molecular markers for microbial ecology studies. Appl. Environ. Microbiol. 73, 278–288.

Chapela, M.-J., Garrido-Maestu, A., Cabado, G.A., 2015. Detection of foodborne pathogens by qPCR: a practical approach for food industry applications. Cogent Food & Agriculture 1, 1013771.

Coton, M., Denis, C., Cadot, P., Coton, E., 2011. Biodiversity and characterization of aerobic spore-forming bacteria in surimi seafood products. Food Microbiol. 28, 252–260.

Durak, Z.M., Fromm, I.H., Huck, R.J., Zadoks, N.R., Boor, J.K., 2006. Development of molecular typing methods for *Bacillus* spp. and *Paenibacillus* spp. isolated from fluid milk products. J. Food Sci. 71, M50–M57.

Ercolini, D., Russo, F., Blaiotta, G., Pepa, O., Mauriello, G., Villani, F., 2007. Simultaneous detection of *Pseudomonas fragi*, *P. lundensis*, and *P. putida* from meat by use of a multiplex PCR assay targeting the *carA* gene. Appl. Environ. Microbiol. 73, 2354–2359.

Errington, J., 2003. Regulation of endospore formation in *Bacillus subtilis*. Nat. Rev. Microbiol. 1, 117–126.

Fangio, M.F., Roura, S.I., Fritz, R., 2010. Isolation and identification of *Bacillus* spp. and related genera from different starchy foods. J. Food Sci. 69, M207–M214.

Fernández-No, C.I., Guarddon, M., Böhme, K., Cepeda, A., Calo-Mata, P., Barros-Velázquez, J., 2011. Detection and quantification of spoilage and pathogenic *Bacillus cereus*, *Bacillus subtilis* and *Bacillus licheniformis* by real-time PCR. Food Microbiol. 28, 605–610.

Filler, D., Furano, V.A., 1981. Duplication of the *tuf* gene, which encodes peptide chain elongation factor Tu, is widespread in gram-negative bacteria. J. Bacteriol. 148, 1006–1011.

Forghani, F., Wei, S., Oh, D.-H., 2016. A rapid multiplex real-time PCR high-resolution melt curve assay for the simultaneous detection of *Bacillus cereus*, *Listeria monocytogenes*, and *Staphylococcus aureus* in food. Journal of Food Protction 79, 810–815.

Gram, L., Ravn, L., Rasch, M., Bruhn, B.J., Christensen, B.A., Givskov, M., 2002. Food spoilage-interaction between food spoilage bacteria. International Journal of Food Microbiology 78, 79–97.

Guinebretiere, M.-H., Berge, O., Normand, P., Morris, C., Carlin, F., Nguyen-The, C., 2001. Identification of bacteria in pasteurized zucchini purées stored at different temperature and comparison with those found in other pasteurized vegetable purées. Appl. Environ. Microbiol. 67, 4520–4530.

Gustavsson, J., Cederberg, C., Sonesson, U., 2011. Global food losses and food waste. In: FAO Report, FAO, Rome, Italy.

Helgason, E., Økstad, O., Caugant, A.D., Johansen, A.H., Fouet, A., Mock, M., Hegna, I., Kolstø, A.-B., 2000. *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*-one species on the basis of genetic evidence. Appl. Environ. Microbiol. 66, 2627–2630.

Helmond, M., Nierop Groot, N.M., van Bokhorst-van de Veen, Hermien, 2017. Characterization of four *Paenibacillus* species isolated from pasteurized, chilled ready-to-eat meals. Int. J. Food Microbiol. 252, 35–41.

Higuchi, R., Fockler, C., Dollinger, G., Watson, R., 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnology 11, 1026–1030.

Hilscher, C., Vahrson, W., Dittmer, P.D., 2005. Faster quantitative real-time PCR protocols may loss sensitivity and show increased variability. Nucleic Acids Res. 33, e182.

Huck, R.J., Woodcock, H.N., Ralyea, D.R., Boor, J.K., 2007. Molecular subtyping and characterization of psychrotolerant endospore-forming bacteria in two New York State fluid milk processing systems. J. Food Prot. 70, 2354–2364.

Huck, R.J., Sonnen, M., Boor, J.K., 2008. Tracking heat-resistant, cold-thriving fluid milk spoilage bacteria from farm to packaged product. J. Dairy Sci. 91, 1218–1228.

Ivy, A.R., Ranieri, L.M., Martin, H.N., den Bakker, C.H., Xavier, M.B., Wiedmann, M., Boor, J.K., 2012. Identification and characterization of psychrotolerant sporeformers associated with fluid milk production and processing. Appl. Environ. Microbiol. 78, 1853–1864.

Jan, S., Brunet, N., Techer, C., Le Maréchal, C., Koné, Z.A., Grosset, N., Cochet, F.M., Gillard, A., Gautier, M., Puterflam, J., Baron, F., 2011. Biodiversity of psychrotrophic bacteria of the *Bacillus cereus* group collected on farm and in egg product industry. Food Microbiol. 28, 261–265.

Janse, I., Hamidjaja, A.R., Bok, M.J., van Rotterdam, J.B., 2010. Reliable detection of *Bacillus anthracis*, *Francisella tularensis* and *Yersinia pestis* by using multiplex qPCR including internal controls for nucleic acid extraction and amplification. BMC Microbiol. 10, 314.

Lathe III, C.W., Bork, P., 2001. Evolution of *tuf* genes: ancient duplication, different loss and gene conversion. FEBS Lett. 502, 113–116.

Leblanc-Maridor, M., Garénaux, A., Beaudeau, F., Chidaine, B., Seegers, H., Denis, M., Belloc, C., 2011. Quntification of *Campylobacter* spp. in pig feces by direct real–time PCR with an internal control of extraction and amplification. Journal of Microbial Methods 85, 53–61.

Lechner, S., Mayr, R., Francis, P.K., Prüss, M.B., Kaplan, T., Wiessner-Gunkel, E., Sterwart, S.A.B.G., Scherer, S., 1998. *Bacillus weihenstephanensis* sp. nov. is a new psychrotolerant species of the *Bacillus* cereus group. Int. J. Syst. Bacteriol. 48, 1373–1382.

Ludwig, W., Weizenegger, M., Betzl, D., Leidel, E., Lenz, T., Ludvigsen, A., Möllenhoff, D., Wenzig, P., Schleifer, H.K., 1990. Complete nucleotide sequences of seven eubacterial genes coding for the elongation factor Tu: functional, structural and phylogenetic evaluations. Arch. Microbiol. 153, 241–247.

Oliwa-Stasiak, K., Kolaj-Robin, O., Adley, C.C., 2011. Development of real-time PCR assays for detection and quantification of *Bacillus cereus* group species: differentiation of *B. weihenstephanensis* and rhizoid *B. pseudomycoides* isolates from milk. Appl. Environ. Microbiol. 77, 80–88.

Pal, N., Bender, S.J., Opriessnig, T., 2010. Rapid detection and differentiation of *Erysipelothrix* spp. by a novel multiplex real-time PCR assay. J. Appl. Microbiol. 108, 1083–1093.

Pereira, A., Sant'Ana, A., 2018. Diversity and fate of spore forming bacteria in cocoa powder, milk powder, starch and sugar during processing: a review. Trends Food Sci. Technol. 76, 101–118.

Polz, F.M., Cavanaugh, M.C., 1998. Bias in template-to-product ratios in multitemplate PCR. Appl. Environ. Microbiol. 64, 3724–3730.

Qi, Y., Patra, G., Liang, X., Williams, E.L., Rose, S., Redkar, J.R., Del Vecchio, G.V., 2001. Utilization of the *rpoB* gene as a specific chromosomal marker gene for real-time PCR detection of *Bacillus anthracis*. Appl. Environ. Microbiol. 67, 3720–3727.

Rodŕiguez-Lázaro, D., Cook, N., Hernández, M., 2013. Real-time PCR in food science: PCR diagnostics. Current Issues Molecular Biology 15, 39–44.

Saikaly, E.P., Barlaz, A.M., de los Reyes III, L.F., 2007. Development of quantitative real-time PCR assays for detection and quantification of surrogate biological warfare agents in building debris and leachate. Appl. Environ. Microbiol. 73, 6557–6565.

Samapundo, S., Heyndrickx, M., Xhaferi, R., de Baenst, I., Devlieghere, F., 2014. The combined effect of pasteurization intensity, water activity, pH and incubation temperature on the survival and outgrowth of spores of *Bacillus cereus* and *Bacillus pumilus* in artificial media and food products. Int. J. Food Microbiol. 181, 10–18.

Shehata, E.T., Duran, A., Collins, B.E., 1971. Influence of temperature on the growth of psychrophilic strains of *Bacillus*. Journal of Daily Science 54, 1579–1582.

Sorokulova, B.I., Reva, N.O., Smirnov, V.V., Pinchuk, V.I., Lapa, V.S., Urdaci, C.M., 2003. Genetic diversity and involvement in bread spoilage of *Bacillus* strains isolated from flour and ropy bread. Lett. Appl. Microbiol. 37, 169–173.

Stenfors, P.L., Granum, E.P., 2001. Psychrotelerant species from the *Bacillus cereus* group are not necessarily *Bacillus weihenstephanensis*. FEMS Microbiol. Lett. 197, 223–228.

Tanaka, Y., Takahashi, H., Simidu, U., Kimura, B., 2010. Design of a new universal real-time PCR system targeting the *tuf* gene for the enumeration of bacterial counts in food. J. Food Prot. 73, 670–679.

Taylor, M.W.J., Sutherland, D.A., Aidoo, E.K., Logan, A.N., 2005. Heat-stable toxin production by strains of *Bacillus cereus*, *Bacillus firmus*, *Bacillus megaterium*, *Bacillus simplex* and *Bacillus licheniformis*. FEMS Microbiol. Lett. 242, 313–317.

Thompson, D.J., Gibson, J.T., Plewniak, F., Jeanmougin, F., Higgins, G.D., 1997. The clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acid Reserach 25, 4876–4882.

Toplak, N., Kovač, M., Piskernik, S., Smole Možina, S., Jeršek, B., 2012. Detection and quantification of *Campylobacter jejuni* and *Campylobacter coli* using real-time multiplex PCR. J. Appl. Microbiol. 112, 752–764.

Trmčić, A., Martin, H.N., Boor, J.K., Wiedmann, M., 2015. A standard bacterial isolate set for research on contemporary dairy spoilage. J. Dairy Sci. 98, 5806–5817.

Untregasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., Leunissen, A.M.J., 2007. Primer3Plus, an enhanced web interface to Primer3. Nucleic Acid Reserch 35, W71–W74.