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Original article

Multiplex PCR for rapid detection of thermophilic *Moorella thermoacetica* and *Geobacillus stearothermophilus* from canned foods and beverages

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Summary A multiplex PCR assay was developed to simultaneously detect the thermophilic spore-forming bacteria *Geobacillus stearothermophilus* and *Moorella thermoacetica*, which are major sources of spoilage in the canned food and beverage industries because of their thermophilic persistence. A total of 294 samples were examined, and none of the samples demonstrated signs of spoilage. Culture-based assays showed that forty-five *M. thermoacetica* isolates were obtained, which was confirmed by multiplex PCR, and *G. stearothermophilus* was not detected in any of the canned food and beverage, but multiplex PCR analysis identified this species in almost all of the samples. Meanwhile, twelve *Bacillus subtilis* isolates were found under both aerobic and anaerobic conditions. Multiplex PCR may allow selective detection of species that are present at low levels in complex matrices, or those that cannot be detected by culture-based methods. Screening for spoilage-causing micro-organisms in ingredients and samples from processing lines, together with traditional culture methods, will help risk management and improve hygiene control.

Keywords Canned food, *Geobacillus stearothermophilus*, *Moorella thermoacetica*, multiplex PCR, spoilage.

Introduction

Microbial spoilage of canned foods is often caused by thermophilic spore-forming bacteria. In particular, aerobic species *Geobacillus stearothermophilus* and obligate anaerobes *Moorella thermoacetica*/*M. thermoautotrophica* and *Thermoanaerobacterium* sp. are often found in canned products. All of these species produce highly heat-resistant endospores (Ashton, 1981; Feeherry *et al*., 1987; Byrer *et al*., 2000) and share high heat-resistance profiles. For example, *G. stearothermophilus* has a decimal reduction time at 121 °C of >1 min in most instances, with *M. thermoacetica* displaying a thermal reduction time of ≥1 h at 124 °C (Byrer *et al*., 2000; Sasaki *et al*., 2000), depending on sporulation conditions. The presence of *M. thermoacetica* in canned foods usually results in strong acidification and can swelling (Ashton & Bernard, 1992; Olson & Sorrells, 1992). The optimal growth temperature for this species is 55–60 °C, and it is considered a model acetogen (Drake & Daniel, 2004). The most frequent cause of spoilage in low-acid canned products is *G. stearothermophilus*, which is characterized by the absence of can swelling and acidification of products (Ashton & Bernard, 1992; Olson & Sorrells, 1992). All of these species can survive high-heat sterilization treatments, with sporulation and spoilage usually occurring after high-temperature incubation. Although none of these bacteria are known human pathogens, they are considered a microbial control risk in the canning industry (Durand *et al*., 2015).

Soil and other environmental sources act as reservoirs of thermophilic spore-forming spoilage bacteria. On average, thermophilic spores are present in crop soil at a rate of 3 log CFU g^{-1} (Drake & Daniel, 2004). Spore contamination of food processing facilities occurs via the presence of soil dust in open areas, adhesion of soil to unprocessed food materials or carriage by employees (Groenewald *et al*., 2009; Sevenier *et al*., 2012). The ingredients themselves are a rich source of thermo-resistant sporeformers, and ingredients that are rich in divalent metal ions exert a strong influence on sporulation (Oomes *et al*., 2007).

Recently, André *et al.* surveyed bacterial species responsible for the instability of low-acid canned food products following prolonged incubation at 55 °C. Results showed that thermophilic bacteria, including *G. stearothermophilus*, *M. thermoacetica*/*M. thermoautotrophica* and

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Thermoanaerobacterium species, were involved in canned food spoilage (André & Remize, 2013). In addition, other thermophilic bacteria belonging to the genera *Bacillus*, *Thermoanaerobacter*, *Caldanaerobius*, *Anoxybacillus*, *Paenibacillus* and *Clostridium* were detected. Bacteria belonging to these genera can survive heat treatment at 100 °C for 10 min and have previously been associated with canned food spoilage (Feig & Stersky, 1981; Raso *et al*., 1995; André & Remize, 2013). To guarantee microbiological stability, an additional heat treatment step has been added to commercial food sterilization processes by many manufacturers; however, this is a costly operation in terms of energy and impact on the organoleptic quality and nutritional value of the product (Rigaux *et al*., 2014).

To simultaneously detect the presence of thermophilic spore-forming bacterial species *M. thermoacetica*/*M. thermoautotrophica* and *G. stearothermophilus* in canned foods and their ingredients, and to enable microbial risk assessment during industrial processing, a rapid and sensitive microbial detection technique is needed to complement existing methods. Traditional culture-based methods require multiple culturing steps for isolation, which makes them time-consuming, and these techniques are not always successful if bacteria fail to grow. PCR-based identification is a suitable alternative because it is comparatively easy and can be completed within several hours.

PCR-restriction fragment length polymorphism (RFLP) and randomly amplified polymorphic DNA (RAPD) analyses have been successfully used for the identification of micro-organisms from different sources. PCR-based molecular methods are generally characterized by their simplicity, speed, cost-effectiveness and reliability. Multiplex PCR, in which several species-specific primer sets are combined in a single PCR assay, enables rapid, simultaneous detection of multiple micro-organisms in a single amplification assay and can even be used to examine more than one locus. Thus, multiplex PCR can be used to detect multiple target organism and to valuate community dynamics. Additionally, depending on the established detection limit, multiple PCR could be considered a semi-quantitative technique.

Previously, we used a quantitative PCR method to identify *M. thermoacetica*/*M. thermoautotrophica* contamination of commercial canned coffee beverages (Nakano, 2015a). In addition, we developed a qPCR assay for identification and quantification of *G. stearothermophilus* from commercial canned food (Nakano, 2015b). These methods successfully detected highly heat-resistant spore-forming bacteria in canned food and beverages. The first aim of this study was to investigate the biodiversity of the contaminating thermophilic spore-forming microbes from commercial canned foods and beverages, which were stored at room temperature or 55 °C for 1 week, using culture-based methods. A total of 294 commercial samples, including 176 canned food and 118 beverages, were subjected to microbiological investigation. Secondly, we aimed to develop new primer pairs for simultaneous detection of thermophilic bacterial species *M. thermoacetica*/*M. thermoautotrophica* and *G. stearothermophilus*, and finally, we applied a direct multiplex PCR assay to screen for highly heat-resistant sporeformers in canned foods and beverages. These species are regularly identified as the most common cause of low-acid spoilage in canned food and beverages (Ashton, 1981; André & Remize, 2013). Compared with conventional PCR, multiplex PCR assays allow simultaneous detection of several genes or species, which is advantageous for large-scale screening. The assay developed in this study is highly reliable and can be completed within several hours. Therefore, the assay could be useful for screening and quantification of highly heat-resistant thermophilic bacteria and major sporeformers in food materials and ingredients.

Materials and methods

Bacterial strains and growth conditions

All strains used in this study are listed in Table 1. Type and reference strains were obtained from various culture collections and maintained in the laboratory according to the reference information. *Moorella* species were incubated at 55–60 °C in modified thioglycolate (mTGC) medium (Nissui Pharmaceutical Co., Tokyo, Japan) with an AnaeroPack (Mitsubishi Gas Chemical Company, Tokyo, Japan) in a sealed container to maintain anaerobic conditions. Agar (1.5%, w/v) was added to mTGC broth medium for plate preparation. *Geobacillus* species were incubated at 55–60 °C in nutrient broth (Eiken Chemical Co., Tokyo, Japan). *Moorella thermoacetica* JCM 9319^{T} and *G. stearothermophilus* NBRC 12550^{T} were used to standardize the multiplex PCR assay. All other aerobic and anaerobic thermophilic strains were incubated at 55–60 °C according to the reference growth information. Trypticase soy broth (BD, LePont de Claix, France) was used to grow other unrelated species.

Isolation and identification of wild-type strains from canned food and beverages

A total of 294 samples (176 canned food and 118 beverages) were subjected to microbiological analysis. Half of samples were stored at room temperature, while the remaining half were incubated at 55 °C for 7 days prior to sample preparation. To obtain aerobic total viable plate counts for the 176 canned food samples, 1 mL of finely ground sample using a stomacher machine (Exnizer 400, ORGANO CORPORATION,

Table 1 Representative strains for multiplex PCR analysis along with reference strains to evaluate the specificity of the assay

Species	Strain and source	PCR results	
		Gv2F/Gv3R-M (157 bp)	v3F/v4R (296 bp)
Geobacillus stearothermophilus	NBRC 12550^T (ATCC 12980)	+	–
Geobacillus stearothermophilus	NBRC 13737 (ATCC 7953)	+	–
Geobacillus stearothermophilus	NBRC 100862^T (DSM 1550)	+	–
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)	–	–
Moorella thermoacetica	JCM 9319^T (ATCC 35608)	–	+
Moorella thermoacetica	JCM 9320^T (ATCC 39073)	–	+
Moorella thermoacetica	24-1 (our collection)	–	+
Moorella glycerini	DSM 11254^T	–	–
Moorella humiferrea	DSM 23265^T	–	–
Moorella mulderi	DSM 14980^T	–	–
Moorella stamsii	DSM 26217^T	–	–
Moorella perchloratireducens	ATCC BAA-1531	–	–
Moorella thermoautotrophica	DSM 1974^T	–	+
Ammonifex sp.	NBRC 100904	–	–
Caldanaerobacter subterraneus subsp. *tengcongensis*	NBRC 100824^T	–	–
Carboxydothermus pertinax	NBRC 107576^T (DSM 23698)	–	–
Tepidanaerobacter syntrophicus	NBRC 100060^T (DSM 15584)	–	–
Thermoaneromonas toyohensis	NBRC 101528^T (DSM 14490)	–	–
Thermanaerobacter cellulolyticus	NBRC 14436	–	–
Clostridium acetobutylicum	NBRC 13948^T (ATCC 824)	–	–
Clostridium clariflavum	NBRC 101661^T (DSM 19732)	–	–
Clostridium kluyveri	NBRC 12016^T (DSM 555)	–	–
Clostridium thermocellum	NBRC 103400^T (ATCC 27405)	–	–
Bacillus subtillis	NBRC 13719^T (ATCC 6051)	–	–
Bacillus coagulans	ATCC 80078	–	–
Bacilus licheniformis	NBRC 12200^T (ATCC 14580)	–	–
Paenibacillus polymyxa	NBRC 15309^T (ATCC 842)	–	–
Staphylococcus aureus	NBRC 100910^T (ATCC 12600)	–	–
Escherichia coli	NBRC 102203^T (ATCC 11775)	–	–

NBRC, NITE Biological Resource Center (Kisarazu, Chiba, Japan); T, type strain; ATCC, American Type Culture Collection (Manassas, VA, USA); DSM, German Collection of Micro-organisms and Cell Cultures (Braunschweig, Germany). References in parentheses indicate the corresponding reference number in an alternative collection. Gv2F/Gv3R-M and v3F/v4R are the primer pairs used to identify *Geobacillus stearothermophilus* and *Moorella thermoacetic*, respectively. The numbers below the primer names indicate the expected product sizes. + and – indicate the presence and absence of PCR products, respectively.

Tokyo, Japan) at 200 r.p.m. for 1–6 min depending on the type of sample was inoculated onto a Compact Dry TC plate (Nissui Pharmaceutical) and incubated at 55 °C for 4 days. A 100-μL aliquot of each sample was also spread onto an mTGC plate and incubated anaerobically at 55 °C for 7 days. Identical plate assays were carried out for the 118 beverage samples, using aliquots of the beverage liquid. Individual samples were examined in triplicate.

Any resultant colonies on the aerobic plates were streaked to isolation in preparation for further analyses. The colonies grown anaerobically were picked directly for isolation. Genomic DNA was extracted from all isolated bacterial samples using an UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) and then used as template for 16S rRNA amplification using two sets of universal primers: 27f (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 783r (5′-ACC MGG GTA TCT AAT CCK G-3′), and com1 (5′-CAG CAG CCG CGG TAA TAC-3′) and 1522r (5′-AAG GAG GTG ATC CAN CCR CA-3′). Each 30-μL reaction mixture

contained 15 μL of EmeraldAmp PCR Master Mix (Takara Bio, Otsu, Japan), 1 μL of each primer (10 μM concentration) and 1 μL of genomic DNA. Assays were conducted in an iCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA) using a protocol of 95 °C for 4 min, 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s, and a final extension of 7 min at 72 °C. After confirming correct amplification using agarose gel electrophoresis, PCR products were purified using a PCR Clean-up Gel Extraction Kit (MACHEREY-NAGEL GmbH & Co. KG, Duren, Germany). Sequencing was carried out using an Applied Biosystems 3730*xl* DNA Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). The sequencing data were combined from individual primer pairs, then >1 kb of each 16S rRNA gene sequence was used for BLAST analysis against the GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Extraction of genomic DNA from target strains for preparation of the multiplex PCR assay

Genomic DNA from *M. thermoacetica* JCM 9319 and *G. stearothermophilus* NBRC 12550 was used as template to estimate the efficacy of the multiplex PCR assay. *M. thermoacetica* JCM 9319 and *G. stearothermophilus* NBRC 12550 were grown to an OD_{600} of 0.3–0.4 (approximately 1×10^8 CFU mL^{-1}) and 0.2–0.3 (approximately 5×10^7 CFU mL^{-1}), respectively. Genomic DNA was extracted using an UltraClean Microbial DNA Isolation Kit following the manufacturer's instructions, with minor modification for extraction of DNA from spores (Rose *et al*., 2011; Nakano, 2015a,b). The concentration of the genomic DNA was determined using a spectrophotometer (μQuant; BioTek Instruments Inc., Winooski, VT, USA) and then diluted to the appropriate concentration prior to use.

Primer design for multiplex PCR

Species-specific 16S rRNA gene primers for *M. thermoacetica*/*M. thermoautotrophica* and *G. stearothermophilus* were designed based on those previously described for qPCR assays (Nakano, 2015a,b). In addition, several novel primer pairs were designed to develop the multiplex PCR assay. Primer sequences used in this study are shown in Table S1.

The specificity of the PCR assay for the detection of *M. thermoacetica*/*M. thermoautotrophica* and *G. stearothermophilus* was evaluated using 100 pg of DNA extracted from pure cultures of each of the reference strains (Table 1). PCR assays were performed in 15-μL reaction mixtures containing 7.5 μL of EmeraldAmp PCR Master Mix, 1 μL of each of the primers (10 μM concentration) and 1 μL of genomic DNA. Reactions were carried out in an iCycler Thermal Cycler at 95 °C for 4 min, followed by 26–34 cycles of 95 °C for 30 s, 58–66 °C (individual assays performed with a 2 °C increase in annealing temperature in each subsequent assay) for 30 s, and 72 °C for 90 s, and a final extension of 7 min at 72 °C. The presence of PCR products and correct target amplicon size were confirmed by agarose gel electrophoresis.

Multiplex PCR amplification

To optimize the multiplex PCR assay for simultaneous detection of *M. thermoacetica*/*M. thermoautotrophica* and *G. stearothermophilus*, 10-fold serial dilutions of genomic DNA (1.6 ng to 1.6 fg) extracted from *M. thermoacetica* JCM 9319^T and *G. stearothermophilus* NBRC 12550^T were prepared in sterile distilled water. Combinations of the species-specific forward and reverse primers were also examined to optimize the sensitivity and productivity of the multiplex PCR for the representative strains *M. thermoacetica* and *G. stearothermophilus*.

Multiplex PCR was carried out in 25-μL reaction mixtures containing 0.2 μM of each primer, 0.125 μL of Multiplex PCR Mix 1, 12.5 μL of Multiplex PCR Mix 2 (reaction buffer containing dNTPs and 2 mM Mg^{2+}; Takara Bio) and 1-μL volumes of the serially diluted genomic DNA templates prepared above. The optimal cycling parameters were 94 °C for 4 min, followed by 28 cycles of 94 °C for 30 s, 64 °C for 60 s and 72 °C for 90 s, with a final extension of 72 °C for 10 min using a Thermal Cycler S1000 (Bio-Rad).

Extraction of microbial genomic DNA from canned food and beverages

Microbial genomic DNA was extracted from half of the samples (176 canned food and fifty-nine beverages) immediately after purchase and then from the remaining samples after they had been incubated at 55 °C for 7 days. To prepare the samples for DNA extraction, the entirety of a can of food (canned volume: 85–350 g) was finely ground using a stomacher machine at 200 r.p.m. for 1–6 min depending on the type of sample. Genomic DNA was then extracted from 1 to 1.5 g of the ground samples using NucleoSpin Food kit (MACHEREY-NAGEL) as per the manufacturer's instructions. Phosphate-buffered saline (PBS; pH 7.4) was added if necessary, depending on the food type. For genomic DNA extraction from beverage samples, microbial pellets were collected as follows. Approximately 185–500 mL of sample was centrifuged at 7800 × ***g*** for 20 min at 4 °C, then the pellets were washed three times with PBS, and the microbial DNA was extracted as described previously (Nakano, 2015a,b).

Molecular detection of *M. thermoacetica* and *G. stearothermophilus* in canned food and beverages

The multiplex PCR assay was then used to screen for the presence of *M. thermoacetica* and *G. stearothermophilus* species in the canned food and beverage microbial genomic DNA samples. Individual reaction mixtures (final volume of 40 μL) were prepared for each sample, with each mixture containing 0.2 μM of each primer, 0.2 μL of Multiplex PCR Mix 1, 20 μL of Multiplex PCR Mix 2 (reaction buffer containing dNTPs and 2 mM Mg^{2+}) and 4–8 μL of the extracted DNA. The presence of PCR products was verified by agarose (2% w/v) gel electrophoresis. Any samples that returned a negative result from the multiplex PCR assay were subjected to a second round of PCR using single primer pairs. The second PCR assay was performed using 15-μL reaction mixtures containing 7.5-μL EmeraldAmp PCR Master Mix, 1 μL of each of the primers (10 μM concentration) and 1 μL of the multiplex PCR product. Reactions were carried out at 95 °C for 4 min, followed by 28 cycles of 95 °C for 30 s, 64 °C for 30 s and 72 °C for 90 s, and a final extension of 7 min at 72 °C in an iCycler Thermal Cycler. The presence of PCR products and target amplicon sizes were confirmed by agarose gel electrophoresis.

Results

Isolation and identification of thermophilic microbes from canned food and beverages

Microbiological analyses are summarized in Table 2. None of the samples displayed signs of spoilage, including acidification or can swelling, despite half of the 294 samples being incubated at 55 °C for 7 days prior to analysis. However, thermophilic bacterial growth was observed in five of the 147 (3.4%) samples stored at room temperature and in seven of the 147 (4.7%) pre-incubated samples under anaerobic conditions. Under aerobic growth conditions, three (2.0%) of the room temperature samples and four (2.7%) of the pre-incubated samples were positive for the growth of thermophilic bacteria after 7 days. The contaminated samples included whole corn, young corn, green peas, mushrooms, pink salmon, Japanese little neck clams, cooked shellfish, green tea and coffee. Among the contaminated samples, the bacterial yield was in the range of 1–3 CFU g^{-1} or mL, except for one green pea and whole corn sample with a yield of approximately 10^2 CFU g^{-1}. No contamination was found in any of the twenty-eight canned fruit samples.

Of the thermophilic bacteria isolated from the samples, *M. thermoacetica* isolates were identified under anaerobic growth conditions, while *B. subtilis* isolates were identified under both growth conditions. Forty-five *M. thermoacetica* isolates were identified from ten of the samples, with four isolates obtained from the samples stored at room temperature and the remaining forty isolates obtained from four pre-incubated canned vegetable samples. None of the samples examined in this study contained *G. stearothermophilus*. Meanwhile, *B. subtilis* was isolated under both aerobic and anaerobic conditions. Three isolates were obtained from the room temperature samples, while nine isolates came from the pre-incubated samples. Among the isolates, seven were isolated from three canned food samples, while five were isolated from four beverage coffee.

Specificity of the *M. thermoacetica* and *G. stearothermophilus* primers

Several primer pairs (Table S1) were examined in this study to improve the specificity and sensitivity of the PCR assay. By altering the PCR conditions (annealing temperature and cycle number), bands of the expected molecular weight were obtained for several target species as well as other non-related species, along with several non-specific bands. Consequently, the primer pairs v3F/v4R and v1-1F/v4R were selected as candidate species-specific primers for *M. thermoacetica*, while Gv2F/Gv3R-M and Gv1F-M/Gv3R–M were chosen for *G. stearothermophilus* (Table 1).

Development of the multiplex PCR assay

Among the primer combinations tested, v3F/v4R and Gv2F/Gv3R-M were successfully used for specific multiplex PCR detection of *M. thermoacetica* and *G. stearothermophilus*, respectively. This primer combination produced amplicons of 296 bp for *M. thermoacetica* and 157 bp for *G. stearothermophilus*. The sensitivity of the assay was determined using a dilution series of target genomic DNA. Results showed that the multiplex PCR assay had a detection limit of 1.6 pg for *M. thermoacetica* and 160 fg for *G. stearothermophilus*, which corresponded to approximately 10^2 and 10^1 CFU mL^{-1}, respectively (Fig. 1). The other primer pair for *Moorella* species, v1-1F/v4R, was less sensitive than v3F/v4R and had a detection limit of 16 pg for *M. thermoacetica*, corresponding to approximately 10^3 CFU mL^{-1} (data not shown). Meanwhile, *Geobacillus* primer pair Gv1F-M/Gv3R-M produced a smeared band under the target band and was therefore not selected.

Molecular detection of *M. thermoacetica* and *G. stearothermophilus* in canned food and beverages

To simultaneously detect the two target species in canned food and beverage samples, genomic DNA extracted from the samples was used as template for

Table 2 Microbiological analysis isolated with canned foods and beverages

Products	Sample stored condition	Total no. of samples tested	Total no. of sample strains grown[†]		Total no. of isolates[‡]			
			Anaerobic plating culture	Aerobic plating culture	*Moorella thermoacetica, M. thermo-autotrophica*	*Geobacillus stearo-thermophilus*	Anaerobic *Bacillus subtilis*	Aerobic
Canned food								
Vegetables								
Sweetcorn (whole, cream),	Normal temperature	46	3	2	3	n.d.[§]	n.d.	2
young corn, mushroom, white asparagus, green peas, mixed beans, other beans, tomato	55 °C 7 days	46	4	n.d.	39	n.d.	n.d.	n.d.
Fruites								
Cranberry, peach, pear, pineapple,	Normal temperature	14	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
blueberry, orange, cherry, olive	55 °C 7 days	14	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Fish, meat								
Trout, mackerel, crab, bonitos,	Normal temperature	28	1	n.d.	1	n.d.	n.d.	n.d.
sardines, squid, tuna, short-neck clams, shell, quail egg, beef, chicken meat	55 °C 7 days	28	2	2	1	n.d.	n.d.	5
Beverages								
Coffee, black tea, green tea,	Normal temperature	59	1	1	1	n.d.	n.d.	1
vegetable juice, tomato juice	55 °C 7 days	59	1	2	n.d.	n.d.	2	2
Total		294	12	7	45	n.d.	2	10

[†]Presented the number of detected with microbiological analysis.

[‡]The >1 kbp sequences were assembled sequences combined from results from 27f/783r and com1/1522r and obtained using the online tool BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

[§]Represented not detected.

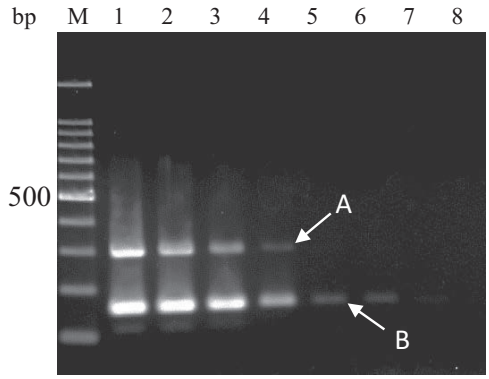

Figure 1 Reference strains *Moorella thermoacetica* and *Geobacillus stearothermophilus* tested by multiplex PCR assays. Lane M, 100-bp DNA ladder marker, lanes 1 and 7, 1.6 ng, 160 pg, 16 pg, 1.6 pg, 160 fg, 16 fg, 1.6 fg of genomic DNA, and lane 8, negative control. 1.6 pg corresponded approx. 10^2 mL^{-1}. The arrows A and B indicated the 296-bp from *M. thermoacetica* and 157-bp from *G. stearothermophilus*, respectively.

the multiplex PCR assay. Partial multiplex PCR results are shown in Fig. 2, and corresponding results are summarized in Table 3. In addition, results obtained from multiplex PCR and the subsequent PCR assay for *M. thermoacetica*/*M. thermoautotrophica* are summarized in Table 4. According to the results shown in Table 4, the number of samples positive for *M. thermoacetica*/*M. thermoautotrophica* was much lower than that for *G. stearothermophilus* contamination in all canned food beverages with multiplex PCR assay. Based on the second PCR analysis, >60% of canned food samples and >90% of beverages returned positive results for *M. thermoacetica*/*M. thermoautotrophica*, suggesting that these species are prevalence in canned and beverages. A realistic hypothesis is that the detection limit for *M. thermoacetica*/*M. thermoautotrophica* was lower than that of *G. stearothermophilus*.

Discussion

Low-acid canned foods are thermally processed to ensure sterility of the food products at ambient temperature for long-term storage. Thermal processing usually results in complete inactivation of vegetative bacteria, but will not eliminate all heat-resistant spores. The most commonly used thermal treatment consists of a F_0 of >5 min (>5 min at 121 °C) at the coldest spot in the products and is based on the botulinum cook (Esty & Meyer, 1922). The canned food F_0 values used in the food industry are strongly related to the heat resistance of the strains isolated. Recent studies have reported the isolation of the highly heat-resistant sporeformers *M. thermoacetica*/*M. thermoautotrophica* from canned products that had been treated at a F_0 of >20 min, while *G. stearothermophilus* is regularly isolated from products treated at moderate or high heat levels (F_0 between 5 and >20 min). These results show that thermophilic bacteria are a good indicator for thermal process settings (Burgess *et al.*, 2010; André & Remize, 2013).

In our study, the absence of spoilage indicators such as acidification and can swelling, while, thermophilic bacterial growth was observed in eleven pre-incubated (3.7%) and seven (2.3%) room temperature canned food and beverage samples (Table 2). These

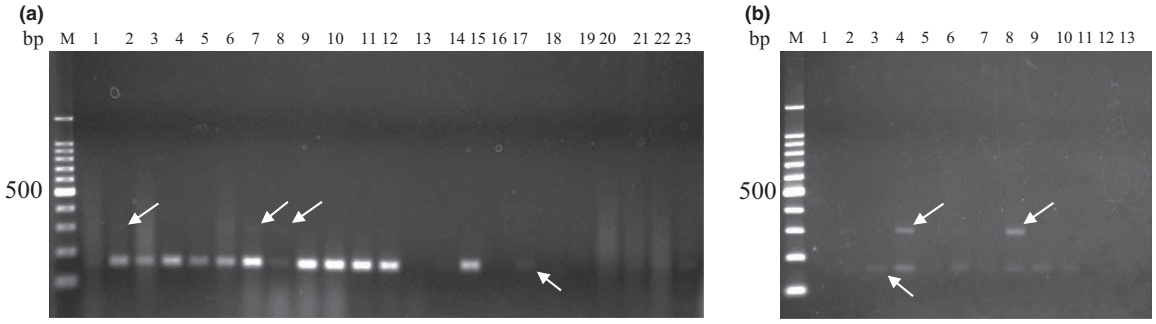

Figure 2 Multiplex PCR results for direct detection from canned food stored at room temperature (a) and beverages incubated at 55 °C for 7 days (b). Canned food samples represented as follows; lane 1: whole corn; lane 2: whole corn; lane 3: cream style corn; lane 4: whole corn; lane 5: whole corn dry pack; lane 6: whole corn; lane 7: young corn; lane 8: mushroom; lane 9 to lane 11: green pea; 12: adzuki beans (*Vigna angularis*); 13: syrup of cherries; 14: canned olives; 15: syrup of nata de coco; 16: syrup of aloe; lane 17 to lane 19: Japanese little neck clam; lane 20: cooked shellfish; lane 21 and lane 22: pink salmon; lane 23: mackerel. Beverage samples represented as lane 1 to lane 8: canned coffee; lane 9 to lane 11: black tea; lane 12: green tea and lane 13 represented negative control. The upper arrow showed 296-bp band expected from *M. thermoacetica*/*M. thermoautotrophica* and under arrow showed 157-bp band expected from *Geobacillus stearothermophilus*.

Table 3 Multiplex PCR assay and second PCR analysis for *Moorella thermoacetica*/*M. thermoautotrophica* from canned foods and beverages

		Multiplex PCR primer pair		Second PCR for detection of *Moorella thermoacetica/ M. thermoautotrophica*
Products	**Stored condition**	**v3F/v4R for *Moorella thermoacetica/ M. thermoautotrophica***	**Gv2F/Gv3R-M for *Geobacillus stearothermophilus***	**v3F/v4R**
Canned food				
Vegetables				
Sweetcorn (whole)	Normal temperature	−	+	+
Sweetcorn (whole)	Normal temperature	+	+	+
Sweetcorn (cream)	Normal temperature	−	+	+
Sweetcorn (whole)	Normal temperature	−	+	+
Sweetcorn (whole, dry pack)	Normal temperature	−	+	+
Sweetcorn (whole)	Normal temperature	−	+	+
Young corn	Normal temperature	+	+	+
Mushroom	Normal temperature	+	+	+
Green peas	Normal temperature	−	+	+
Green peas	Normal temperature	−	+	+
Adzuki beans (*Vigna angularis*)	Normal temperature	−	+	+
Adzuki beans (*Vigna angularis*)	Normal temperature	−	+	+
Cherry	Normal temperature	−	−	+
Olive	Normal temperature	−	−	+
Nata de coco	Normal temperature	−	+	+
Aloe	Normal temperature	−	−	+
Japanese littleneck (*Ruditapes philippinarum*)	Normal temperature	−	+	+
Japanese littleneck (*Ruditapes philippinarum*)	Normal temperature	−	−	+
Japanese littleneck (*Ruditapes philippinarum*)	Normal temperature	−	+	+
Shell	Normal temperature	−	−	+
Pink salmon	Normal temperature	−	+	−
Pink salmon	Normal temperature	−	+	+
Mackerel	Normal temperature	−	−	+
Beverages				
Coffee	55 °C 7 days	−	−	+
Coffee	55 °C 7 days	−	+	+
Coffee	55 °C 7 days	−	+	+
Coffee	55 °C 7 days	+	+	+
Coffee	55 °C 7 days	−	−	+
Coffee	55 °C 7 days	−	+	+
Coffee	55 °C 7 days	−	−	+
Coffee	55 °C 7 days	+	+	+
Black tea	55 °C 7 days	−	+	+
Black tea	55 °C 7 days	−	+	+
Black tea	55 °C 7 days	−	+	+
Green tea	55 °C 7 days	−	+	+

thermophilic bacteria were identified as *M. thermoacetica* and *B. subtilis*, but did not detect *G. stearothermophilus* in any of the canned food and beverage samples with culture-based assay (Table 2), while multiplex PCR analysis identified this species in almost all of the samples (Tables 3). In general, the spores of thermophilic spoilage bacteria can germinate during an incubation step, such as elevated temperature during storage, and/or under favourable growth conditions, such as in the presence of certain media components, leading to their multiplication. However, our results revealed a relatively low isolation rate for these thermophilic bacteria. Therefore, a realistic hypothesis is that treatment process used in production of the commercial canned products and beverages examined in this study satisfactorily inactivates *G. stearothermophilus*, but did not completely eliminate the most heat-resistant species *M. thermoacetica*/

Table 4 Summary of results obtained by multiplex PCR and subsequent single 2nd PCR analysis

Products		Sample storage condition	Ratio of positive samples (%)		
			Multiplex PCR		Single 2nd PCR
			Moorella thermoacetica/ M. thermoautotrophica	*Geobacillus stearothermophilus*	*Moorella thermoacetica/ M. thermoautotrophica*
Canned food	Vegetables, beans	Normal temperature	5.3	98	76
		55 °C 7 days	2.6	88	62
	Fruits	Normal temperature	0	21	43
		55 °C 7 days	0	7.1	36
	Fish, meat, shellfish	Normal temperature	0	74	81
		55 °C 7 days	0	67	70
Beverages		Normal temperature	33	58	96
		55 °C 7 days	11	23	91

M. thermoautotrophica and *B. subtilis*. It is likely that the contaminating *G. stearothermophilus* spores germinate at some point during food processing, losing their heat resistance, and becoming relatively easy to kill. In addition, dormant *G. stearothermophilus* spores that did not germinate during the 1-week incubation period would still yield DNA for the PCR assay. The yields obtained in the current study for *M. thermoacetica* and *B. subtilis* were $\leq 10^2$ CFU g^{-1}, regardless of whether or not the samples were subjected to the pre-incubation step. This suggested no signs of spoilage of the products. In general, D values of *G. stearothermophilus* were still far lower than those of *M. thermoacetica*/*M. thermoautotrophica*.

Sevenier *et al.* surveyed the prevalence of thermophilic spore contamination in two types of canned vegetables (carrots and green beans) that are widely used in the French canning industry. Prevalence rates of 1.6% for *M. thermoacetica*/*M. thermoautotrophica* in green beans and 8.6% for either *G. stearothermophilus* or *Thermoanaerobacterium* spp. in carrot samples were obtained (Sevenier *et al.*, 2012). The food processing lines are continually flowed-in by highly heat-resistant spores but contamination levels are largely dependent on the kind of raw materials (Sevenier *et al.*, 2012). Thus, control of highly heat-resistant bacteria is much more efficiently approached by considering prevalence and initial concentrations of contaminants prior to thermal treatment to achieve suitable risk reduction. Control of bacteria in canned foods depends on the initial number of spores in the product and the parameters used for thermal processing (Leguerinel *et al.*, 2007; Membre & van Zuijlen, 2011). In this study, the PCR results implied a high prevalence of thermophilic sporeformers, including both *M. thermoacetica*/*M. thermoautotrophica* and *G. stearothermophilus* in canned food and beverages. This suggests that these highly heat-resistant species are present in soil particles and marine products and regularly enter canned food processing lines. Soil is a major habitat for many sporeformers, including *M. thermoacetica*, which is present in anoxic microzones (Drake & Daniel, 2004).

Among the species belonging to the aerobic spore-forming bacterial genus *Bacillus*, *B. cereus* is well known not only for its role in food spoilage, but also for its potential to cause food poisoning (Ehlinf-Schulz *et al.*, 2004). Other *Bacillus* species such as *B. licheniformis*, *B. amyloliquefaciens* and *B. pumilus* also produce toxic compounds that may play a role in food poisoning (Suominen *et al.*, 2001; Mikkola *et al.*, 2004); however, none of these species were identified in this study. Under aerobic and anaerobic growth conditions, seven and five *B. subtilis* isolates, respectively, were obtained from canned food and beverage samples in the current study (Table 2). This is in accordance with our previous results showing that the spores of both thermophilic and mesophilic bacteria were highly heat-resistant (Lucking *et al.*, 2013). A recent report confirmed the presence of *B. subtilis* in cocoa powder production lines despite heat treatment at 110 °C for 5 min (Lima *et al.*, 2012). Another study showed that *B. subtilis* was the predominant highly heat-resistant spore-forming species associated with product spoilage in industrial processing environments, accounting for 26% of all isolates (Lucking *et al.*, 2013). Several other studies reported the isolation of *B. subtilis* and *B. amyloliquefaciens* from various food products, including cocoa powder, canned soup ingredients and bread (Ahn *et al.*, 2007; Oomes *et al.*, 2007). There is also evidence to suggest that the isolation of highly heat-resistant sporeformers such as *Bacillus* and *Clostridium* species, which are associated with food contamination, might be favourably influenced by the

use of certain food ingredients and food processing technologies (Postollec *et al*., 2012).

Beverages such as canned coffee with milk and soup are maintained at 55–60 °C in hot vending machines. To avoid food spoilage caused by germination of contaminating bacterial spores, hydrophobic food emulsifiers such as sucrose monopalmitate and sucrose monooleate have been used commercially in the food and beverage industries, to remarkable effect (Nakayama *et al*., 1982; Moriyama *et al*., 1996). However, such additives are less common in canned food manufacturing. In addition, the bacteriostatic effects of heat treatment are limited in comparison with the emulsifiers used in the beverage industry.

The species-specific primers designed by Prevost *et al*. (2010) for the detection of *G. stearothermophilus* were subsequently modified for use in other studies (Pennacchia *et al*., 2014). Pannacchia *et al*. developed a multiplex PCR assay for rapid identification of *G. stearothermophilus* and *A. flavithermus* targeting the internal transcribed spacer region 16S–23S rRNA region and the *rpoB* gene sequence. The assay had a limit of detection of 5 and 50 pg of DNA for the two gene regions, respectively, and was successfully used to assess contamination of milk powder (Pennacchia *et al*., 2014). In our study, the multiplex PCR assay had a detection limit of 1.6 pg of DNA for *M. thermoacetica* and *G. stearothermophilus*, corresponding to approximately 10^2 CFU mL^{-1}. These results confirm that multiplex PCR is likely to be sensitive enough to detect low level contamination and allow monitoring of contamination in food materials.

It is difficult to confirm whether positive results from PCR-based assays represent the presence of live bacteria, or whether they are actually false positives derived from amplification of DNA from dead or damaged cells or from extracellular sources. Inhibitors of PCR can also be found in microbial DNA solutions extracted soil, food and general environmental samples. This is occasionally interpreted as a weak point of PCR, but can be irrelevant for food safety considerations. Regardless of whether the micro-organisms are viable, a positive result indicates the occurrence of possible contamination events at some point during food material treatment and processing, and/or food production. However, it should be remembered that DNA from cells that are killed during processing would also be detected by PCR-based techniques, making it appear that there is a greater level of contamination than is detected using culture-based methods.

Conclusion

Highly heat-resistant thermophilic sporeformers can contaminate canned food and beverage products by entering processing lines with raw materials and ingredients. This multiplex PCR method may allow selective detection of bacteria that are present at low levels in complex matrices, as well as that can remain and undetected by culture-based method. We used this multiplex PCR method to quantify thermophilic sporeformers commercial canned food and beverage products and showed that the current approach could be useful for screening bacterial contaminants in various food materials and ingredients. Together with traditional culture-based methods, screening raw materials and ingredients, as well as samples from processing lines, could help in risk management strategies and improve initial safety controls. The multiplex PCR assay is simple and economically advantageous for guaranteeing the hygiene of food materials and may therefore be suitable for use by material and ingredient suppliers as well as the food processing industry.

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Conflict of interests

The authors declare no conflict of interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primer sequences used in this study.