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ABSTRACT

Spoilage of low-acid canned food during prolonged storage at high temperatures is caused by heat resistant thermophilic spores of strict or facultative bacteria. Here, we performed a bacterial survey over two consecutive years on the processing line of a French company manufacturing canned mixed green peas and carrots. In total, 341 samples were collected, including raw vegetables, green peas and carrots at different steps of processing, cover brine, and process environment samples. Thermophilic and highly-heat-resistant thermophilic spores growing anaerobically were counted. During vegetable preparation, anaerobic spore counts were significantly decreased, and tended to remain unchanged further downstream in the process. Large variation of spore levels in products immediately before the sterilization process could be explained by occasionally high spore levels on surfaces and in debris of vegetable combined with long residence times in conditions suitable for growth and sporulation. Vegetable processing was also associated with an increase in the prevalence of highly-heat-resistant species, probably due to cross-contamination of peas via blanching water. *Geobacillus stearothermophilus* M13-PCR genotypic profiling on 112 isolates determined 23 profile-types and confirmed process-driven cross-contamination. Taken together, these findings clarify the scheme of contamination pathway by thermophilic spore-forming bacteria in a vegetable cannery.

1. Introduction

Sealed and sterilized canned vegetables remain microbiologically stable for years at ambient temperature as the heat process inactivates mesophilic microorganisms. Spoilage in low-acid (pH > 4.5) canned vegetables occurs mainly at high incubation temperatures (>40 °C) and is caused by the survival and further multiplication of thermophilic spore-forming bacteria. These bacteria are not known to be pathogenic to humans but are considered an industrial risk that warrants proper control by canning industries. For instance, the stability testing of canned foods after 7-day incubation at 55 °C (French standard NF V08-408: AFNOR, 1997) is used for detection of a lack of hygiene during fabrication and as a recommendation for export to high-temperature climate zones in line with EU regulations on safe and wholesome food in trading. The percent non-stability of canned green beans recorded for instance by the French canning industry over a 10 year period was close to 1.5% (Rigaux et al., 2014). *Geobacillus stearothermophilus*,

Moorella thermoacetica/thermoautotrophica and *Thermoanaerobacterium* spp. are regularly identified as the most common causes of low-acid spoilage in canned foods, including canned vegetables, representing up to 75% of the species responsible for non-stability at 55 °C (André et al., 2013; Ashton and Bernard, 1992; Carlier et al., 2006; Carlier and Bedora-Faure, 2006; Dotzauer et al., 2002). These three bacteria share high heat resistance, with D-values (decimal reduction times) at 121 °C greater than 1 min for *G. stearothermophilus* in most instances and up to 111 min for *M. thermoacetica* (André et al., 2013; Byrer et al., 2000; Matsuda et al., 1982). Other moderately thermophilic bacteria such as *Bacillus coagulans*, *Bacillus licheniformis*, *Anoxybacillus* spp., *Paenibacillus* spp., *Thermoanaerobacter* spp. and *Clostridium thermobutyricum/thermopalmarium* that are able to survive heat treatment at temperatures over 100 °C for 10 min have also been identified as canned food spoilage microorganisms (André et al., 2013; Feig and Stersky, 1981; Raso et al., 1995).

Food processing facilities are exposed to a range of contamination sources (Carlin, 2011). Soil and natural environments are reservoirs of spore-forming bacteria (Groenewald et al., 2009; Guinebretiere and Nguyen-The, 2003; Logan and De Vos, 2009; Zeigler, 2014). Spore contamination into food processing facilities is vectored by soil dust in open areas, adhesion to unprocessed food material, or carriage by employees (Groeneweld et al., 2009; Sevenier et al., 2012). The process of canning vegetables combines several operations resulting in contamination with

micro-organisms. Freshly-harvested vegetables are washed, trimmed and cut, then blanched by steaming or dipping in hot water for a few minutes at temperatures close to 100 °C, filled into cans and covered with a hot cover brine, and finally sterilized. However, between blanching and sterilization, vegetables remain at relatively high temperatures that may allow thermophilic bacteria to grow and eventually sporulate. Moreover, spore adhesion to industrial materials and spore resistance to cleaning and disinfection favor spore persistence along the chain (Parkar et al., 2001). A delay in spore release from debris and surfaces may occur, and cross-contamination may emerge long after initial contamination (André et al., 2012; Sànchez et al., 2009; Seale et al., 2008; Tauveron et al., 2006). To better understand contamination pathways and to explore the eventuality of spore formation in the processing chain is therefore necessary for better control of canned food quality.

There are a number of classical and molecular methods for detecting and quantifying spore-forming bacteria from processing lines (Postollec et al., 2012; Prevost et al., 2010). For instance, *Bacillus cereus* strains isolated at different steps in a processing line of zucchini purée were typed by M13 sequence-based PCR (M13-PCR), an amplification method derived from for Random Amplified Polymorphic DNA (RAPD; Guinebretiere et al., 2003; Guinebretiere and Nguyen-The, 2003). The method clustered strains according to genetic fingerprint and was able to identify contamination pathways: cultivation soil of zucchini and ingredients such as milk proteins and starch were proven to be a major source of primary contamination in zucchini purée. However, the *B. cereus* genotypes detected in ingredients were not considered a concern in processed products properly stored at cold temperatures. The three genotyping methods RAPD, *rpoB* sequencing, and multiple loci variable number of tandem repeats analysis (MLVA) applied to *B. licheniformis* thermophilic isolates from milk powder processing line samples and commercial retail samples established that this bacterium derives from non-factory sources and is not subjected to significant clonal selection within processing plants (Dhakal et al., 2013). High-resolution melt analysis of MLV recently applied to *Geobacillus* species isolated from milk found that several genotypes can coexist in a single processing run, on top of the presence of the same genotypes in samples taken at a 17-year interval (Seale et al., 2012).

The aim of this work was to determine changes in population levels of thermophilic spore-forming bacteria at different steps of the processing chain for canned green peas and carrots. The thermophilic species most frequently involved in spoilage were specifically screened from the processing line. M13-PCR typing was then applied on *G. stearothermophilus* isolates to clear up the contamination pathways involved. The method was previously described for genetic group differentiation in this species (Durand et al., 2014).

2. Material and methods

2.1. Sampling

A total of 341 samples (263 in 2011 and 78 in 2012) taken for microbiological analyses were collected near or in a single Western-France processing plant during June and July in 2011 and in 2012, over 5 weeks corresponding to the pea harvest period. Samples were obtained from 8 and 13 different days in 2011 and 2012 respectively. The nine soil samples (SL) were collected during the production period of green pea crops intended for processing, and the remaining 332 samples were collected in a French cannery producing canned green peas and a canned mix of green peas and carrots. These samples corresponded to vegetables at different steps of processing (Fig. 1), process liquids, and surface samples from processing equipment. Raw unprocessed green peas were washed by successive immersions in water, then blanched in hot water (95 °C) and convey to the can filler. Carrots were washed with water, peeled, then added to cans previously part-filled with green peas. At this stage, cover brine (ca. 2% salt wt/vol and 2% sugar wt/vol in water) was poured into the filled cans. The cans

were sealed and heat-sterilized by for an equivalent 121 °C heating time (F_0) of over 20 min before further storage at ambient temperature. Solid and liquid samples were collected with sterile instruments and directly poured into sterile plastic vials. Surfaces of approximately 100 cm² were wiped with sterile dry sponges moistened with 10 mL pharmacopeia-grade sodium thiosulfate thinner (VWR BDH Prolabo, Fontenay-sous-Bois, France). Sample numbers and the corresponding sampling stages are reported in Table 1. Raw green peas (R-P) and raw carrots (R-C) were sampled at the reception deck. Prepared green peas (P-P) were collected immediately before blanching. Blanching water (W) was sampled directly from the blancher. Blanched green peas were sampled immediately after blanching in the processing flow (W-P) as well as lateral debris just after blanching (W-P-OF). Lateral debris during transfer on the conveyor (T-P-OF) was also sampled. Peeled carrots (P-C) were sampled immediately before filling. Both green peas and carrots were collected at the filling machine (T-P/C), again after cover brine was added (B), and again immediately before jarring, aluminum-lidding and sterilization (B-P/C). The cover brine (B) was separately sampled from the pipe recycling excess brine back to the brine tank. In the 2011 campaign, the temperature of each sample was recorded with a digital thermometer (EcoScan Temp5, Fisher, Illkirch, France) immediately after sampling. Mean temperatures are listed in Table 1. In 2011, samples were collected three times a day, i.e. at the beginning (approx. 9–10 am), in the middle (between 2 pm and 3 pm) and 1 h before the end of processing (approx. 9 pm). In 2012, samples were collected once a day, in the middle of processing. Surface samples were collected on the wall of the blancher (W-S), on the conveyor belts used for transfer (T-S), and on the wall of the tank of cover brine (B-S). The surface samples were collected after the day's production run and immediately before the processing line was cleaned. During the 1-month production period, 9 SL samples were collected from the green pea crops. All samples were immediately chilled at 4 °C post-collection and reached the laboratory within two days, where they were then frozen and stored at −20 °C until analysis.

2.2. Sample preparation for microbiological analysis

Samples were thawed at 4 °C one day before the assay. A quantity of 10 g of solid samples was mixed with 90 mL Rosenow broth modified by supplementation with 1 g/L starch (mRB) (BioRad, Marnes-la-Coquette, France) in a sterile BagFilter™ (Interscience, Saint-Nom-la-Bretèche, France), and the diluted samples were homogenized for 30 s using a BagMixer™ (Interscience). The BagFilter™ filtrate was used for further enumeration and detection of target microorganisms. Liquid samples were analyzed without any special preparation. Sponges used for surface sampling were placed in sterile bags and covered with 90 mL mRB, then homogenized for 30 s. The filtrate was recovered after manually wringing the sponge.

2.3. Anaerobic spore counts

An aliquot fraction of sample filtrate was placed in a 2.2 mL sealed glass ampoule and heat-treated for 10 min at 106 °C in a temperature-regulated oil bath to select highly-heat-resistant (HHR) thermophilic spores. Another fraction was poured into an 1.7 mL Eppendorf tube and heat-treated for 10 min at 100 °C (André et al, 2013) in a Stuart® SBH130DC Digital Block Heater (Bibby Scientific, Staffordshire, United Kingdom) to select spores of thermophilic bacteria.

Serial dilutions of heat-treated samples in tryptone salt broth were plated (1 mL) on meat liver glucose agar (Biokar Diagnostic, Beauvais, France) modified by supplementation with 2 g/L yeast extract (mMLGA) (Sevenier et al, 2012). Plates were incubated at 55 °C for 5 days in anaerobic jars using Genbox anaer generators (Biomerieux, Craponne, France). Spore counts were calculated with a detection threshold of 10 thermophilic anaerobic spores (TAS)/g and 10 HHR TAS/g for solid samples, 1 TAS/mL and 1 HHR TAS/mL for liquid samples,
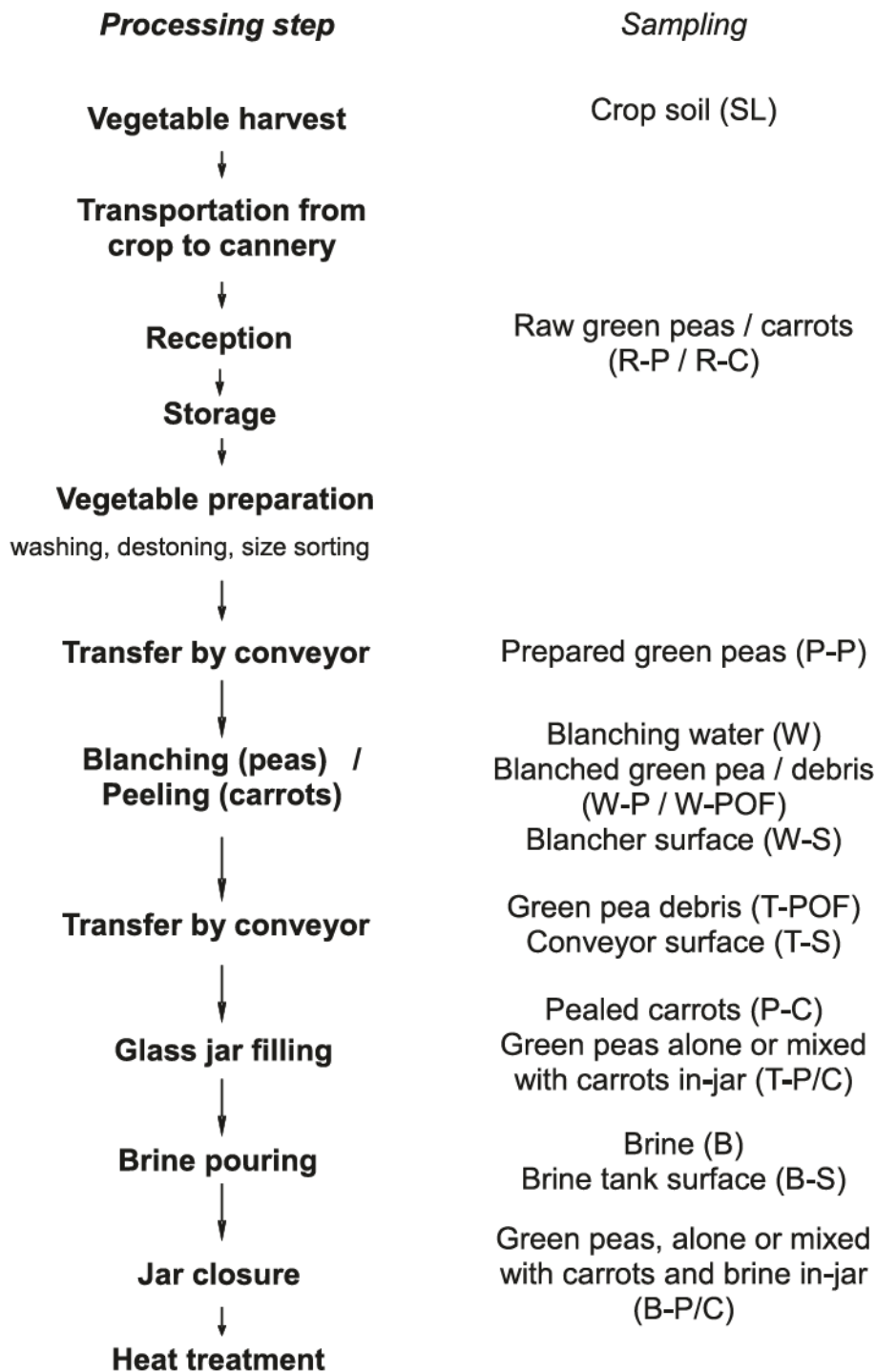
Processing step	**Sampling**
Vegetable harvest	Crop soil (SL)
↓	
Transportation from crop to cannery	
↓	
Reception	Raw green peas / carrots (R-P / R-C)
↓	
Storage	
↓	
Vegetable preparation washing, destoning, size sorting	
↓	
Transfer by conveyor	Prepared green peas (P-P)
↓	
Blanching (peas) / Peeling (carrots)	Blanching water (W) Blanched green pea / debris (W-P / W-POF) Blancher surface (W-S)
↓	
Transfer by conveyor	Green pea debris (T-POF) Conveyor surface (T-S)
↓	
Glass jar filling	Pealed carrots (P-C) Green peas alone or mixed with carrots in-jar (T-P/C)
↓	
Brine pouring	Brine (B) Brine tank surface (B-S)
↓	
Jar closure	Green peas, alone or mixed with carrots and brine in-jar (B-P/C)
↓	
Heat treatment	

Fig. 1. Flow diagram charting green peas and carrots processing steps in the surveyed vegetable cannery.

and 1 TAS/cm^2 and 1 HHR TAS/cm^2 for surface samples. This enumeration method counts both strict and facultative thermophilic anaerobes, including *G. stearothermophilus* and some *Bacillus* spp.

2.4. Prevalence of G. stearothermophilus, M. thermoacetica/ thermoautotrophica and Thermoanaerobacterium spp.

Samples diluted in mRB were submitted to a 100 °C heat selection during 30 min then further incubated for 4 days at 55 °C for enrichment. The germination and outgrowth of *G. stearothermophilus* spores after heat-activation were shown to be slightly inhibited or not affected by anaerobiosis (Feeherry et al, 1987; Periago et al,

1998). A 1 mL volume of the sample was then used to extract total DNA with the InstaGene™ matrix following the manufacturer's instructions (Bio-Rad, Marnes-la-Coquette, France). *G. stearothermophilus, M. thermoacetica/thermoautotrophica* or *Thermoanaerobacterium* spp. were specifically detected using the specific PCR technique SporeTraQ™, as previously developed (Sevenier et al., 2012). Discrimination of the species *M. thermoacetica* from *M. thermoautotrophica* is challenging and *M. thermoautotrophica* could be considered as a sub-species of *M. thermoacetica* (Collins et al., 1994). Briefly, this method amplifies a DNA region corresponding to rRNA internal transcribed sequence 16S–23S for *G. stearothermophilus* and amplifies 16S rRNA coding regions for the other bacteria detected. Amplification product sizes were

Table 1

List and number of samples subjected to microbiological analyses and temperature measurements, collected in 2011 and 2012 along the canned vegetable processing chain, and temperatures recorded in 2011.

Sample	Sample code	Number of samples collected in 2011	Number of samples collected in 2012	2011 temperature (mean ± SD)[b]
Soil of the green peas crop	SL	8	–[a]	25.4 ± 2.5
Raw carrots (reception deck)	R-C	16	–	22.6 ± 0.4
Peeled carrots	P-C	12	–	nd
Raw green peas (reception deck)	R-P	22	–	25.2 ± 1.7
Prepared green peas	P-P	22	13	17.9 ± 1.1
Blanching water	W	23	13	85.7 ± 1.1
Blanched green peas	W-P	22	13	83.9 ± 4.7
Blanched green peas, lateral debris	W-POF	22	–	46.6 ± 5.3
Blancher surface	W-S	8	–	nd
Green pea debris on conveyor	T-POF	23	13	41.8 ± 9.2
Conveyor belt surface	T-S	8	–	nd
Green peas, alone or mixed with carrots, conveyed to the can filling machine	T-P/C	23	–	43.1 ± 6.6
Brine	B	23	13	69.3 ± 7.3
Brine tank surface	B-S	8	–	nd
Green peas, alone or mixed with carrots, in cans with cover brine	B-P/C	23	13	50.2 ± 4.3

nd: not determined.

[a] No sample analyzed.

[b] Mean ± standard deviation. Temperature was measured in the center of the sample immediately after collection.

302 bp, 467 bp and 487 bp corresponding to *G. stearothermophilus*, *M. thermoacetica/thermoautotrophica* and *Thermoanaerobacterium* spp., respectively. All amplification steps were performed in a total volume of 25 μL with a GeneAmp® PCR System 9700 (Applied Biosystems, USA). PCR products were directly analyzed by electrophoresis in Tris-Acetate-EDTA (TAE) buffer 1% agarose gel. The electrophoretic migration was performed during 30 min at 150 mV. This extraction/amplification method enables a detection threshold of 10 cells per mL enrichment broth (Prevost et al., 2010). Threshold of detection was estimated at one spore per g sample enriched (Sevenier et al., 2012).

2.5. G. stearothermophilus M13-PCR typing

Isolation of *G. stearothermophilus* strains was performed from samples tested positive with SporeTraQ™. A droplet (10 μL) of the positive sample was deposited on the bromocresol purple (BCP) surface and incubated for 24 h at 55 °C, then 3–5 colonies were isolated on BCP and incubated for 24 h at 55 °C. One loopful of bacterial colony was used to extract total DNA with the InstaGene™ (Bio-Rad) matrix following the manufacturer's instructions. *G. stearothermophilus* was specifically detected by SporeTraQ™ PCR as described above. Then, DNA of positively-testing bacterial isolates was extracted with chloroform/phenol and amplified with PM13 primer as described previously, with modifications (Durand et al., 2014; Guinebretiere and Nguyen-The, 2003). A negative control containing Milli-Q water instead of DNA and a positive control with the DNA of *B. cereus* strain ATCC 14579 producing a regular banding pattern were included in each PCR experiment. A *G. stearothermophilus* isolate recovered from spoiled canned green peas in 2010 was included in the panel and labeled #124 (Durand et al., 2014).

PCR products from M13-PCR were separated by electrophoresis on 1.5% agarose gels in 0.5× TBE buffer (20 mM Tris, 10 mM acetic acid, 0.5 mM EDTA, pH 8) using Eurogentec Smart Ladder. The electrophoretic migration was performed successively with one step at 120 mV during 15 min and then a second step at 80 mV during 3 h. Gels were stained with ethidium bromide and digitized using a UV gel imager (Molecular Imager® Gel Doc™ XR System 170-8170, BioRad, Marnes-la-Coquette, France). A dendrogram was constructed from the similarity of M13-PCR banding patterns (Dice's coefficient) and using the unweighted pair group method with arithmetic mean (UPGMA). Band detection, similarity coefficient and dendrogram were realized using BioGene 99.04 software (Vilber Lourmat, Marne-la-Vallée, France). The confidence interval on band size was adjusted to obtain 100% similarity between independent replicates of control strains.

2.6. Statistical analyses

All statistical analyses were carried out with XLSTAT software (Addinsoft™, Paris, France). Statistical analyses were performed on $\log_{10}$ transformed counts. Numeration data below the threshold of detection were set to threshold divided by square root of two (Hornung and Reed, 1990). Data were analyzed by one-way ANOVA. P-value was set at 0.05. The Bonferroni test was applied to detect significant differences between means. The confidence interval of proportions at 95% was determined as the value corresponding to 1.96× standard deviation (SD). Proportion comparisons were performed according to Fleiss et al. (2004).

3. Results

3.1. Enumeration of thermophilic anaerobic spores (TAS) and highly-heat-resistant (HHR) TAS

TAS and HHR TAS were enumerated in all samples collected in 2011 and 2012 (Table 2 and Fig. 2). The highest TAS count means found in 2011 were in soil (SL) and raw carrots (R-C), at $3.2 \pm 0.5 \log_{10}$ cfu/g and $3.1 \pm 1.0 \log_{10}$ cfu/g, respectively. Globally, HHR TAS counts were lower than TAS counts, but the highest HHR TAS were also found in SL and R-C samples, at $2.7 \pm 0.2 \log_{10}$ cfu/g and $2.3 \pm 0.8 \log_{10}$ cfu/g, respectively. These population levels significantly differed from other counts. Raw pea (R-P) TAS and HHR TAS populations ($2.0 \pm 0.4 \log_{10}$ cfu/g and $1.2 \pm 0.5 \log_{10}$ cfu/g, respectively) were significantly lower ($P < 0.05$) than the population counts found in soil samples and raw carrots.

Spore counts of vegetables decreased during the vegetable preparation process chain at ambient temperature until pea blanching (W-P samples) and carrot peeling (P-C samples), then remained unchanged through to final processing in T-P/C and B-P/C samples. TAS and HHR TAS counts from washed-and-peeled carrots (P-C) were $0.4 \pm 0.0 \log_{10}$ cfu/g and $0.5 \pm 0.0 \log_{10}$ cfu/g, respectively. TAS and HHR TAS counts from prepared peas (P-P) were $1.3 \pm 0.6 \log_{10}$ cfu/g and $0.6 \pm 0.1 \log_{10}$ cfu/g respectively, with HHR TAS counts significantly lower ($P < 0.05$) than raw pea (R-P) counts. From the pea blanching step (W-P) to in-can brining (B-P/C), there was no detectable variation in mean population. Furthermore, mean TAS counts were lowest in blanched peas (W-P), peas alone or mixed with carrots and filled in-can (T-P/C), blanching water (W), and brine (B), and ranged from $0.3 \pm 0.7 \log_{10}$ cfu/g or/mL to $0.6 \pm 0.4 \log_{10}$ cfu/g or /mL. HHR TAS

Table 2
Thermophilic anaerobic spore (TAS) and highly-heat-resistant (HHR) TAS counts from vegetable samples collected in 2011 and 2012 from the canned vegetable processing chain.

Samples[a]	Microbiological counts (log$_1$ cfu/g or mL or cm^2)[b] (mean ± SD) in year	
	2011	2012
Thermophilic anaerobic spores		
SL	3.2 ± 0.5 A	–[c]
R-C	3.1 ± 1.0 A	–
P-C	0.4 ± 0.0 E	–
R-P	2.0 ± 0.4 BC	–
P-P	1.3 ± 0.6 CD	0.6[d] ± 0.3 A
W	0.6 ± 0.5 E	0.7 ± 0.6 A
W-P	0.5 ± 0.3 E	0.5 ± 0.2 A
W-POF	2.4 ± 0.9 AB	–
W-S	0.9 ± 0.7 DE	–
T-POF	1.2 ± 0.4 D	0.5[d] ± 0.2 A
T-S	0.0 ± 0.4 F	–
T-P/C	0.5 ± 0.4 E	–
B	0.3 ± 0.7 E	0.2 ± 0.5 A
B-S	1.1 ± 1.2 DE	–
B-P/C	1.0 ± 0.8 DE	1.8 ± 1.0 B
Highly-heat-resistant thermophilic anaerobic spores		
SL	2.7 ± 0.2 A	–
R-C	2.3 ± 0.8 AB	–
P-C	0.6 ± 0.0 EF	–
R-P	1.2 ± 0.5 D	–
P-P	0.6 ± 0.1 EF	0.6[d] ± 0.1 A
W	0.4 ± 0.5 F	0.8 ± 0.7 A
W-P	0.6 ± 0.0 EF	0.8 ± 0.3 A
W-POF	2.0 ± 0.9 BC	–
W-S	0.0 ± 0.5 F	–
T-POF	0.9 ± 0.2 DE	0.7[d] ± 0.3 A
T-S	−0.1 ± 0.0 G	–
T-P/C	0.6 ± 0.1 EF	–
B	0.3 ± 0.5 F	0.8 ± 1.2 A
B-S	1.4 ± 0.9 CD	–
B-P/C	0.6 ± 0.1 EF	1.1 ± 0.6 A

[a] The different samples were SL, Soil; R-C, Raw carrot; P-C, Peeled carrot; R-P, Raw green peas; P-P, Prepared green peas; W, Blanching water; W-P, Blanched green peas; W-POF, Blanched green peas, lateral debris; W-S, Blancher surface; T-POF, Green pea debris on conveyor; T-S, Conveyor belt surface; T-P/C, Green peas, alone or mixed with carrots, transferred to the can filling machine; B, Brine; B-S, Brine tank surface; B-P/C, Green peas, alone or mixed with carrots, in cans with cover brine.

[b] Values are expressed as log$_{10}$ cfu/g for solid samples, log$_{10}$ cfu/mL for liquid samples and log$_{10}$ cfu/cm^2 for surface samples. Different letters between samples of the same year correspond to significant differences (P < 0.05).

[c] No sample analyzed.

[d] Significant difference between samples collected in 2011 and 2012 at P < 0.05.

counts were also lowest in W-P, T-P/C, W and B samples, and were in the same range as mean TAS counts. For the whole transformation process, a ca. 10-fold decrease of spore contamination was observed both for TAS and for HHR TAS, as populations of sporeforming bacteria in canned peas (B-P/C) were 0.6 to 1.0 log cfu/g lower than the population in the raw material (R-P). Interestingly, beside the variations of count means, extreme values of counts increased considerably from blancher step (W and W-P) to brine filling step (T-P/C and B-P/C) (Fig. 2). Amplitude of log$_{10}$ spore counts in blanching water (W), cover brine (B), and canned product before heat treatment (B-P/C) varied from 0.65 to 2.45 log$_{10}$ cfu/g or /mL.

The TAS and HHR TAS populations determined in debris (W-POF and T-POF) ranged from 1.2 ± 0.4 log$_{10}$ cfu/g to 2.4 ± 0.9 log$_{10}$ cfu/g for TAS and from 0.9 ± 0.2 log$_{10}$ cfu/g to 2.0 ± 0.9 log$_{10}$ cfu/g for HHR TAS. The number of TAS collected on equipment surfaces (W-S, T-S and B-S) at the close of the production day ranged from 0.0 log$_{10}$ cfu/cm^2 to 1.1 ± 1.2 log$_{10}$ cfu/cm^2, and the number of HHR TAS ranged from 0.0 log$_{10}$ cfu/cm^2 to 1.4 ± 0.9 log$_{10}$ cfu/cm^2. Log$_{10}$ spore counts varied strongly according to surface sample, and the amplitudes were up to 1.15 log$_{10}$ cfu/cm^2.

3.2. Temperature measurement along the processing chain

Temperatures measured in 2011 throughout the processing chain (Table 1) ranged from 17.9–25.4 °C (ambient temperature) to 85.7 ± 1.1 °C (blanching water). Pea temperature remained high post-blanching, at 83.9 ± 4.7 °C. Temperatures of T-P/C, B-P/C and B samples were 43.1 ± 6.6 °C, 50.2 ± 4.3 °C and 69.3 ± 7.3 °C, respectively. Temperatures of W-POF and T-POF lateral debris were 46.6 ± 5.3 °C and 41.8 ± 9.2 °C, respectively. Note that standard deviation on all sample temperatures increased sharply after the blanching step.

3.3. Prevalence of M. thermoacetica/thermoautotrophica, Thermoanaerobacterium spp. and G. stearothermophilus

A total of 333 samples collected in 2011 and 2012 and anaerobically enriched for analysis were screened to test for the thermophilic species *M. thermoacetica/thermoautotrophica*, *Thermoanaerobacterium* spp. and *G. stearothermophilus* (Fig. 3). Depending on nature of the sample, the prevalence of *G. stearothermophilus* ranged from 0% to 100% and the prevalence of *M. thermoacetica/thermoautotrophica* ranged from 0% to 91%. In contrast, *Thermoanaerobacterium* spp. was never detected in samples except in 17% of blanching water from year-2011 samples, and was therefore considered a minor microbiological contaminant in the processing plant at the time of survey (data not shown). The prevalence of *G. stearothermophilus* was (prevalence % [0.95 confidence interval %]) 5% [1; 22] in raw peas (R-P) and 13% [3; 36] in raw carrots (R-C). The prevalence of *M. thermoacetica/thermoautotrophica* was 9% [3; 28] in raw peas and 0% [0; 19] in raw carrots. Both these bacteria progressively increased in prevalence in green pea samples along the process. *G. stearothermophilus* was detected in 18% [7; 39], 59% [39; 77] and 65% [45; 81] of P-P, W-P and T-P/C samples, respectively. *M. thermoacetica/thermoautotrophica* was detected in 23% [10; 43], 82% [61; 93] and 61% [41; 78] of P-P, W-P and T-P/C samples, respectively. A peak in *M. thermoacetica/thermoautotrophica* prevalence, i.e. 91% [73; 98], was found in the in-can brine-covered product, with or without carrots (B-P/C). Cover brine (B) showed low prevalence values of *G. stearothermophilus* and *M. thermoacetica/thermoautotrophica*. Interestingly, a peak in *G. stearothermophilus* prevalence was in blanching water (W), where it reached 100% [86; 100]. *G. stearothermophilus* prevalence was significantly different between raw and blanched peas and between prepared peas and blanching water (Suppl. Table 1). *M. thermoacetica/thermoautotrophica* prevalence was significantly different between R-P and B-P, between P-P and B-P, and between R-P or R-C and T-P/C samples.

Surfaces from the blancher (W-S) and transfer conveyor (T-S) and samples from out-of-flow peas as lateral debris (W-POF and T-POF) showed high prevalence values of *G. stearothermophilus* spores at up to 100%. *M. thermoacetica/thermoautotrophica* prevalence in surface, debris and conveyed samples was in the range 13–50%. The brine tank surface (B-S) showed moderate prevalence values of 25% [7; 59] for *G. stearothermophilus* and 13% [2; 47] for *M. thermoacetica/thermoautotrophica*.

G. stearothermophilus and *M. thermoacetica/thermoautotrophica* prevalence values did not differ significantly between year-2011 and year-2012 samples, except for *M. thermoacetica/thermoautotrophica* prevalence in B-P/C and *G. stearothermophilus* prevalence in T-POF.

3.4. Analysis of the diversity among G. stearothermophilus isolates with M13-PCR

A total 112 (87 in 2011 and 25 in 2012) *G. stearothermophilus* strains were isolated and genotyped by M13-PCR. An electrophoresis pattern was obtained for each isolate. Number of 200 to 4000-bp DNA bands varied from 10 to 17. A similarity threshold set at 97% resulted in 9 groups and 14 singletons (Fig. 4 and Suppl. Fig. 1). In parallel, visual observation of M13-PCR patterns allowed to identify only 14 profile-types
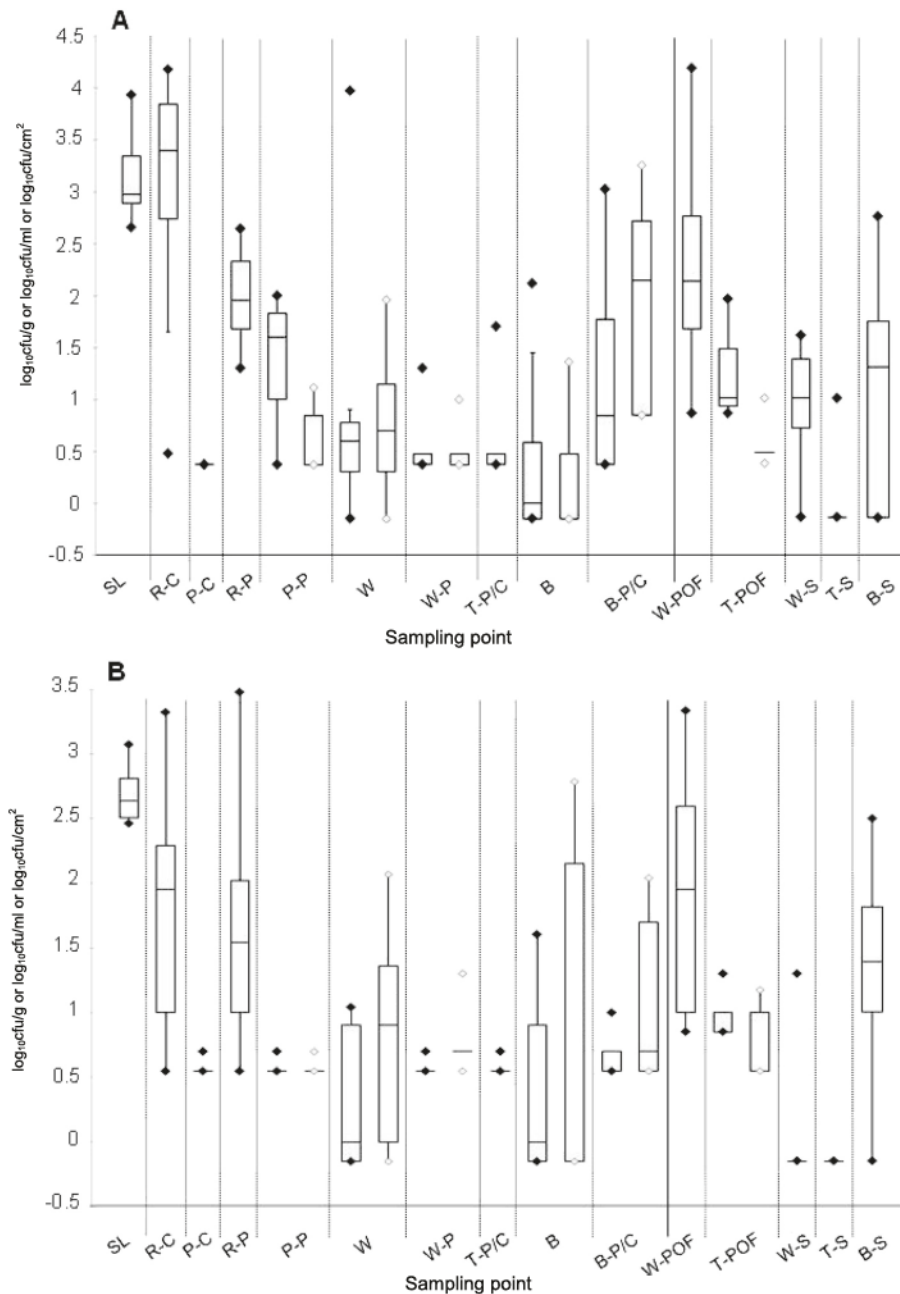
Fig. 2. Distribution of thermophilic anaerobic spore levels according to process sampling. Distribution is represented by box-and-whisker plots for thermophilic anaerobic spore counts (A) and highly-heat-resistant thermophilic anaerobic spore counts (B). The samples were as follows: SL, soil under the green pea crop; R-C, Raw carrot; P-C, Peeled carrot; R-P, Raw green peas; P-P, Prepared green peas; W, Blanching water; W-P, Blanched green peas; T-P/C, Green peas, alone or mixed with carrots, conveyor-transferred to a can filling machine; B, Brine; B-P/C, Green peas, alone or mixed with carrots, in cans filled with cover brine; W-POF, Blanched green peas, lateral debris; T-POF, Green pea debris on the conveyor; W-S, Blancher surface; T-S, Conveyor-belt surface; B-S, Brine tank surface (See Table 1). Counts are expressed as $\log_{10}$ cfu/g, $\log_{10}$ cfu/mL or $\log_{10}$ cfu/cm². In the box-and-whisker plots, the central box covers the middle 50% of the data values between the lower and the upper quartiles. The central line in the box shows the median. Whiskers extend to 1.5 times the box length (inter-quartile range). Extreme values are plotted as diamonds. For some samples, black diamonds (♦) correspond to 2011 data and white diamonds (◊) to 2012 data.

classifiable in 8 groups and 6 singletons. A global coherence with the dendrogram groups and singletons was clearly observed but 10% of strains (8 singletons and group No. 5) were not allocated to the true group due to errors generated by the automatic band detection system, in the present experimental conditions (Suppl. Fig. 1). Thus, the erroneous singletons 3, 4, 15, 17, 18, 19, 20, 21 and group No. 5 were reclassified in the true remaining groups (see Suppl. Fig. 1) as presented in Table 3.

Four major groups were recorded at different step on the processing line (Table 3), among those group 6 was the largest (45% of all isolates). Group 6 was representative of year 2011 only and was not recovered in the final product despite its large dominance in debris and surfaces.

Among the three other major groups (groups 12, 1, 10), groups 1 and 10 were also representative of year 2011, whereas group 12 isolates were recovered from both 2011 and 2012. Groups 1, 22 and 7 were also recovered in the final product as well as group 12, and it appeared that these final contaminations originated from blanching water and blanched peas and further from post-blanching samples (transfer debris (T-POF), brine (B)).

Within the profile-types, certain bands were specific to a given group and were characterized by higher fingerprint intensity and/or a specific size (Suppl. Fig. 1). Each electrophoretic pattern from groups 1, 12, 10 and 14 exhibited one characteristic marker-band at respectively 420 bp, 730 bp, 470 bp and 2800 bp. Note that, by means of 730 bp
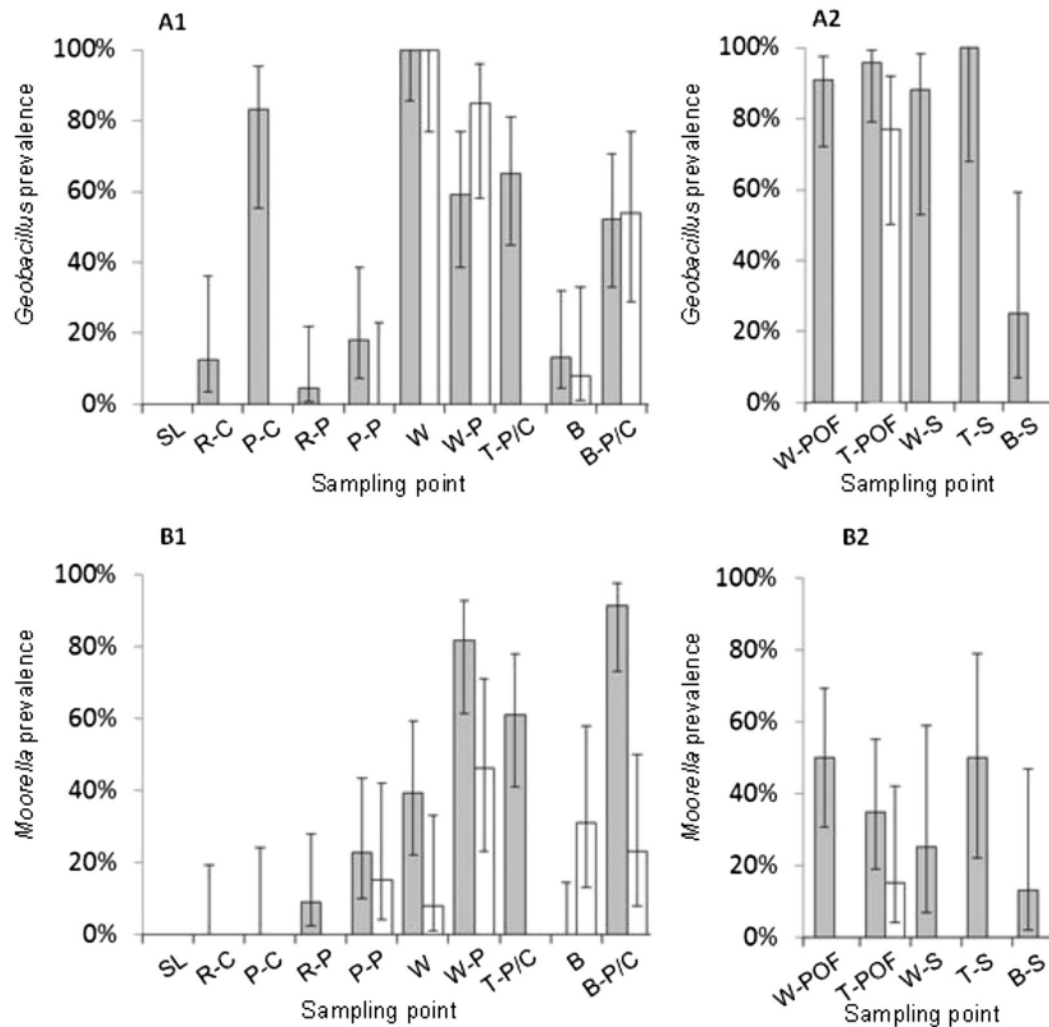
Fig. 3. Prevalence of *G. stearothermophilus* and *M. thermoacetica/thermoautotrophica* throughout the process line. A: *Geobacillus stearothermophilus* prevalence in samples (% positives) collected in the flow of conveyed vegetables (A1) or in lateral debris of vegetables and from surfaces (A2); B: *Moorella thermoacetica thermoautotrophica* prevalence in samples (% positives) collected in the flow of conveyed vegetables (B1) or in lateral debris of vegetables and from surfaces (B2). The samples were: SL, Soil; R-C, Raw carrot; P-C, Peeled carrot; R-P, Raw green peas; P-P, Prepared green peas; W, Blanching water; W-P, Blanched green peas; T-P/C, Green peas, alone or mixed with carrots, transferred to the can filling machine; B, Brine; B-P/C, Green peas, alone or mixed with carrots, in cans filled with cover brine; W-POF, Blanched green peas, lateral debris; T-POF, Green pea debris on the conveyor; W-S, Blancher surface; T-S, Conveyor belt surface; B-S, Brine tank surface. For some samples and sampling points, gray (white) bars correspond to 2011 (2012) data. Error bars cover the 95% confidence interval of calculated proportions.

marker-band, the profile-type of group 12 was easily recovered on several *G. stearothermophilus* strains involved in canned vegetables spoilage (before 2010), one of which (#124; Durand et al., 2014) was independently isolated in 2010 from spoiled canned green peas produced at the same cannery as surveyed here (Fig. 4).

4. Discussion

Microbiological non-stability of canned food due to thermophilic bacteria causes significant economic costs in industrial canning. Thermophilic spores surviving heat treatment is the main cause of spoilage of canned foods. Better knowledge of spore contamination levels and of the prevalence of the main spoilage-induced spore former species throughout the processing-line is vital in order to better rationalize the heat treatment and hygiene procedures. In addition, such species could be of interest as biological indicators for new thermal technological treatments. Here we examined several zones of the production line from raw vegetables to in-can product before heat treatment. The prevalence of the three thermophilic bacteria most frequently implicated in canned food spoilage (André et al., 2013) was determined after sample enrichment using a sensitive method developed previously (Prevost

et al., 2010). *Thermoanaerobacterium* spp. was rarely detected in this study, thus confirming its low prevalence in canned vegetables and spoilage (ca. 5% of cases in canned vegetables according to André et al. (2013)).

In this study, the average population of thermophilic spores in crop soil was 3 $\log_{10}$ cfu/g. The highest spore population was found in raw carrots, where the average population was close to the figure reported by Sevenier et al. (2012) in the same product (2.6 ± 0.1 $\log_{10}$ cfu/g), but with a much higher dispersion of values in our study. However, raw vegetables showed low prevalence levels (0–24%) of *G. stearothermophilus* and *M. thermoacetica/thermoautotrophica*, as previously observed (Sevenier et al., 2012). Soil can be therefore considered a highly probable source of spore contamination in the industrial plant studied. Direct contact with soil could explain the higher spore populations on carrots than on peas which grow in aerial part of plants. Raw materials were thus a major vector of HHR spores into the processing line. Similar conclusions were reached for pathways of zucchini contamination by *B. cereus* (Guinebretiere et al., 2003).

The marked decrease in spore counts in the course of processing operations at ambient temperature, especially between reception of the raw vegetables and after preparation for blanching, could be explained
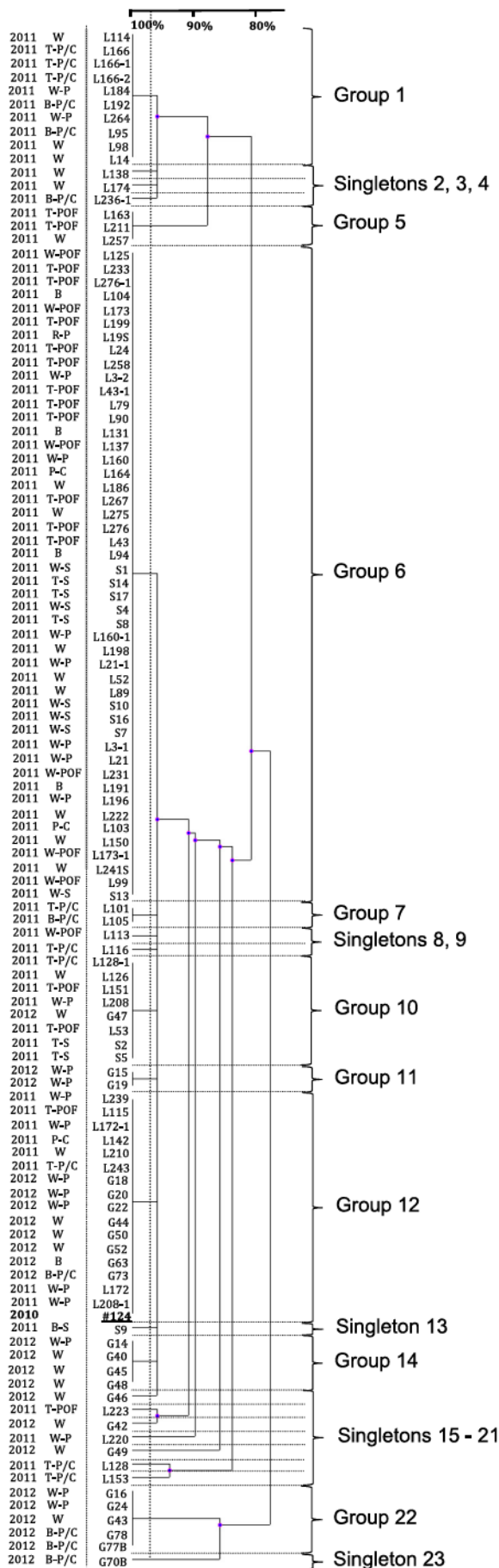
by multiple washing steps, as previously suggested (Sevenier et al., 2012). Raw vegetables were prepared at room temperature (8 °C to 25 °C) which likely impairs the multiplication and sporulation of thermophilic bacteria (Scott et al., 2007). After vegetable preparation, spore populations at entry to the high-temperature processing (i.e. >41 °C) zone were low (0.4 to 1.3 log cfu/g). At this stage of the process, prevalence levels were 3–39% for *G. stearothermophilus* and 0–43% for *M. thermoacetica/thermoautotrophica*.

The range of temperatures supporting the growth of thermophilic spore-formers, including *G. stearothermophilus* and *M. thermoacetica* (Coorevits et al., 2012; Fontaine et al., 1942; Wiegel, 2009), suggests that the multiplication and ultimate sporulation of thermophiles may only occur in a few niches along the processing line. Here, these niches were probably out-of-flow peas with prolonged residence times, recycled brine, peas in cans, alone or mixed with carrots, with or without brine, and probably also process-line surfaces, as the temperatures were in the range 41 °C–69 °C. The residence time of properly conveyed vegetables seems to be short enough to prevent significant growth or sporulation, as vegetable TAS and HHR TAS populations remained unchanged after blanching. In contrast, high residence time (over several hours) of contaminated food material is a common feature on equipment surfaces, in processing liquid samples, and in debris not conveyed in the main vegetable stream. For instance, accumulated vegetable debris on the lateral side of the chain were only eliminated during cleaning at the end of the production day. Nutrients are available in the processed food itself or in recycled brine by diffusion of food constituents, which could enable local growth and eventually sporulation. In addition, all these niches are encountered after blanching and the blanching step may induce germination of spores. Spores accumulated on surfaces and in vegetable debris may be randomly released in the course of production, leading to high variations in product spore counts before thermal treatment. This is confirmed by *G. stearothermophilus* genotypic analysis which showed the presence of isolates from groups 10 and 12 at different accumulation locations and in-can vegetables.

One striking observation was the large increase in prevalence of *G. stearothermophilus* and *M. thermoacetica/thermoautotrophica* at the blanching step. Blanching temperature is not compatible with spore formation or vegetative cell multiplication. This process step is designed to inactivate enzymes and vegetative cells and to fix green pea color. As expected, blanching inactivates many thermophilic anaerobic spores but much less HHR TAS. This is observed from count differences between TAS and HHR TAS which became null in post-blanching samples. The heat treatment applied in the blanching water may select HHR spore-formers such as *G. stearothermophilus* and *M. thermoacetica/thermoautotrophica*. The blancher in the surveyed processing chain worked with continuous water recycling and steam production during vegetable processing. A very likely hypothesis to explain the prevalence increase is that HHR spores entered the blancher with prepared green peas and were partly released in the vegetable water, where they accumulated and then cross-contaminated peas that later went through the blanching tank.

Interestingly, most of *G. stearothermophilus* genetic groups determined in this study were found in blanching-water isolates. Isolates from blanching water and blanched peas were distributed in the same genetic groups and at similar proportions. Three out of the four groups detected in the final product before heat treatment also include isolates from the blancher step, both in water and blanched peas, and to a lesser extent isolates from transfer debris, brine or transfer surfaces. This clearly shows that the blanching step contributed to the dissemination of strains to the product before heat treatment. Group 6 isolates were

Fig. 4. Dendrogram of *G. stearothermophilus* isolate profile-types obtained with the M13-PCR method. Percent similarity represented by the horizontal scale was calculated using Dice's coefficient, and clustering was performed by UPGMA. Profile-types obtained at a set 97% similarity threshold are separated with the vertical scale and labeled with their group or singleton numbers.

Table 3
Distribution of *G. stearothermophilus* isolates collected in 2011 and 2012 according to sampling origin and M13-PCR groups.

Sample origin[a]	Number of isolates	Number of isolates by profile-type[b]								Ungrouped profile-types[c]
		6	12	1	10	22	14	7	11	
R-P	1	1								
W	27	8	4 (3)	4	2 (1)	1 (1)	6 (6)			1
W-P	23	7	8 (3)	2	1	2 (2)	1 (1)		2 (2)	1 (1)
W-POF	7	6								1
W-S	6	6								
P-C	2	2								
T-POF	17	11	1		2					1
T-S	5	3			2					
T-P/C	10		2	3	3			1		1
B	5	4	1 (1)							
B-S	1									1
B-P/C	8		1 (1)	3		2 (2)		1		
Total (2012)[b]	112 (25)	51	17 (8)	12	10 (1)	5 (2)	7 (7)	2	2 (2)	6 (1)

[a] The samples were collected at different stages of the processing line: R-P, Raw green peas; W, Blanching water; W-P, Blanched green peas; W-POF, Blanched green peas, lateral debris; W-S, Blancher surface; P-C, Peeled carrot; T-POF Green pea debris on conveyor; T-S, Conveyor belt surface; T-P/C, Green peas, alone or mixed with carrots, transferred to the can filling machine; B, Brine; B-S, Brine tank surface and B-P/C, Green peas, alone or mixed with carrots, in cans with cover brine.

[b] In brackets, number of isolates collected in 2011.

[c] singletons.

evenly distributed in the processing chain between debris, surface, brine and blancher water, but in higher proportions compared to conveyed blanched peas. The absence of group 6 isolates in the final product supports the assumption that final product contamination mostly reflects in-flow pea and carrot contamination rather than accumulation and release events. However, accumulation and release events may explain individual cases of microbiologically unstable canned food despite monitoring of F_0 values. The isolates found in the canned product, with or without brine, belonged to four groups, numbered 1, 7, 12 and 22. In this study, marker-bands were specifically detected for groups 1, 10, 12, 14 and 23. Moreover, a *G. stearothermophilus* isolate from spoiled canned green peas at the same cannery presented one of these marker-bands. Naturally, the clonal status of independent isolates collected during a long time interval has to be confirmed by a second typing method. If the presence of this marker-band is confirmed by further M13-PCR typing of isolates implicated in spoilage cases, and provided that this band exhibits the same sequence in different strains, the sequence of this band could be further used for specific pre-heat-treatment detection of canned food spoilage-related isolates.

We examined the persistence of profile-types over a one year interval. Genetic groups 1, 6 and 7 were specifically detected in 2011 whereas groups 11, 14 and 22 were 2012-specific. Group 12 isolates were found in both 2011 and 2012. Group 10, essentially detected in 2011, also had one representative in the 2012 isolates. This temporal distribution indicates annual variations in the most frequent isolates from vegetables despite geographically identical crop location and processing environment. The presence of the same profile-types in two consecutive years could be explained by persistence of certain bacterial strains either in soil or in the processing plant, but the broad renewal of profile-types observed over two consecutive years suggests that the location effect is minor. Further examination of soil and equipment contamination between several production campaigns is necessary to elucidate this point.

The observed cross-contamination of vegetables during the blanching step together with HHR spore selection, the presence of zones of vegetable accumulation supporting spore level increases, and the occasional release of spores to conveyed vegetables gives evidence that blancher and conveyors are critical points for microbial contamination of food before thermal treatment. In conclusion, processing plant modification should be made a priority in order to reduce the incidence of microbiological non-stability in canned food.

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