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Contamination pathways of spore-forming bacteria in a vegetable cannery

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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.

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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 (Groenewald et al., 2009; Sevenier et al., 2012). The process of canning vegetables combines several operations resulting in contamination with

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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 cm^2 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,

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