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PCR Detection of Thermophilic Spore-Forming Bacteria Involved in Canned Food Spoilage

S. Prevost, S. Andre, F. Remize

Abstract Thermophilic bacteria that form highly heat-resistant spores constitute an important group of spoilage bacteria of low-acid canned food. A PCR assay was developed in order to rapidly trace these bacteria. Three PCR primer pairs were designed from rRNA gene sequences. These primers were evaluated for the specificity and the sensitivity of detection. Two primer pairs allowed detection at the species level of *Geobacillus stearothermophilus* and *Moorella thermoacetica/thermoautrophica*. The other pair allowed group-specific detection of anaerobic thermophilic bacteria of the genera *Thermoanaerobacterium*, *Thermoanaerobacter*, *Caldanerobium* and *Caldanaerobacter.* After a single enrichment step, these PCR assays allowed the detection of 28 thermophiles from 34 cans of spoiled low-acid food. In addition, 13 ingredients were screened for the presence of these bacteria. This PCR assay serves as a detection method for strains able to spoil low-acid canned food treated at 55°C. It will lead to better reactivity in the canning industry. Raw materials and ingredients might be qualified not only for quantitative spore contamination, but also for qualitative contamination by highly heat-resistant spores.

Introduction

The thermal process applied during canning results in products, which remain stable for long periods at ambient temperature. The minimum levels of heat treatment were determined on safety concerns to prevent the development of *Clostridium botulinum* [10]. However, this thermal treatment is not always sufficient to inactivate all spore-forming bacteria, especially those that are highly heat-resistant and non-pathogenic [13].

Spore-forming thermophilic bacteria are recognized as the main cause of spoilage of canned food [3, 8, 18]. The typical species of thermophilic bacteria found in canned products are *Thermoanaerobacterium thermosaccharolyticum* and *Moorella thermoacetica* for obligate anaerobes, plus *Geobacillus stearothermophilus* for facultative anaerobes [2, 5, 9]. *G. stearothermophilus* is the typical species responsible of "flat sour" spoilage, due to the production of acids but not gas from saccharides [7]. Thermophilic anaerobes usually produce abundant gas, which result in swelling of cans. *M. thermoacetica* produces acetate as main fermentation product, and as such is termed acetogenic and used for biotechnological applications [7, 14]. These bacteria share the characteristic of being able to form highly heat-resistant spores [2, 4, 11]. The decimal reduction times is well established for *G. stearothermophilus* to a few min at 121°C [20, 26]. The spores of *M. thermoacetica* strains, which survive autoclaving, have decimal reduction times between 23 and 111 min at 121°C depending on sporulation conditions [4].

Thermal process setting requires taking into account spore heat resistance and population level, together with their ability to grow under ambient storage conditions [13, 17, 19].

Therefore, not only the number of heat-resistant spores but also the nature of bacteria is an important parameter regarding the potentially high heat resistance of endospores. In addition, identification of the bacteria, which cause canned food spoilage, can implement corrective and preventive action, as the thermal resistance of the spores

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and the conditions of growth and sporulation are known. These elements are then used in the processing lines, e.g. to control the thermal treatment applied, or to reduce the level of spore contamination at different steps of the process before heat treatment, through enhancement of hygiene procedures.

Nowadays, identification is performed by 16S rRNA gene sequencing, a method that requires multiple culture steps to achieve isolation and therefore is time-consuming and sometimes unsuccessful if the bacteria fail to grow. The improvement of industrial reactivity requires the design of molecular methods for fast detection of contaminants. In case of spoiled canned food, molecular tools need to detect thermophilic spore-forming bacteria. Specific primers that amplify 16S rRNA gene and 16S–23S rRNA gene internal transcribed spacer (ITS) have been widely used as a target, and have given promising results [16, 21, 27]. In this study, three PCR primer pairs were developed from publicly available sequences to detect the most frequent spoilage thermophilic spore formers: *M. thermoacetica*, *G. stearothermophilus*, *Thermoanaerobacterium* spp. The specificity and the sensitivity of the primer sets were evaluated on DNA extracted from thermophilic bacteria obtained from collections and isolated from canned food samples. In addition, the potential of the PCR assay was assessed with non-stable canned food and ingredients.

Materials and Methods

Bacterial Strains and Culture Conditions

The strains used for specificity assays are shown in Tables 1 and 2. Types and reference strains were obtained from various culture collections and maintained in the laboratory. Other strains were collected and isolated from non-stable canned food and were identified by 16S rRNA gene sequencing.

Aerobic bacteria were grown in brain–heart infusion (BHI) broth or in BCP glucose agar (dextrose tryptone agar), whereas anaerobic bacteria were grown in Rosenow Cysteine broth complemented with brain fragments and marble chips or in meat-liver glucose agar complemented with 2% yeast extract.

Thermophilic strains were incubated at 55°C. *Geobacillus* and *Thermoanaerobacterium* cultures were incubated for 2 days, while *Moorella* cultures were incubated for 5 days.

Other bacteria were incubated at 37°C for 48 h.

PCR Primers Design

Target sequences were selected from the National Centre for Biotechnology Information database. The sequences of the 16S rRNA gene and ITS 16S–23S regions were aligned in order to search for specific primer sites. A pair of oligonucleotides for each bacterial group was selected and synthesized by Eurogentec. The sequences and positions of oligonucleotides are presented in Table 3.

DNA Preparation and PCR Optimization

DNA was prepared from picked colonies or from 1 ml liquid culture with the InstaGene^{TM} (Bio-Rad, Marnes-la-Coquette, France) matrix following manufacturer's instructions.

All amplification steps were performed in 25 μl tubes with an Applied Biosystem GeneAmp PCR system 9700. The reactions were performed with 5 μl of DNA matrix, 0.2 mM dNTPs, 1× *Taq* reaction buffer, 2 units of *Taq* polymerase AmpliTaq (Applied Biosystem, Courtaboeuf, France), 0.3 μM of each primer and 1.5–3 mM $MgCl_2$. The PCR was cycled once at 94°C for 5 min, 40 repetitions at 94°C for 1 min, 65°C as indicated for 1 min, 72°C for 1 min, and once at 72°C for 10 min. On completion of cycling, amplicons were directly analysed by electrophoresis in TAE 1% agarose gel.

The specificity was optimized by adjusting annealing temperature and magnesium chloride concentration.

16S rRNA gene amplification was performed with primers FD1 and RD1 [29]. Sequencing was carried out by Eurofins MWG Operon (Ebersberg, Germany).

Sensitivity Assays

The sensitivity was determined for each of the three PCR primer pairs with DNA from different strains. The level and purity of the purified DNA were evaluated by measuring OD 260 and 280 nm and confirmed by amplification with universal primers. The level of DNA was adjusted to 1 μg for 5 μl and serially diluted in triplicate in ultrapure water. Each DNA extract corresponding to one dilution level was tested for PCR amplification in duplicate, so that at least six PCR tests were obtained for each concentration. The lowest amount of DNA with a positive amplification in most tests was chosen as the sensitivity limit.

Examination of Canned Food

Non-stable canned products were detected after an incubation of 7 days at 55°C according to NF V08-408 [1]. Samples of 25 or 50 g of non-stable products were diluted twofold in peptone water and homogenized with a stomacher for 1 min. The stomacher bag filtrate was used to inoculate broth, which was then incubated at 55°C for 2–5 days. Identification was performed from positive

Table 1 List of strains used for *G. stearothermophilus* detection specificity tests

Species	Bacterial strains	No of strains	Source	PCR Gbs[a]
G. stearothermophilus	DSM 1550, 2804 001, 2804 017, 2804 019, 2804 030, 2804 033, 2804 037, 2804 038, 2804 039, 2804 052, 2804 053, 2804 054, 2804 055, 2804 056, 2804 057, 2804 116, 2804 129, 2804 144, 2804 145	19	DSMZ CTCPA	+
Geobacillus kaustophilus	DSM 7263	1	DSMZ	–
Geobacillus subterraneus	DSM 13 552	1	DSMZ	–
Geobacillus thermocatenulatus	DSM 730	1	DSMZ	–
Geobacillus thermodenitrificans	DSM 465	1	DSMZ	–
Geobacillus thermoglucosidasius	DSM 2 542	1	DSMZ	–
Geobacillus thermoleovorans	DSM 5 366	1	DSMZ	–
Geobacillus uzenensis	DSM 13 551	1	DSMZ	–
Geobacillus caldoxylosilyticus	2801 003	1	CTCPA	–
Geobacillus sp	2810 004	1	CTCPA	–
Bacillus amyloliquefaciens	3101 006	1	CTCPA	–
Bacillus cereus	ATCC 14579	1	ATCC	–
Bacillus circulans	3104 002	1	CTCPA	–
Bacillus coagulans	3105 003, 3105 034	2	CTCPA	–
Bacillus licheniformis	3107 028	1	CTCPA	–
Bacillus smithii	3108 002, 3108 004, 3108 017	3	CTCPA	–
Bacillus subtilis	3111 032, 3111 033, 3111 035, 3111 038	4	CTCPA	–
Paenibacillus graminis	2910 001	1	CTCPA	–
Paenibacillus macerans	2903 006, 2903 009, 2903 012, 2903 014	4	CTCPA	–
Paenibacillus turicensis	2907 001	1	CTCPA	–
Lactobacillus sakei	3009 005	1	CTCPA	–
Enterococcus faecalis	ATCC 29 212	1	ATCC	–
Escherichia coli	DSM 30 083	1	DSMZ	–
Listeria monocytogenes	ATCC 19 115	1	ATCC	–
M. thermoacetica	DSM 521	1	DSMZ	–
M. thermoautotrophica	DSM 1974	1	DSMZ	–
M. thermoacetica/ thermoautotrophica	1901 056, 1901 057, 1901 058, 1901 059, 1901 060	5	CTCPA	–
Thermoanaerobacterium thermosaccharolyticum	2506 006, 3224 001	2	CTCPA	–
Clostridium novyi	3210 014, 3210 017, 3213 011	3	CTCPA	–
Clostridium sporogenes	DSM 1734, 3213 018, 3213 020, 3222 004	4	DSMZ CTCPA	–
Clostridium thermopalmarium/ thermobutyricum	3219 001, 3226 006	2	CTCPA	–

[a] To detect *G. stearothermophilus*

culture broth tubes, preferentially anaerobic tubes if available. It was further confirmed by 16S rRNA gene sequencing of DNA extracted from isolated colonies obtained from the same broth tube by plating onto the adequate medium.

Examination of Ingredients

Samples of 25 g of ingredients were diluted 10-fold in peptone water and homogenized with a stomacher for

Table 2 List of strains used for *Moorella* and *Thermoanaerobacterium* detection specificity tests

Species	Bacterial strains	No. of strains	Source	PCR Moo[a]	PCR Thm[b]
M. thermoacetica	DSM 521	1	DSMZ	+	−
M. thermoautotrophica	DSM 1974	1	DSMZ	+	−
M. thermoacetica/thermoautotrophica	1901 043, 1901 056, 1901 057, 1901 058, 1901 059, 1902 051	6	CTCPA	+	−
M. thermoacetica/thermoautotrophica	1901 003, 1901 066, 1901 067, 1901 071, 1901 044	5	CTCPA	+	nd
M. thermoacetica/thermoautotrophica	1901 060, 1902 006	2	CTCPA	+	+
Moorella glycerini	DSM 11 254	1	DSMZ	−	nd
Moorella mulderi	DSM 14 980	1	DSMZ	−	nd
Thermoanaerobacter mathranii/ thermocopriae	2105 001, 3220 003, 2503 001	3	CTCPA	−	+
Thermoanaerobacterium aciditolerans/ aotearoense	2502 001, 2503 020, 2503 018, 2503 008, 2501 001	5	CTCPA	−	+
T. aciditolerans	DSM 16 487	1	DSMZ	+	+
T. aciditolerans/aotearoense	2503 014, 2503 019	2	CTCPA	+	+
Thermoanaerobacterium saccharolyticum	2505 001, 2506 007, 2503 012	3	CTCPA	−	+
Thermoanaerobacterium sp	2503 009	1	CTCPA	−	+
Thermoanaerobacterium thermosaccharolyticum	DSM 571	1	DSMZ	−	+
T. thermosaccharolyticum	2506 006, 3224 001, 2503 010	3	CTCPA	−	+
T. thermosaccharolyticum	2503 006, 2503 015, 2506 011	3	CTCPA	+	+
Thermoanaerobacterium zeae	2507 001	1	CTCPA	−	+
Thermoanaerobacterium sp	2506 012	1	CTCPA	−	−
Thermoanaerobacterium saccharolyticum	DSM 7060	1	DSMZ	−	−
Caldanaerobium fijensis	2503 013	1	CTCPA	−	+
Clostridium bifermentans	DSM 630	1	DSMZ	−	−
Clostridium cochlearium	3206 002	1	CTCPA	−	−
Clostridium novyi	3210 014, 3210 017, 3210 011	3	CTCPA	−	−
Clostridium sporogenes	3213 018, 3213 020, 3222 004, DSM 1734	4	DSMZ	−	−
Clostridium thermobutyricum/ thermopalmarium	3219 001, 3226 006	2	CTCPA	−	−
Clostridium thermobutyricum/ thermopalmarium	3226 004, 3226 005	2	CTCPA	nd	−
Clostridium thermopalmarium	DSM 2544	1	DSMZ	nd	−
Bacillus amyloliquefaciens	3110 006	1	CTCPA	−	−
Bacillus coagulans	3105 034	1	CTCPA	−	−
Bacillus coagulans	3105 003	1	CTCPA	−	nd
Bacillus coagulans	DSM1	1	DSMZ	nd	−
Bacillus licheniformis	3107 028	1	CTCPA	−	−
Bacillus licheniformis	3107 001, 3107 014	2	CTCPA	−	nd
Bacillus smithii	3108 017	1	CTCPA	−	−
Bacillus subtilis	3111 032, 3111 033, 3111 038	3	CTCPA	−	−
G. stearothermophilus	2804 144, 2804 145	2	CTCPA	−	−
Paenibacillus macerans	2903 006, 2903 014	2	CTCPA	−	−
Lactobacillus sakei	3009 005	1	CTCPA	−	−

[a] To detect *M. thermoacetica*; [b] To detect the *Thermoanaerobacterium* group; *nd* not determined

1 min. The obtained stomacher bag filtrate was treated for 10 min at 100°C to eliminate vegetative cells and then used for serial dilutions in peptone salt broth. For CFU determination, the dilutions were poured into two media. BCP glucose agar was used to determine aerobic thermophilic counts and was incubated for 2 days at 55°C. Meat-

Table 3 Primers used in this study

Reaction	Region	Name	Sequence
PCR *Moorella* F	rRNA 16S	FD1	AGAGTTTGATCHTGGCTCAG
PCR *Moorella* R	rRNA 16S	R3	AAAGGCTATTCGCCTTTAAGAC
PCR *Thm* F	rRNA 16S	772F2	TGGCGAAAGCGGCTCTCTGG
PCR *Thm* R	rRNA 16S	1239R	CCCCACCTTCCTCCGTG
PCR *Geobacillus* F	rRNA ITS 16S–23S	Fits2	GGGGAAGCGCCGCGTTCGG
PCR *Geobacillus* R	rRNA ITS 16S–23S	Rits2	GTGCAAGCACCCTTGCAGGCGAAGA
Universal PCR F	rRNA 16S	FD1	AGAGTTTGATCHTGGCTCAG
Universal PCR R	rRNA 16S	RD1	GGMTACCTTGTTACGAYTTC
Sequencing R	rRNA 16S	S6-16S	GTATTACCGCGGCTGCTG

liver glucose agar was used to determine anaerobic thermophilic counts and was incubated for 5 days at 55°C. For PCR assay, up to 10 colonies if available were randomly picked prior to DNA isolation.

Results

PCR Specificity for *G. stearothermophilus*

A PCR assay was developed from available sequence data to detect the aerobic thermophile *G. stearothermophilus*. A specific primer pair was designed in the rRNA internal transcribed sequence (ITS) 16S–23S (Table 3). The assay optimization resulted in the choice of an annealing temperature of 65°C with 1.5 mM $MgCl_2$.

The specific amplification of a 302-bp fragment was obtained for 19 *G. stearothermophilus* isolates, whereas no amplicon was obtained from nine strains belonging to other *Geobacillus* species (Table 1). Forty-one other non-*Geobacillus* strains were not detected by the assay. Among them, the most closely related species were the thermophilic species *Bacillus coagulans*, *Bacillus licheniformis* and *Bacillus smithii* belonging to *Bacillaceae* family.

PCR Specificity for the Detection of *M. thermoacetica/thermoautotrophica* and *Thermoanaerobacterium* Group

The PCR assay developed to detect *M. thermoacetica/thermoautotrophica* detection triggered the 16S rRNA gene region (Table 3). From a genetic point of view, *M. thermoacetica* and *M. thermoautotrophica* present differences in 16S rRNA gene sequences of less than 1% [5]. The forward primer FD1 was described by Weisburg et al. [29] for use as a universal 16S rRNA coding region amplification primer. A reverse primer R3 was designed by sequence alignments. The use of these primers allowed the amplification of a single 488-bp fragment at an annealing temperature of 65°C with 3 mM $MgCl_2$.

PCR specificity was assayed against 15 *M. thermoacetica/thermoautotrophica* isolates, 1 *Moorella glycerini* and 1 *Moorella mulderi* (Table 2). All *M. thermoacetica/thermoautotrophica* strains were detected, while other species of the same genus were not. Fifty-one other isolates were used for exclusivity assays. Among them, anaerobic thermophilic bacteria accounted for 33 of the isolates. Unexpectedly, three *Thermoanaerobacterium aciditolerans/aotearoense* and three *T. thermosaccharolyticum* strains generated positive signals. Sequencing of the 16S rRNA coding region of these strains did not show higher similarity with the R3 primer than did DNA from other *Thermoanaerobacterium* strains like DSM 7060, which did not generate positive signals with the *M. thermoacetica/thermoautotrophica* detection assay.

Primers were designed in the 16S rRNA gene region by alignment from all *Thermoanaerobacterium* sequences available (Table 3). A maximal score was obtained for DNA regions from the following genera: *Thermoanaerobacter*, *Thermoanaerobacterium*, *Caldanaerobium* and *Caldanaerobacter*. These primers were also checked against other spore-forming bacteria sequences. PCR optimization was performed with cycle conditions identical to the PCR runs for *M. thermoacetica/thermoautotrophica* and *G. stearothermophilus*. It showed that 1.5 mM $MgCl_2$ was optimal regarding specificity. Twenty-two isolates of the industrial and collection strains of *Thermoanaerobacterium* spp, three isolates of *Thermoanaerobacter thermocopriae/mathranii* and one isolate of *Caldanaerobium fijensis* were assayed. Two of them, DSM 7060 and 2506 012, were not detected by this assay (Table 2), but were amplified by universal PCR. Sequence analysis from the former did not reveal any mismatch. Ten isolates of the closely related species *M. thermoacetica* were tested and two of these led to positive detection. As expected, the 29 isolates from other species, including anaerobic

thermophiles such as *Clostridium novyi* and *Clostridium thermopalmarium*, did not lead to positive detection.

The PCR test to detect *M. thermoacetica/thermoautotrophica* isolates was able to detect the species. The other PCR assay detected *Thermoanaerobacterium* and its closest relatives, and was named the *Thermoanaerobacterium* group detection assay. For two *M. thermoacetica/thermoautotrophica* and six *Thermoanaerobacterium* sp strains, a positive signal was obtained with the two PCR assays that trigger anaerobic bacteria rRNA gene. In these cases, complementary experiments such as sequencing of 16S rRNA coding region are necessary to confirm the identification.

Sensitivity of PCR Detection

Sensitivity assays were performed with the three PCR assays herein described as well as with the universal PCR assay (Fig. 1). The most sensitive PCR was the one aiming to detect *G. stearothermophilus*. The sensitivity level of this assay was evaluated at 0.5 pg. The least sensitive assay was that for *M. thermoacetica* detection, with a level of 500 pg. The detection limit for the *Thermoanaerobacterium* group PCR assay was estimated at 50 pg. It was identical to the latter for universal PCR assay.

Detection of Thermophilic Bacteria in Non-Stable Canned Products

In order to test whether the PCR assays were appropriate for the investigation of non-stable canned food, we tested 34 products, which had failed the stability test performed at 55°C (Table 4). After a single enrichment step and quick DNA extraction, the samples were tested with the three PCR assays.

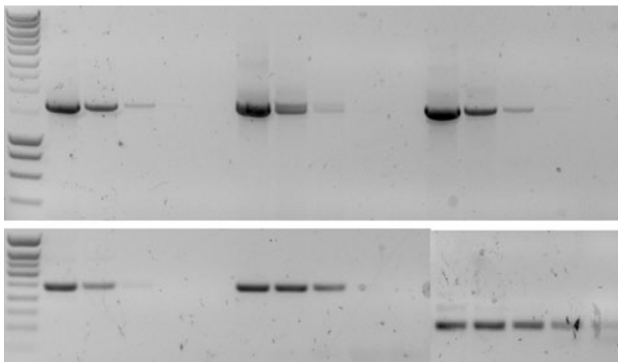

Fig. 1 Sensitivity of the PCR assays. *Upper part*: 16S rRNA gene amplification; *lower part*: specific detection PCR. *From left to right*: ladder, five lanes *M. thermoacetica* DNA as template, five lanes *T. thermoasaccharolyticum* DNA as template, five lanes *G. stearothermophilus* DNA as template. The five lanes correspond to 5 ng, 500 pg, 50 pg, 5 pg and 0.5 pg of template DNA

Geobacillus stearothermophilus was detected in seven non-stable samples. Fourteen samples tested positive for *M. thermoacetica/thermoautotrophica* and seven were positive for *Thermoanaerobacterium* group bacteria. Seven samples generated negative PCR tests even though the cultures were visually positive. These samples were used to isolate and identify bacteria by 16S rRNA gene sequencing. Identification showed the presence of two strains of *B. licheniformis*, one *B. smithii*, one *C. novyi*, one *C. thermopalmarium* and one *Paenibacillus graminis*.

Detection of Thermophilic Bacteria in Ingredients

Ingredients may be the entrance point for highly heat-resistant bacteria in food [19]. In particular, milk powders, which are produced by a heating process, are known to present thermophilic bacteria spore contamination occasionally [6, 22]. Thermophilic bacteria spores were counted in 13 ingredients such as spices, milk powder and aromas. DNA was extracted from randomly picked colonies and used in PCR assays. *G. stearothermophilus* or *Thermoanaerobacterium* group bacteria were detected by the assay in several samples (Table 5). No correlation was observed between colony counts and positive PCR tests. This was expected because heat-resistant spores present a remarkable diversity in milk powders and spices [19, 22].

Discussion

Moorella thermoacetica, *G. stearothermophilus* and *Thermoanaerobacterium* spp are among the most frequent contaminants causing low-acid canned food spoilage [2, 5, 9]. These thermophilic bacilli are of hygienic concern to the manufacturers and processors of low-acid canned food, and the ability to monitor these contaminants before or after thermal processing would have economic benefits. Remarkably, the first reports on the presence of thermophilic bacteria causing flat souring or swelling of canned food date from about 80 years [28], but rapid microbiology tools were rarely reported in spite of their recognized value [9, 15]. This study describes the development of a PCR assay to detect thermophilic bacteria forming highly heat-resistant spores that may pass through retort process of low-acid canned food.

An extensive specificity study is reported with both collection strains and strains collected from industrial environments. The possibility that strains from the same taxonomical group but isolated from different sources result in a negative result with a detection PCR is well documented for *Bacillus sporothermodurans* [12, 27]. A high specificity level was established for our PCR assay designed at the species level for *G. stearothermophilus*. A

Table 4 Detection of thermophilic bacteria from 55°C non-stable canned food

Sample	Product	PCR positive detection and/or rRNA gene sequencing identification[a]
CT196	Canned fish-based course	*G. stearothermophilus*[a]
CT197	Canned meat	*G. stearothermophilus*[a]
CT198	Canned fish-based course	*G. stearothermophilus*[a]
CT199	Meat dumpling	*G. stearothermophilus*[a]
CT248	Canned meat	*G. stearothermophilus*[a]
CT260	Sweet corn	*G. stearothermophilus*
CT263	Sweet corn	*G. stearothermophilus*
D8137	Fish dumpling	*M. thermoacetica/thermoautotrophica*[a]
D8161	Pre-cooked meal with meat	*M. thermoacetica/thermoautotrophica*[a]
D8162	Cooked vegetables	*M. thermoacetica/thermoautotrophica*[a]
CT090	Pre-cooked meal with meat	*M. thermoacetica/thermoautotrophica*[a]
CT027	Pre-cooked meal with chicken	*M. thermoacetica/thermoautotrophica*
CT071	Green peas and carrots	*M. thermoacetica/thermoautotrophica*[a]
CT073	Green peas	*M. thermoacetica/thermoautotrophica*[a]
CT089	Green peas	*M. thermoacetica/thermoautotrophica*
CT141	Mixed vegetables	*M. thermoacetica/thermoautotrophica*[a]
CT201	Peas	*M. thermoacetica/thermoautotrophica*[a]
CT217	Peas	*M. thermoacetica/thermoautotrophica*
CT256	Peas	*M. thermoacetica/thermoautotrophica*
CT274	Pre-cooked meal with meat	*M. thermoacetica/thermoautotrophica*
CT195	Pre-cooked meal with meat	*M. thermoacetica/thermoautotrophica*[a]
CT086	Peas	*Thermoanaerobacterium* group[a]
CT109	Peas	*Thermoanaerobacterium* group[a]
CT124	Peas	*Thermoanaerobacterium* group
CT125	Pre-cooked meal with meat	*Thermoanaerobacterium* group[a]
CT149	French beans	*Thermoanaerobacterium* group[a]
CT070	Peas	*Thermoanaerobacterium* group[a]
CT072	Paté	*Thermoanaerobacterium* group[a]
CT135	Soup	*Bacillus licheniformis*[a]
CT087	Paté	*Bacillus licheniformis*[a]
CT213	Paté	*Bacillus smithii*[a]
CT228	Pre-cooked meal with meat	*Clostridium novyi*[a]
CT169	Paté	*Clostridium thermopalmarium*[a]
CT017	Pre-cooked meal with fish	*Paenibacillus graminis*[a]

[a] Corresponds to strains, which were isolated and further identified by 16S rRNA gene sequencing

species-specificity level was obtained for the *M. thermoacetica/thermoautotrophica* detection assay, whereas the PCR assay for the *Thermoanaerobacterium* group detected all tested *Thermoanaerobacterium* species, but also the closest relatives belonging to *Thermoanaerobacter* and *Caldanaerobium* genera. These two PCR assays, which trigger thermophilic obligate anaerobes, presented some overlaps regarding the detected isolates.

All three PCR assays were able to detect low levels of DNA. However, high sensitivity is not an absolute requirement since these assays were developed with an enrichment step from canned food or from colonies picked from ingredients.

This assay showed its relevance for the identification of bacteria responsible for low-acid canned spoilage at 55°C. It allowed the cause to be detected rapidly and without isolation of bacteria in more than 80% of examined cases.

In addition, this assay was useful for ingredient qualification. Indeed, thermophilic spore counts, without species consideration, are an important issue for risk assessment [23–25]. It takes into account the large diversity of spore formers in food environment. However, also the detection of highly heat-resistant species is important. Adjustment of food retort settings takes into account historic data collection about spore contamination levels of ingredients and raw materials. In order to optimize sterilization processes,

Table 5 Spore enumeration and PCR assays of ingredients and raw materials

Product	Thermophilic anaerobic spores counts (cfu/g)	Thermophilic aerobic spores counts (cfu/g)	PCR positive detection
Cocoa and dry milk	1,700	220	*G. stearothermophilus*
Milk powder	20	<10	*G. stearothermophilus*
Milk powder	1,200	400	*G. stearothermophilus*
Spices	100	nd	*G. stearothermophilus*
Cocoa and dry milk	1,800	220	*G. stearothermophilus*
Milk powder	1,000	50	*G. stearothermophilus*
Pre-cooked meal with meat	<10	40	*G. stearothermophilus*
Milk powder	1,000	50	*G. stearothermophilus* and *Thermoanaerobacterium* group
Milk powder	nd	400	None
Dry garlic	nd	1,000	None
Spices	nd	40	None
Pre-cooked meal with meat	<10	320	None

the heat resistance of contaminating spores must be considered. The tools developed here enable ingredients to be qualified. Similarly, PCR methods were developed for *B. sporothermodurans* and were further used to explore possible contamination routes [27].

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