Prevalence of Clostridium botulinum and thermophilic heat-resistant spores in raw carrots and green beans used in French canning industry

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Prevalence of *Clostridium botulinum* and thermophilic heat-resistant spores in raw carrots and green beans used in French canning industry

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Two categories of vegetables (carrots and green beans) that are widely used in the manufacture of canned food were surveyed for their spore contamination. Samples were recovered from 10 manufactures spread over all producing areas in France. Two samples over 316 raw vegetables collected were found positive for botulinum neurotoxin producing *Clostridia* spores as tested by PCR-based GeneDisc assay. Both positive samples tested positive for the type B neurotoxin gene (*bont/B*). In parallel, heat-resistant spores of thermophilic bacteria that are likely to be associated with canned food spoilage after prolonged incubation at 55 °C were surveyed after specific enrichment. Prevalence varied between 1.6% for *Moorella thermoacetica/thermoautotrophica* in green bean samples and 8.6% for either *Geobacillus stearothermophilus* or *Thermoanaerobacterium* spp. in carrot samples. Vegetable preparation, *e.g.* washing and edge cutting, considerably reduced spore contamination levels. These data constitute the first wide examination of vegetables specifically cultivated for industrial purposes for their contamination by spores of thermophilic bacterial species.

1. Introduction

Proteolytic *Clostridium botulinum* is historically the reference pathogen for low acid canned food (Esty and Meyer, 1922). Epidemiological data show a relatively low incidence of 0.2 to 0.5 food-borne botulism cases per 1 million people, which is stable since 1991 in France and cases caused by industrially preserved canned food (Brown, 2000; Carlier et al., 2007; McLauchlin et al., 2006) are not common. An outbreak of botulism occurred in south-east and northern France in early September 2011. The source of infection was considered to be a ground green olive paste. Botulinum type A toxin was identified in seven cases and in the incriminated olive paste (Pingeon et al., 2011). Incorrect sterilization techniques were observed at the artisanal producer's workshop. These episodes highlight the potential public health threat of *C. botulinum* linked to inadequate sterilization of food products. In October 2011 in Finland, two persons fell ill with symptoms compatible with botulism after having eaten conserved olives stuffed with almonds. One of these two died. *C. botulinum* type B and its neurotoxin were detected in the implicated olives by PCR and mouse bioassay, respectively. The olives were traced back to an Italian manufacturer and withdrawn from the market (Jalava et al., 2011). In two outbreaks, the Finnish and the French, defects potentially explaining the contamination were identified. In the Finnish outbreak, seals in other jars from the same batch were found to have defects, although none was found to be contaminated. In the French outbreak an improper sterilization process was identified. These outbreaks demonstrate that even modern industrialized production and distribution methods can occasionally allow contamination by botulinum toxin and prompt some important questions.

Spores that are much more resistant than *C. botulinum* spores might be present in the food before retorting. Spoilage caused by mesophilic spore formers is very rare as regulatory control of retorting efficiency and canned food safety is based in many countries on stability tests performed between 30 and 37 °C (Ababouch, 1999). The most heat-resistant spores are formed by thermophilic bacteria (Ashton and Bernard, 1992; Byrer et al., 2000; Olson and Sorrells, 1992). Among the thermophilic group, the most heat-resistant spores belong to *Geobacillus stearothermophilus* and *Moorella thermoacetica* species. They exhibit a decimal reduction time, named D-value, of several minutes at 121 °C and more than 1 h at 124 °C respectively (Byrer et al., 2000; Feeherry et al., 1987; Leguerinel et al., 2007; Sasaki et al., 2000; Wiegel et al., 1981). Persistence of spores in retorted foods may cause spoilage after an extended incubation at high temperature. The most described spoilage of low acid canned food, which is caused by *G. stearothermophilus* is flat sour, while

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M. thermoacetica is responsible for strong acidification and can swelling (Ashton and Bernard, 1992; Olson and Sorrells, 1992).

Despite a long history of description of these bacteria, the origin of spores in canned food is poorly investigated. In cooked food, a high number of ingredients are involved and may carry microbial contaminants (Membre and van Zuijlen, 2011). As an example, *G. stearothermophilus* is known to frequently contaminate dehydrated milk powders (Brent Seale et al., 2011; Ruckert et al., 2004; Ruckert et al., 2005). In single vegetables canned with brine, very few food components are involved. Therefore, spore contamination in these products probably comes from soil or may result from sporulation during food preparation (Carlin, 2011). The aim of this study was to evaluate how frequently spores from *C. botulinum* and thermophilic spoilage species may enter the vegetable canned food process lines, and if vegetable preparation such as washing and cutting was efficient to limit the entry of thermophilic spores in the process zone of transformation at elevated temperature.

2. Material and methods

2.1. Vegetable samples

Sampling was performed from six manufactures for carrots (*Daucus carota*) and eight manufactures for green beans (*Phaseolus vulgaris*). The manufactures were located in different production areas of the French territory. The harvest period occurred between the 15th of June and the end of July 2010 for carrots, and between the 15th of July and the beginning of October 2010 for green beans. Twice a week, three 250 g-samples were randomly picked from each manufacture which collected the vegetable: one in the morning and one in the afternoon from raw vegetable, plus a sample after vegetable preparation, *e.g.* washing, edge cutting, possible peeling, just before the vegetable enters the plant zone where treatments at high temperature occur such as blanching and then retorting. A total of 128 raw carrots samples and 64 prepared carrots samples were collected over 6 weeks. For green beans, 188 raw samples and 93 prepared green beans were collected over 12 weeks. Samples were sent to the laboratory by rapid cold road transport and were processed on arrival. Samples were aseptically cut in 3–4 cm slices and 10 g were weighted into sterile pouches under a laminar flow hood.

In parallel, manufacturers performed stability tests at 37 °C on canned vegetables according to their own sampling plants. All tests performed from canned food corresponding to sampling days of this study revealed 100% stable products.

2.2. Heat shock, enrichment procedure and DNA extraction

Raw samples were assayed for anaerobic mesophilic heat-resistant spore counts, anaerobic thermophilic bacteria counts and anaerobic thermophilic heat-resistant spore counts (Fig. 1). In addition, presence of *C. botulinum* and anaerobic thermophilic heat-resistant spore in 10-g samples was determined. Prepared vegetable samples were assayed for anaerobic thermophilic bacteria counts and anaerobic thermophilic heat-resistant spore counts.

For anaerobic mesophilic spore counts, samples were homogenized with an identical weight of peptone water in a stomacher (AES Chemunex, Ivry sur Seine, France) for 1 min. 10 mL were collected into a glass tube and a thermal treatment of 10 min at 80 °C in a water bath was applied prior to mesophilic anaerobic spore count determination. Mesophilic anaerobic spores were enumerated by pouring 1 mL on meat-liver glucose agar (Biokar diagnostics, Beauvais, France) complemented with 2 g/L yeast extract and incubated for 48 h at 37 °C under anaerobiosis. The detection limit was thus 2 cfu/g. Anaerobiosis was ensured by an anaerobiosis generator system (anaerogen 3.5 L, Oxoid, Dardilly, France) in a locked jar. Anaerobiosis was verified with anaerobic indicators (Biomerieux, Craponne, France).

For *C. botulinum* detection, samples were enriched in anaerobic conditions as previously described (Braconnier, 2001; Braconnier et al., 2001). First, 10-g samples were 10-fold diluted in TGY (tryptone-glucose-yeast extract) medium containing cycloserine 200 mg/L. The 100-mL stomacher filtrate was transferred in a 120-mL bottle and subsequently submitted to a heat shock in a water bath corresponding to an effective incubation of the suspension of 10 min at 80 °C. The cooled down treated bottle was incubated for 72 h at 30 °C with a paraffin stopper to maintain anaerobiosis.

For thermophilic bacterial spores, another 10-g sample was 10-fold diluted in Rosenow (Bio-Rad, Marnes la Coquette, France) medium and homogenized. The resulting 100-mL stomacher filtrate was collected for subsequent use. Three milliliters were directly

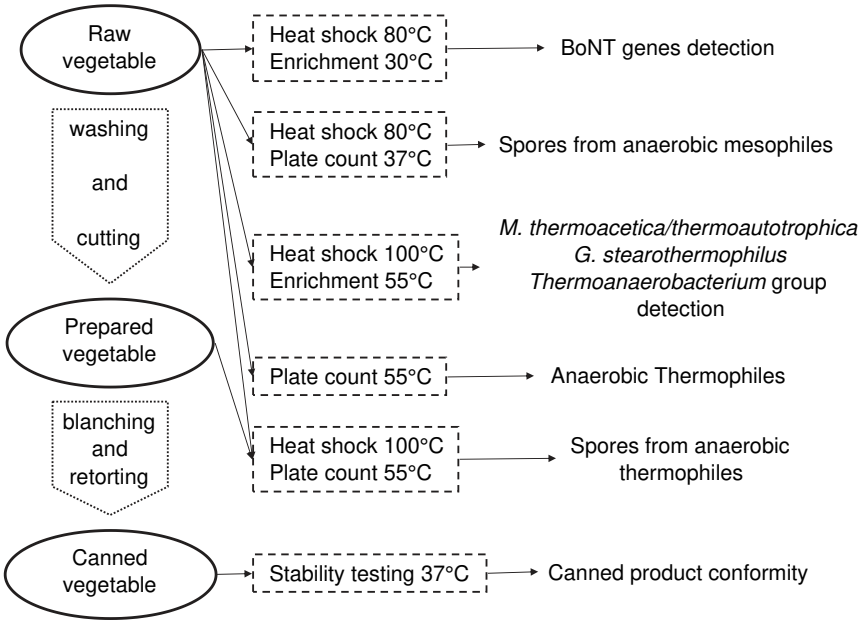

Fig. 1. Schematic representation of sample preparation and testing.

used for anaerobic thermophilic bacteria counts by pouring 1 mL directly or after decimal dilutions on meat-liver glucose agar complemented with 2 g/L yeast extract and incubated for 4 days at 55 °C under anaerobiosis. Consequently, the detection limit for thermophilic anaerobic bacteria was 10 cfu/gL. The remaining 97-mL sample was treated in a boiling water bath such as the sample was kept effectively for 10 min above 95 °C. It was directly used for anaerobic thermophilic spore count determination by pouring 1 mL on agar medium. The detection limit for anaerobic thermophilic spore counts was 2 cfu/g as five plates were inoculated with 1 mL from each sample. For anaerobic thermophile detection, an enrichment of 4 days at 55 °C with a paraffin stopper was performed from the remaining heat-treated suspension. After enrichment, both for *C. botulinum* in TGY medium and for anaerobic thermophiles in Rosenow broth, 1 mL was sampled from each enrichment condition, centrifuged (3000 $\times g$, 5 min) and cell pellets after supernatant elimination were stored at −20 °C.

DNA was extracted from cell pellets with the InstaGene® lysis system following the manufacturer's instruction (Bio-Rad, Marnes-la-Coquette).

2.3. Detection of the bont (Botulinum neurotoxin) genes using the BoNT GeneDisc® array

Detection of *bont*-genes was performed from 30 °C-TGY enrichments of raw vegetables heat-treated at 80 °C for 10 min (Fig. 1). The BoNT GeneDisc® array has been designed for simultaneous examination of six different samples, each being tested for four *bont*-specific gene targets, together with negative and inhibition controls. It has the following settings: microwell 1) PCR inhibition control, microwell 2) *bont*/A, microwell 3) *bont*/B, microwell 4) *bont*/E, microwell 5) *bont*/F, and microwell 6) negative PCR control. The oligonucleotide primers and gene probes used in the GeneDisc® have been described previously (Fach et al., 2009). All oligonucleotides were purchased from Sigma-Aldrich (St. Quentin Fallavier, France). GeneDisc® spotting and manufacturing was performed by Pall-GeneSystems (Bruz, France). PCR analyses with the GeneDisc® array were performed as described by Fach et al. (2011). Thirty six microliters of DNA extract was tested with the GeneDisc® array.

2.4. Highly heat-resistant thermophilic spore forming bacteria detection

Detection of highly heat-resistant spore (HRS) forming bacteria was performed from 55 °C-Rosenow broth enrichments of raw vegetables heat-treated for 10 min at 100 °C (Fig. 1). A group-specific detection PCR was used according to Prevost et al. (2010) with some modifications. *G. stearothermophilus* was detected by a specific 302-bp amplicon corresponding to rRNA ITS 16S–23S region. The group corresponding to the two species *M. thermoacetica* and *M. thermoautotrophica*, which share a 16S rRNA identity of more than 99%, was detected as a 486-bp amplicon. The reverse primer sequence was modified such as a 10-fold decrease of the detection limit from DNA was obtained. To achieve this, two 5′ nucleotides were removed, leading to a better PCR yield without changes in specificity of detection (data not shown). The new primer sequence was 5′-AGGCTATTCGCCTTTAAGAC-3′. The last detected group corresponded to the genus *Thermoanaerobacterium* plus *Caldanaerobius* and *Thermoanaerobacter*, which all belong to the Thermoanaerobacterales order. A 468-bp amplicon was expected.

PCR was performed with 0.2 mM dNTP, either 1.5 mM or 3 mM magnesium chloride respectively for *G. stearothermophilus* plus *M. thermoacetica*/*thermoautotrophica* or *Thermoanaerobacterium* group, *Taq* polymerase (Diamond *Taq* polymerase, Eurogentec, Seraing, Belgium) 2 U and DNA 5 µL in a 25-µL final volume (Prevost et al., 2010). The thermal profile involved 40 cycles with annealing at 65 °C and elongation time of 1 min. Amplicons were detected after 1% Seakem agarose (Lonza, Paris, France) gel electrophoresis in TAE buffer and ethidium bromide staining (Prevost et al., 2010). Ladders were purchased from Eurogentec (Seraing, Belgium).

2.5. Statistics

Statistical analysis was carried out with XLSTAT Version 2010.3.02 (Addinsoft, Paris). Correlation tests were performed by linear regression and Pearson coefficient calculation. Mean comparisons were performed by a *t*-test in case of two-means comparison, and by a two-factors analysis in other cases. To point out significant differences between means, a Bonferroni test was done. Confidence interval for incidence was estimated as 1.96 standard deviation interval. Proportion comparisons were performed according to Fleiss (1981).

3. Results

3.1. Enumeration of spores

Results from vegetative cells and/or spore counts from raw vegetables are presented in Table 1 and Fig. 2. Contamination levels ranged between <1 log cfu/g for thermophilic anaerobic spores to 6.1 log cfu/g for mesophilic anaerobic spores. Examination of the different microbial group populations determined on raw vegetables showed that the highest contamination levels were observed for mesophilic spores and the lowest for thermophilic spores. Relationships between the determined populations, expressed as log cfu/g, were further investigated. Correlation analysis showed that enumeration values for mesophilic spores were not correlated to thermophilic spore counts (p-value = 0.021 but Pearson coefficient < 0.06). Nevertheless, a positive correlation was detected by linear regression (p-value < 0.0001) between thermophilic spore counts and thermophiles (vegetative cells plus spores) counts. The slope confidence interval at 95% was [0.36; 0.70]. However, the square of Pearson coefficient was 0.29, indicating a weak correlation.

For each microbial group considered, contamination levels, expressed as log cfu/g, were compared between the two vegetables. A *t*-test showed, with p-values < 0.0001 and risk α of 0.05, that each of anaerobic mesophilic spore, anaerobic thermophile and anaerobic thermophilic spore counts significantly differed between both vegetables: whatever the population considered, raw carrots exhibited systematically higher contamination levels than raw green beans (Table 1; Fig. 2). In addition, prepared carrots were also significantly more contaminated by thermophilic anaerobic spores than prepared green beans.

Table 1
Vegetative or heat-resistant spore enumeration from vegetable samples.

Vegetable	Number of tested samples	Minimum	Mean ± standard dev	Maximum
Mesophilic anaerobic spore counts (log cfu/g)				
Raw carrot	121	1.7	3.8 ± 0.1 A	6.1
Raw green bean	172	0.6	3.0 ± 0.1 B	5.8
Anaerobic thermophiles counts (log cfu/g)				
Raw carrot	117	1.0	3.3 ± 0.1 A	>5.5*
Raw green bean	187	<1*	2.5 ± 0.1 B	>5.5*
Thermophilic anaerobic spore counts (log cfu/g)				
Raw carrot	126	<1*	2.7 ± 0.1 A	4.6
Raw green bean	188	<1*	1.3 ± 0.1 B	3.8
Prepared carrot	63	<0.3*	0.5 ± 0.1 C	2.8
Prepared green bean	92	<0.3*	0.2 ± 0.0 D	1.1

* For mean and mean standard deviation calculation, counts below detection limit were considered as 0 and counts above detection limit were used at maximal value. Different letters correspond for each microbial group to significant differences (p-value < 0.001).

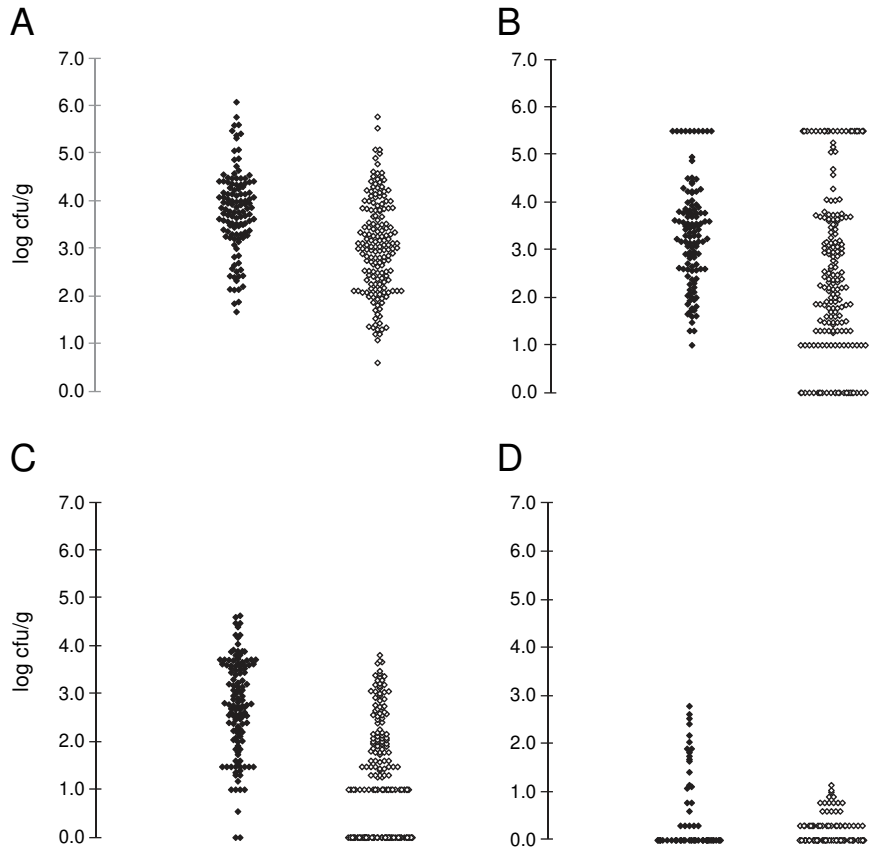

Fig. 2. Distribution of microbial group populations from carrots and green beans. (A) anaerobic mesophilic spores from raw vegetables; (B) anaerobic thermophiles from raw vegetables; (C) anaerobic thermophilic spores from raw vegetables; (D) anaerobic thermophilic spores from prepared vegetables. Black symbols correspond to carrot analyses; open symbols to green beans. Enumeration values below detection limit were drawn as 0 and counts above detection limit were marked at maximal value.

The effect of the nature of the vegetable and state of sampling (raw or prepared) was determined by two-factors variance analysis applied to anaerobic thermophilic spore counts. A conservative Bonferroni test showed a significant difference (p-value$<$0.0001) between means for both factors. Thus, vegetable preparation was efficient to reduce thermophilic spore counts in each vegetable considered. An average decrease of 1.5 log cfu/g was observed. However, differences between vegetables were noticed (Fig. 2). Indeed, the confidence interval at 95% for mean differences was [1.9; 2.5] for carrots and [0.8; 1.3] for green beans, which means a more pronounced decontamination effect for carrots than for green beans. This difference is related to the initial contamination level, *i.e.* low initial contamination levels cannot be decontaminated as effectively as a high initial level. Another way to estimate decontamination effect is to compare the proportion of samples from each vegetable and each state of sampling that exhibit anaerobic thermophilic populations above the detection limits. 98.4% of raw carrots and 62.2% of raw green beans were contaminated by thermophilic anaerobic spores, with a detection limit of 10 cfu/g (Fig. 2). The contaminated proportions were decreased respectively to 38.1% and 42.4% for the two vegetables, after vegetable preparation and with a detection limit of 2 cfu/g (Fig. 2).

3.2. Prevalence of C. botulinum spores

From the analysis of 316 raw vegetable sample enrichments, spores of *C. botulinum* were detected in two samples, one from raw carrot and one from raw green beans (Table 2). Both corresponded to *C. botulinum* type B. As a result, 0.8% and 0.5% of incidence for BoNT producing spores were calculated respectively for carrots and green beans. A global incidence with 95% confident interval of [0; 1.5] was calculated.

3.3. Prevalence of thermophiles

316 raw vegetable sample anaerobic enrichments performed after heat selection at 100 °C were used to detect three groups of highly HRS: *M. thermoacetica/thermoautotrophica, G. stearothermophilus* and *Thermoanaerobacterium* group (Table 2). Incidence ranged between 1.6% in green beans for *M. thermoacetica/thermoautotrophica* and 8.6% in carrots for both *G. stearothermophilus* and *Thermoanaerobacterium* group. Surprisingly, the incidence of thermophilic spore species was not significantly higher for carrots than for green beans, excepted for *G. stearothermophilus* (p-value$<$0.008). Whatever the sample category, the detection frequency was the lowest for *M. thermoacetica/thermoautotrophica* and the highest for *Thermoanaerobacterium* group.

Table 2
Incidence and 95% confidence interval (in brackets) of *C. botulinum* and highly HRS in raw vegetable samples.

	Raw carrots	Raw green beans
Number of tested samples	128	188
C. botulinum	0.8% [0; 2.3]	0.5% [0; 1.6]
M. thermoacetica/thermoautotrophica	4.7% [1.0; 8.3]	1.6% [0; 3.4]
G. stearothermophilus	8.6% [3.7; 13.4]	2.1% [0.1; 4.2]
Thermoanaerobacterium group	8.6% [3.7; 13.4]	4.3% [1.4; 7.1]
Highly HRS assayed	19.5% [12.7; 26.4]	8.0% [4.1; 11.9]

4. Discussion

C. botulinum is considered as the reference pathogenic bacterium for canned food. In industrial canneries, *C. botulinum* risk is well-controlled by good manufacturing practices. The inactivation by retorting of the most heat-resistant mesophilic bacteria is checked by a product stability test after prolonged incubation at 37 °C, frequently called "ambient commercial sterility test". In this study, thanks to a rapid new molecular tool (Fach et al., 2011), we examined for the first time the incidence of this bacteria in raw vegetables cultured specifically for the French canning industry. The determined incidence values fall into usual ranges for *C. botulinum* in foods containing vegetables or raw vegetables, as positive samples usually represented 0 to 0.36% of all samples tested in previous studies (Braconnier et al., 2001; Gibbs et al., 1994; Insalata et al., 1969; Lilly et al., 1995). The highest contamination levels are described for fishes and *C. botulinum* type E is the main type described in this kind of food products. For raw vegetables, *C. botulinum* types B and A are the main types described, with type B as the most common in Europe (Carlin et al., 2004). Our data confirm previous observations, as type B was the only type found.

A second objective of the study was to examine the levels of anaerobic thermophilic bacteria in vegetables intended for industrial canning and to evaluate the incidence of some groups of highly heat-resistant spore-forming bacteria, well-known as spoilage bacteria in canned food. To achieve "elevated-temperature commercial sterility", including during storage in extreme temperature conditions, thermophile spores which exhibit particularly high heat-resistance have to be considered by manufacturers. Spores of highly heat-resistant bacteria can contaminate the product by entering the processing line with raw materials and ingredients and/or by sporulation during the high-temperature food-preparation steps (Carlin, 2011). We show here that process lines are continually flowed-in by highly heat-resistant spores but contamination levels are largely dependent on the kind of raw material. The higher contamination level of carrots compared to green beans was expected due to the presence of soil particles on the root vegetable. *Thermoanaerobacterium* group was the most frequently detected, which was expected as this group gathers many species and several genera. On the whole, almost 20% of raw carrots exhibited highly-HRS-species contamination and 8% of raw green beans did. This means that highly-HRS-species regularly enter canned-food processing lines. Soil is the habitat of many spore-forming bacteria, including *M. thermoacetica* in anoxic microzones (Drake and Daniel, 2004; Gossner et al., 1999). The ability of highly heat-resistant strains to persist in diverse environments is linked to the longevity of their spores.

The thermophilic spore load after cold-zone vegetable preparation was low, but still approx. 40% of the samples analyzed at this state exhibited contamination above the detection limit of 2 cfu/g. After cold-zone preparation, vegetables enter an elevated-temperature transformation zone which starts with blanching. Afterwards, vegetables are poured into cans with a hot-juice, mainly constituted of salt, sometimes sugar, and water, afterwards, cans are sealed before retorting. During vegetable processing at elevated temperature, the environmental conditions become favorable for multiplication of thermophiles and sporulation. Effective multiplication and sporulation would depend on various factors, such as process duration, temperature profile and atmosphere. Taking into account the continuous flow of thermophilic spores entering the elevated-temperature zone, control of sporulation during this step is probably critical to achieve "high temperature commercial sterility". Further studies should aim to elucidate this hypothesis.

Control of bacteria in canned food depends on the initial amount of spores in the product and thermal process setting parameters (Leguerinel et al., 2007; Membre and van Zuijlen, 2011). However, retort process settings cannot be indefinitely increased unless products are overcooked. Highly heat-resistant bacteria control is consequently much more efficiently approached by considering prevalence and concentration before thermal treatment, and suitable preparation treatments, likely to reduce and control the spore contamination before blanching and retorting. Knowledge of heat-resistant spore-forming bacterial load and incidence are crucial to elaborate new strategies to minimize retort process setting parameters that ensure canned product quality.

Acknowledgments

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