Thermophilic spore-forming bacteria isolated from spoiled canned food and their heat resistance. Results of a French ten-year survey

Stéphane André, François Zuber, Fabienne Remize

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S. André*[,1], F. Zuber[1], F. Remize[1,2]

[1] CTCPA Centre Technique de la Conservation des Produits Agricoles, Site Agroparc, ZA de l'Aéroport, BP 21 203, F-84 911 AVIGNON cedex 9, France.

[2] Present address: Laboratoire de Chimie des Substances Naturelles et Sciences des Aliments, ESIROI Agroalimentaire, Université de la Réunion, Parc Technologique Universitaire, 2 rue Joseph Wetzell, F-97490 Sainte-Clotilde, France

Running title: Thermophilic spores from spoiled canned food

* Corresponding author: Stéphane ANDRÉ, CTCPA Centre Technique de la Conservation des Produits Agricoles, Site Agroparc, ZA de l'aéroport, BP 21 203, F-84 911 AVIGNON cedex 9, France. Tel +33 490 84 17 09; Fax +33 490 84 17 26; email: sandre@ctcpa.org

Highlights:

- 455 samples of non-stable Low-Acid Canned Food (LACF) thah showed signs of spoilage after storage at 55°C from 122 canneries were collected over 10 years.
- From 93% of samples only one species was isolated.
- Two species, *Moorella thermoacetica/thermoautotrophica* and *Geobacillus stearothermophilus*, represented 69% of spoilage cases.
- Different hygienic indicators are proposed for different food categories.

Abstract

Thermal processing of Low Acid Canned Foods (LACF), which are safe and shelf-stable at ambient temperature for several years, results in heat inactivation of all vegetative microorganisms and the partial or total inactivation of spores. Good Manufacturing Hygienic Practices include stability tests for managing the pathogen risk related to surviving mesophilic bacterial spores. LACF are also often submitted to additional incubation conditions, typically 55°C for 7 days, to monitor spoilage by thermophiles. In this study we identified the bacterial species responsible for non-stability after prolonged at 55°C incubation of LACF from 455 samples collected from 122 French canneries over 10 years.

Bacteria were identified by microsequencing or a recent developed tool for group-specific PCR detection (SporeTraQ™). A single species was identified for 93% of examined samples. Three genera were responsible for more than 80% of all non-stability cases: mostly *Moorella* (36%) and *Geobacillus* (35%), and less frequently *Thermoanaerobacterium* (10%). The other most frequent bacterial genus identified were *Bacillus, Thermoanaerobacter, Caldanaerobius, Anoxybacillus, Paenibacillus* and *Clostridium*.

Species frequency was dependent on food category, i.e. vegetables, ready-made meals containing meat, seafood or other recipes, products containing fatty duck, and related to the intensity of the thermal treatment applied in these food categories. The spore heat resistance parameters (D or δ and **z** values) from 36 strains isolated in this study were determined. Taken together, our results single out the species most suitable for use as indicators for thermal process settings. This extensively-documented survey of the species that cause non-stability at 55°C in LACF will help canneries to improve the management of microbial contamination.

Abbreviations: *A.*: *Anoxybacillus, B.*: *Bacillus*, *Cr.*: *Caldanaerobacter, Ca.*: *Caldanaerobius*, *C.*: *Clostridium*, *Ge.*: *Gelria*, *G.*: *Geobacillus*, *M.*: *Moorella, P.*: *Paenibacillus*, *Th.*: *Thermoactinomyces*, *Tr.*: *Thermoanaerobacter*, *Tm.*: *Thermoanaerobacterium*

Introduction

Low Acid Canned Foods (LACF according to Codex Alimentarius, 1979) are thermally processed to ensure “commercial sterility” of the food product at ambient temperature for long-term storage. The biological stabilization process requires sufficient heat treatment, at temperatures above 100°C at every point of the container. This process results in the total inactivation of all vegetative bacteria and partial or total inactivation of spores. The pathogen risk related to surviving of mesophilic bacterial spores is managed according to Good Manufacturing Hygienic Practice guidelines (Codex Alimentarius, 1979, CTCPA, 2012a; CTCPA, 2012b). In addition, stability tests involving food container incubation are widely used to detect the possible development of surviving spores. The Codex Alimentarius recommends that food containers should be incubated for 10 or 14 days at 37°C (Codex Alimentarius, 1979). Under French standards NF V08-401 (Afnor, 1997a) and NF V08-408 (Afnor, 1997b), samples are incubated at 37°C for 7 days or 32°C for 21 days. It has been shown that these conditions allow surviving mesophilic spores to germinate and grow in the canned food. Spoilage resulting from microbial growth is then detected by gas production (container swelling), abnormal odours/colours or pH variation, and possibly microscopy examination. If samples test positive, the canned food batch is destroyed to prevent food safety issues. Industrial canning industry processes are consequently designed to reach sterilization values (F_o, min) that ensure “commercial sterility” and therefore microbiological food safety.

The simulation of excessive temperature conditions during storage (transport and retail, especially in relation to exports to the countries with high ambiant temperatures) uses other incubation conditions to test canned food stability, typically 55°C for different durations according countries guidelines. Although thermophilic spore-forming bacteria are not described as pathogenic, their presence may impair the commercial viability of products stored at high ambient temperatures. In addition, LACF non-stability detected after prolonged 55°C incubation reflects insufficient control of hygiene during the end-to-end food processing chain, mainly due to: i) insufficient heat treatment and/or ii) the presence of highly heat-resistant spores on processing lines and raw materials, even at low concentrations.

Therefore, global hygiene management on industrial-line processes essentially relies on surveys of the thermophilic spores that contaminate food before the can sterilization step. Consequently the canning industry needs better knowledge of thermophilic spore-forming bacteria and their origin on processing lines in order to ensure better process control of hygiene conditions.

Only a handful of now outdated studies have addressed the identification and occurrence of spore-forming bacteria responsible for canned food spoilage (Richardson, 1972; Pflug et al., 1981; Matsuda et al., 1985b), and most of these studies remained limited to a single product category (Vicini, 1986) or a

single group of microorganisms (Matsuda et al., 1985a; Dotzauer et al., 2002). Taxonomy has since evolved to integrate new species definitions, and strain isolation and identification techniques have made great strides forward. The aims of this study were: i) to identify the bacterial species responsible for spoilage in 55°C-incubated LACF and ii) to bring insight on the possible causes of canned food non-stability and the species present in spoiled products. The species detected were characterized to help develop better monitoring protocols and detect emerging and/or poorly described species. Spoiled canned food samples were collected in France over a ten-year period.

1. Material and methods

1.1 Sampling

From 2001 to 2010, French canners sent samples of spoiled LACF detected after incubation tests performed at 55°C to the CTCPA (French Technical Center for the Preservation of Agricultural Products) for laboratory analysis in order to isolate and identify the microorganisms present in the cans.

Samples were grouped into three recipe categories: vegetables (21 different recipes); ready-made meals containing essentially meat (15 different recipes), seafood (6 different recipes) or other courses (7 different recipes); products containing fatty duck (two different recipes). The heat treatments used for the canned food were grouped into three categories : low-heat treatments levels with a Fo of less than 5 min ; moderate-heat treatments levels with Fo values between 5 and 20 min ; and high-heat treatments levels with a Fo of over 20 min.

Samples were detected spoiled after incubation at 55°C for 7 days as proposed by French standard NF V08-408 (Afnor, 1997b). According to this standard, non-stability is primarily detected by a change in packaging aspect. After aseptically opening the container, the odor and appearance of the food product were recorded and pH was determined to detect variation between 55°C-incubated samples and room temperature-incubated controls. Microscopy analysis was performed if required.

1.2 Culture of bacteria

AFNOR-CNERNA guidelines were used to revivify viable bacteria from spoiled products (Bouvier et al., 1982) by homogenizing 10 g samples with an 90 ml of peptone water (AES Chemunex, Ivry-sur-Seine, France) in a stomacher for 1 min. Around 10 mL of the stomacher bag filtrate were collected into a glass tube and treated at 10 min at 100°C in a water bath was applied to select heat-resistant spores by killing all vegetatives cells and favoring spore germination. Both non-heated and heated 1 mL-homogenized-

and-filtered samples were used to inoculate broths described in Bouvier et al. (1982). Incubation lasted 7 days at 37°C or 55°C and aerobically or anaerobically by the use of a paraffin stopper.

For isolation, bacterial cultures were plated on dextrose tryptone agar or meat-liver glucose agar (Biokar, Beauvais, France) and incubated at the same temperature and atmosphere as the earlier liquid culture. The same culture conditions were used for enumeration using decimal dilution method.

1.3 DNA preparation

A colony was suspended in 100µL of sterile water, or 1 mL of broth culture was centrifugated (13000 *x g* for 5 min), washed in sterile water, centrifigated again (13000 *x g* for 5 min) and suspended in 100µL of sterile water. And the InstaGene® lysis system according to the manufacturer's instructions (Bio-Rad, Marnes-la-Coquette, France) was used to extract DNA.

1.4 PCR detection of specific bacterial group (SporeTraQ™)

Geobacillus stearothermophilus, Moorella thermoacetica/thermoautotrophica and *Thermoanaerobacterium* spp group were screened by PCR assay according to Prevost et al. (2010) after bacterial growth followed revivification performed at 55°C. The reverse primer sequence for *M. thermoacetica/thermoautotrophica* detection was modified to AGGCTATTCGCCTTTAAGAC (Sevenier et al., 2012). All amplifications were performed in a GeneAmp 9700 PCR system (Applied Biosystems, Courtaboeuf, France).

1.5 Identification of bacteria

Identification involved partial sequencing of the 16S rRNA coding region. For this, PCR was performed with primers FD1 and RD1 according to Weisburg et al. (1991). The amplicon was column-purified before sequencing. Sequencing was performed by Eurofins MWG Operon (Ebersberg, Germany) according to Sanger's method. The primers used for sequencing were S6-16S (GTATTACCGCGGCTGCTG) and/or FD1. Longer sequences were obtained for *Thermoanaerobacterium* isolates with a second sequencing primer CCCCACCTTCCTCCGTG. Sequences were checked for quality and further compared against nucleotide databases (GenBank at NCBI) using MEGABLAST with RDP software (Cole et al., 2009).

16S rRNA coding sequences of 0.9 kb minimal length from isolated thermophilic anaerobes were used for sequence analysis and registered in GenBank under accession numbers JX984955 to JX984980. Type-strain sequences from the RDP database corresponded to: *Caldanaerobacter subterraneus* AE012979 (1527 bp), *Cr. subterraneus* AF212925 (1501 bp), *Caldanaerobius fijiensis* EF507903 (1354 bp), *Cs.*

polysaccharolyticus U40229 (1360 bp), *Cs. zeae* U75993 (1449 bp), *Gelria glutamica* AF321086 (1725 bp), *Moorella glycerini* U82327 (1513 bp), *M. thermoacetica* AY656675 (1430 bp), *M. thermoautotrophica* L09168 (1553 bp), *Tr. brockii* L09165 (1513 bp), *Tr. brockii* L09166 (1523 bp), *Tr. brockii* U14330 (1507 bp), *Tr. ethanolicus* L09162 (1740 bp), *Tr. italicus* AJ250846 (1480 bp), *Tr. mathranii* AY701758 (1404 bp), *Tr. mathranii* Y11279 (1507 bp), *Tr. pseudethanolicus* L09164 (1515 bp), *Tr. siderophilus* AF120479 (1561 bp), *Tr. sulfurigignens* AF234164 (1501 bp), *Tr. thermocopriae* L09167 (1522 bp), *Tr. uzonensis* EF530067 (1415 bp), *Tr. wiegelii* X92513 (1464 bp), *Tm aciditolerans* AY350594 (1442 bp), *Tm. aotearoense* X93359 (1478 bp), *Tm. saccharolyticum* L09169 1552 bp), *Tm. thermosaccharolyticum* AF247003 (1452 bp), *Tm. thermosaccharolyticum* EU563362 (1509 bp), *Tm. thermosaccharolyticum* CP002171 (1504 bp), *Tm. thermosaccharolyticum* HM585225 (1457 bp), *Thermoanaerobacterium thermosulfurigenes* L09171 1574 bp), *Tm. xylanolyticum* L09172, *Thermodesulfobium narugense* AB077817 (1363 bp).

These sequences were aligned using the CLUSTAL Omega program at EBI (Goujon et al., 2010). A dendrogram was built by the average distance method using percent identity on JalView software (Waterhouse et al., 2009). Distance expresses the average relative level of divergence between two aligned sequences. On the tree, branch lengths represent distances.

1.6 Spore suspensions

Thirty eight spores suspensions obtained with 36 strains were prepared according to French standard method NF T 72-231 (Afnor, 1988). Cell suspension (5 ml) following incubation at optimal temperature was inoculated onto agar media (140 mm diameter plate). Aerobic bacteria medium consisted of 10 g of beef extract, 2 g of yeast extract, 0.04 g of MnSO4 H2O, and 15 g of agar in 1 liter. Anaerobic bacteria medium consisted of 30 g of tryptone, 5 g of glucose, 20 g of yeast extract, 1 g of sodium thioglycolate, and 15 g of agar in 1 liter. Incubation was performed either 37 or 55°C depending on species and prolonged for 2 to 5 days for aerobic bacteria and 3 to 4 weeks under anaerobic conditions for others. Durations were ajusted by survey of percentage of spores, determined by microscopy observation. When 90% of spores were observed, harvest was decided. For meso-thermophilic species, optimal sporulation temperature was set by the highest quantity of spores obtained. For 2 strains (*B. coagulans* and *B. smithii*), the both temperatures were used. Spores were harvested by adding cold sterile distilled water onto agar and transferred into a sterile centrifugation tube. The spore suspension was centrifuged at 4000 *x g* for 20 min at 4°C and the pellet was washed three times following the same protocol, resuspended in 20 mL sterile distilled water, heat-treated, then stored at 4°C until analysis. Heat treatment was performed

in a water bath for 10 min at 80°C for *Clostridium* and strict-mesophilic *Bacillus* spp and *Paenibacillus* spp isolates and for 10 min at 100°C for other *Bacillus* spp, *Geobacillus stearothermophilus*, *Moorella thermoacetica/thermoautotrophica*, *Thermoanaerobacter pseudothanolicus* and *Thermoanaerobacterium* sp strains. The concentrated heat-treated suspensions exhibited up to 10^8 spores/mL, except for *Moorella*, *Thermoanaerobacter* and *Thermoanaerobacterium* sp. For the latter, spore suspension contained between 10^6 and 10^7 spores/mL.

1.7 Heat resistance of spore suspensions

100 µL capillary tubes (Ringcaps® Duran®) were filled with 50 µL of spore suspension in 0.2 M phosphate buffer pH7. Spores were heat-treated in a thermostated oil bath at temperatures ranging from 82°C to 132°C. For each spore suspension, heat resistance was evaluated on a temperature range of 9 to 13°C over a time range of 30 seconds to 6 hours. After heating, the tubes were immediately cooled in water. Then each end was opened aseptically and the suspension was flushed out with 3 mL of sterile tryptone-salt broth (AES Chemunex, Ivry-sur-Seine, France). Culturable cells were counted as described above. D values were estimated from linear portions of the log plots of surviving population *vs* heating time. δ and p values were calculated according to the Weibull model modified by Mafart et al. (2002) in which δ is the first reduction time that leads to a 10-fold reduction in surviving population, and p is the shape parameter. z values were determined by plotting D or δ values *vs* temperature.

1.8 Statistical analysis

Statistical analysis was performed using XLSTAT software (AddinsoftTM, Paris, France). The correlation between the two heat resistance modeling methods was evaluated with a Pearson test. All tests were interpreted with a p-value of 0.05 (Tukey's honestly significant difference test).

2. Results and discussion

2.1 Each spoilage case is mainly associated to a single species

A total of 455 samples of various recipes from 122 factories were collected over the course of this 10-year survey. An average of 26 vegetables, 17 ready-made meals and 5 samples containing fatty duck were examined each year.

During first years of study then several times during last years, several isolates were collected after enrichment from spoiled samples. Finally, in 99 spoiled samples, 2 to 5 differents isolates were identified

(Table 1). These 99 samples, whith several identifications, among 462 cases, were representative of the food category variety [vegetables (55% of 99 selected samples and 56% of the 462 samples collection), ready-made meals containing meat (27% and 24%), ready-made meals containing seafood (3% and 3%) or other ready-made meal recipes (7% and 10%), and products containing fatty duck (8% and 7%)]. A single species was identified for 93% of these samples. This observation is consistent with the observation that 10% of samples in which bacterial DNA was directly extracted from spoiled food resulted in two mixed sequences on chromatogram (personal communication). For the 7 other samples in which different species were observed, only two species were found: *Geobacillus* was found with either *Thermoanaerobacterium* or *Bacillus,* while *Thermoanaerobacterium* was found with *Moorella* or *Thermoanaerobacter*. For analysis of the identification results, the 7 samples containing two identified species were considered as 14 different identifications. Taking into account the high percentage of samples with a single species identified, a single identification was performed for all other samples examined either if two different isolates were clarely detected.

For the five food categories, i.e. vegetables, ready-made meals based on meat, ready-made meals based on seafood, or other ready-made meal recipes, and products containing fatty duck, isolation resulted, respectively, in 258 (55%), 112 (24%), 15 (4%), 46 (10%) and 31 (7%) different identifications (Table 2). Thus, a total of 462 different identifications were considered in this study.

2.2 Phylogenic relationship between canned-food isolated Thermoanaerobacterium sp. type Thermoanaerobacterium species and other thermophilic anaerobic sporeformers

A large fraction of the thermophilic anaerobes identified in this study belong to phylogenetic groups that were only recently described. Thus we analyzed the 16S rRNA gene sequences to investigate how the 29 isolates obtained here and labeled were related to previously-described *Thermoanaerobacterales*. This order gathers *Thermoanaerobacterium* (Liu et al, 1996; Can et al, 2001; Klubanov et al, 2007), *Thermoanaerobacter* (Fardeau et al, 2004; Carlier et al, 2006), *Caldanaerobius* (Lee et al, 2008), *Caldanaerobacter* (Fardeau et al, 2004), *Moorella* (Pierce et al, 2008) and *Gelria* (Plugge et al, 2002). We collected 32 sequences of type species and type strains from databases to anchor our analysis with (accession numbers starting by JX or as CTT). The percent of identity between each pair of sequence aligned varies between 36% and 100%.

As expected, the obtained phylogenic tree exhibited clusters corresponding to the different genera (Figure 1). Six branches differed by a distance greater than 4. The strain *Thermodesulfobium narugense* clustered independently and was marked branch A. One group, labeled B, corresponded to

Thermoanaerobacter and *Caldanaerobacter*. The genus *Moorella* was clearly separated into a branch labeled C. Two other clusters gathered the *Gelria* genus on one side (group D) and *Caldanaerobius* on the other (group E). The *Thermoanaerobacterium* group was independently clustered in a branch labeled F.

A strain referenced JX984955 originally identified from a partial 16S rRNA gene sequence as *Gelria glutamica* was the closest relative of the type strain, although at a distance of 0.44. Similarly, the isolates JX984962, JX984963 and JX984964 were clearly related to *M. thermoacetica/M. thermoautotrophica* type strains and separated from the species *M. glycerini* by a distance of 2.31. Four isolates, JX984972, JX984980, JX984966 and CTT4, were gatherable with the species *Ca. polysaccharolyticus* and *Ca. fijiensis* and were more distant to *Ca. zeae*. These isolates were identified as *Ca. polysaccharolyticus*.

The isolates identified as *Thermoanaerobacterium* spp were partly separated. One cluster, labeled F1, exhibiting a maximal distance between all isolates of less than 0.96 gathered the species *Tm. aotearoense Tm. aciditolerans* and *Tm. thermosaccharolyticum*. However, within this cluster, three subgroups could be distinguished. An isolate named JX984977 that was related to this group but clustered individually was identified as *Thermoanaerobacterium* sp. A first subgroup was related to the reference strain *Tm. aotearoense* X93359. It gathered the isolates CTT5, JX984960, JX984975, JX984967, JX984969, JX984965 and JX984959. These isolates were then classified as *Tm. aotearoense*. Another cluster corresponding to isolates JX984961, JX984957 and JX984970 was related to *Tm. Aciditolerans* type strain. A second subgroup clustering the isolates JX984956, JX984978, JX984979, JX984976, JX984958, JX984974, JX984971 and JX984968 was closely related to the *Tm. aciditolerans* type strain. Lastly, a cluster labeled F2, gathered isolates CTT3 and JX984973 with *Tm. saccharolyticum* at a distance of 1.11 from *Tm. thermosulfurigenes* and *Tm. xylanolyticum*.

2.3 Patterns of the bacterial species involved in high-temperature spoilage of canned food

Only two genera gathered 71% of bacteria identified from 55°C spoiled canned food samples. *Moorella* and *Geobacillus* were found respectively in 36% and 35% of samples (Table 2). Strikingly, these two genera were mainly represented by a single species: *G. stearothermophilus* represented 94% of *Geobacillus* spp and *M. thermoacetica / thermoautotrophica* 100% of *Moorella* spp. *M. thermoacetica* is an anaerobic sporeformer described as highly resistant to heat (Ashton and Bernard, 1992; Wagner and Wiegel, 2008). Its growth in canned food is reported to result in strong acidification and can swelling (Ashton et al., 1992; Olson and Sorrells, 1992). Its optimal growth temperature is 55-60°C and it is considered as a model acetogen (Drake and Daniel, 2004). *G. stearothermophilus* is typically described

as responsible for flat sour can spoilage (Olson et al., 1992; Moir et al., 2001; Tucker and Featherstone, 2011). The optimal growth temperature of the *Geobacillus* group is 55-65°C (Nazina et al., 2001).

Thermoanaerobacterium spp was identified in 8% of spoiled canned food samples. It shares similar physiological characteristics, such as anaerobic growth and an optimal growth temperature of around 63°C, to *M. thermoacetica*. Except for the species *Tm. thermosaccharolyticum* (formerly *Clostridium thermosaccharolyticum*), the other species were not previously described in food (Ashton et al., 1992; Chapman, 2001).

Bacillus spp was identified in 9% of spoiled samples. *B. coagulans* was the most frequent of the 7 different *Bacillus* species observed, while *B. smithii* and *B. licheniformis* were the two other species frequently detected. *B. coagulans* is commonly involved in the spoilage of moderately-acid canned vegetables like tomato products (Thompson, 1981; Hanlin, 1998; Moir et al., 2001; Tucker and Featherstone, 2011) and in other canned vegetables (Matsuda et al., 1985a; Oomes et al., 2007), and is acid-tolerant. *B. licheniformis* is often isolated in spoiled canned food (Anatskaya and Efimova, 1978; Chang and Lee, 1982; Matsuda et al., 1985b) and in the dairy industry (Anatskaya et al., 1978; Hanlin, 1998; Scheldeman et al., 2005), whereas *B. smithii* had never previously been isolated from canned food and only once in food (Röling et al., 2001).

Other individual genera represented less than 5% of spoilage cases. The lowest frequencies of detection corresponded to *Caldanaerobius* spp, *Gelria glutamica*, *Anoxybacillus* spp, *Paenibacillus* spp, *Thermoanaerobacter* spp, *Clostridium thermopalmarium/thermobutyricum*, *Thermoactinomyces* sp and several species of *Geobacillus* other than *stearothermophilus*. To our knowledge, *Caldanaerobius* spp and *Gelria glutamica* have never been previously described as canned food contaminants. *Anoxybacillus contaminans* has been described in food gelatin batches (De Clerck et al., 2004) while *Anoxybacillus flavithermus* has been described in heat-processed dairy products and biofilms from food processing environments (Rueckert et al., 2005; Burgess et al., 2009; Postollec et al., 2012). *Paenibacillus* species are routinely found in low-acid canned products or dairy products (Anatskaya et al., 1978; Casadei et al., 2000). Several species of *Thermoanaerobacter* were identified from hot environments (Wagner et al., 2008). The biochemical characteristics and phylogenetic relationship of *Thermoanaerobacter* isolates from canning factories was investigated by Carlier and Bedora-Faure (2006) who proposed a new subspecies, *Thermoanaerobacter mathranii* subsp. *alimentarius*. *Clostridium thermopalmarium/thermobutyricum* was the only *Clostridium* species identified in our study. *Cl. thermopalmarium*, originally described from palm wine in Senegal (Soh et al., 1991), is moderately thermophilic with an optimal growth temperature of 50-55°C. It is genetically close to *Cl. thermobutyricum*,

but with distinct physiological traits (Wiegel et al., 1981). This species was identified exclusively in products containing fatty duck. It represented the main bacterium involved in this spoilage food category and was never isolated in other food categories.

The origin of thermophilic anaerobes like *Moorella* spp, *Thermoanaerobacterium* spp and *Thermoanaerobacter* spp, which caused more than half of can spoilage cases in our study, is usually hot environments like geothermal hot springs and hydrothermal vents or sometimes warm environments like compost and manure (Wagner et al., 2008). For Presland et al. (2004), only *Geobacillus* and *Bacillus* aerobic bacteria and *Moorella* anaerobic sporeformers were cited as thermophiles involved in canned food spoilage. *M. thermoacetica* has occasionally been isolated in canned vegetables (Carlier et al., 2006) or in specific spoiled food products such as canned coffee and “shiruko” (Matsuda et al., 1982). Dotzauer et al., (2002) identified *Thermoanaerobacterium* and *Thermoanaerobacter* and not *Moorella*, but the occurrence of the different genera in their thermophilic anaerobes groups contrasts with our results. It could be due to their agar medium on which isolates from this genus was unable to grow. We consider especially difficult to isolate these genus because it requires special agar not used in bibliography and long duration of incubation rarely used for spore forming bacteria culture. In addition, the genus *Desulfutomaculum* was not isolated in our study whereas it was described as a spoilage bacterium of canned foods, such as spoiled canned milk-containing 'shiruko' coffee or low-acid canned vegetables such as sweet corn (Matsuda et al., 1982; Chapman, 2001; Sperber and Doyle 2009).

2.4 Food category breakdown of the occurrence of the main bacterial groups

Differences in the patterns of identified isolates according to product category were observed (Table 3). In spoiled vegetables and ready-made meals, the same dominant species, *G. stearothermophilus* and *M. thermoacetica/thermoautotrophica,* were identified. This correlation between vegetables and ready-made meals could be due to the contribution of vegetables and other plant-based ingredients such as spices which are frequent sources of contamination (Bolton, 2001; Witkowska et al., 2011). This predominance of both species was not observed in the products containing fatty duck, in which the main spoilage species were *Thermoanaerobacterium* and *Clostridium thermopalmarium/thermobutyricum*. This difference could be related to the product category (presence of high concentrations of lipids that could be limiting for spore germination (Dallyn and Everton, 1970; Lekogo et al., 2010)) or to a low level of thermal treatments (products containing fatty duck present F_0 values lower than 5 min; Table 3). The raw materials used were also very different (only fatty liver or duck meat and fat), although spices (usually black pepper) could be a source of the same bacterial species as in vegetables and ready-made meals.

The other main species detected were *Thermoanaerobacterium* spp and *Bacillus* spp. The species distribution is similar between the five food categories, with *Tm. thermosaccharolyticum* one of the most represented within the genus. Dotzauer et al. (2002) observed a similar species distribution, with few *Thermoanaerobacterium* sp and a majority of *Tm. thermosaccharolyticum.*

A deeper analysis was made of the relationship between spoiled canned food products and the bacteria identified by considering recipe and F_0 for each product category (Table 3).

In vegetable-based spoiled canned food, *G. stearothermophilus* was isolated from 18 out of 22 different recipes, whereas *M. thermoacetica/thermoautotrophica* was isolated from 11 different recipes. *M. thermoacetica/thermoautotrophica* was mainly identified from green peas (40% of *Moorella* spoilage cases) and green peas with carrots (22% of *Moorella* spoilage cases). *Moorella* represented more than two thirds of spoilage bacteria identified from these products. In other vegetable-based recipes, *M. thermoacetica/thermoautotrophica* was frequently detected in mushrooms, green beans, spinach and mixed vegetables. *G. stearothermophilus* spoilage was widespread among the different recipes: 11 vegetable-based recipes resulted in at least four (4.5%) independent detections of the bacterium. The recipes that most frequently led to *G. stearothermophilus* development were mixed vegetables, followed by green beans, green peas, and then sweet corn. Green beans, green peas and sweet corn are the leading canned vegetables by volume produced in France (Bernardin et al., 2010). However, *G. stearothermophilus* alone accounted for more than 40% of spoilage cases of green beans and more than 80% of spoilage cases of sweet corn. As *G. stearothermophilus* was found in various recipes containing a single vegetable, it was logically also found in mixes of these products. *G. stearothermophilus* was isolated from canned peas by Georgescu and Bugulescu (1969), from spoiled canned tomatoes by Hernandez and Feria (1971), and from spoiled canned green beans by Baumgart et al. (1983). For other bacteria identified from spoiled vegetable-based canned food, we found no clear distribution pattern according to recipe, except that *A. contaminans* was identified exclusively in soups.

In meat-based ready-made meals, the difference between recipe distribution of spoilage caused by *M. thermoacetica/thermoautotrophica* or *G. stearothermophilus* was less marked than in vegetables. *G. stearothermophilus* was mainly found in meat-based recipes and cassoulet, while *M. thermoacetica/thermoautotrophica* was mainly found in poultry recipes, sausages-and-lentils, and cottage pie. Its presence in cottage pie may be due to *M. thermoacetica/thermoautotrophica* contamination of the heavily processed potatoes flakes widely used for industrial mashed potato preparation. However, the high frequency of *M. thermoacetica/thermoautotrophica* in the sausages-and-lentils recipe could not be directly related to lentil contamination, as we found no cases of caused *M.*

thermoacetica/thermoautotrophica spoilage of lentils in brine. For other bacteria detected as spoilage agents of ready-made canned meals, the only salient feature was *B. coagulans* mainly identified in cassoulet. *G. stearothermophilus* was again the most widespread species. In other ready-made meals as well as in quenelles and dairy dessert recipes. *G. stearothermophilus* was often incriminated in the contamination of powdered milk and dehydrated ingredients (Rueckert et al., 2005; Postollec et al., 2012). Lastly, *A. flavothermus* was exclusively identified from spoiled fish soup.

In products containing fatty duck, *Thermoanaerobacterium* sp was found mainly in foie gras and in fat-preserved duck, whereas *C. thermopalmarium/thermobutyricum* was only found in foie gras.

Based on this analysis, several bacterial groups of spore-formers could thus be singled out as candidate for spoilage indicators in different food categories, in ordrer to establish microbial criteria or minimum processing parameters. These indicators depend on both the ingredients used and the level of heat treatment applied. For canned vegetables and meat-based ready-made meals heat-treated according to traditional process with F_0 values above 20 min, the general indicators are *G. stearothermophilus* and *M. thermoacetica/thermoautotrophica*. *M. thermoacetica/thermoautotrophica* emerges as a specifically relevant indicator for vegetable mixes containing peas containing, while *G. stearothermophilus* is specific to dairy-derived products. *Anoxybacillus* spp is relevant for soups, and to a lesser extent *B. coagulans* is relevant for cassoulet and foie gras. For products containing fatty duck that are treated with F_0 values below 5 min, both *Thermoanaerobacterium* sp and *C. thermopalmarium thermobutyricum* are the most suitable hygienic indicators.

2.5 Heat resistance parameters

Heat resistance parameters were determined on spore suspensions in order to investigate prospective relationships between spoilage bacteria spore heat resistance and the level of heat treatment applied for product stabilization (Table 4). Standardized conditions were required to compare heat-resistance levels between strains. As most heat destruction kinetics were not log-linear, the Weibull model was used (Mafart et al., 2002). However, as $z_{Weibull}$ values were not significantly different to $z_{loglinear}$ values (Pearson test, $p < 0.0001$), we used D values to compare species according to their heat resistance, as D values are a virtual worldwide industry standard whereas Weibull model parameters (δ and p values) are rarely employed. The stability of z values, which are pH and sporulation temperature-independent, was reported by Baril et al. (2012). The indicator species were tested and other mesophilic species were added to expand the overview.

As expected, *M. thermoacetica/thermoautotrophica* was the species exhibiting the highest heat resistance, with D values at 122°C up to 30 min for the three strains tested. Similar results were reported by Byrer et al. (2000) and Mastuda et al. (1982). D values at 121°C obtained for *G. stearothermophilus* corresponded to a few minutes, as usually reported (Bender and Marquis, 1985; Ocio et al., 1996). D values were slightly higher for *Thermoanaerobacterium* than *G. stearothermophilus* but still far less than for *M. thermoacetica/thermoautotrophica*. The D values of *Thermoanaerobacterium* isolates were below those reported by Xezones et al. (1965) who found D values at 121°C up to 50 min.

Contrary to thermophilic strains, all mesophilic strains exhibited D values of 10 min at temperatures below 108°C. This was expected, as mesophiles are often described as less heat-resistant than thermophiles (Sperber and Doyle 2009). The two meso-thermophilic strains that were sporulated either at 37°C or at 55°C (*B. licheniformis* 3107 043 and *B. smithii* 3108 003) exhibited a slightly higher heat resistance for spores produced at 55°C than at 37°C. Leguerinel et al. (2007) and Sala et al. (1997) demonstrated a high impact of sporulation conditions on heat resistance that could partly explain this observation. However, this relationship between spore resistance and sporulation temperature was modulated by the strain used, as different strains of *B. licheniformis* and *B. smithii, Thermoanaerobacterium* sp and *B. coagulans* able to grow at both 37°C and 55°C displayed the most variable D values. Relationship between growth temperature range and spore heat resistance range was found: mesophilic strains were less heat resistant than thermophilic ones. And among the thermophilic species, extreme thermophiles were more heat resistant than others. For these meso-thermophilic species, D values of 10 min were observed on a temperature range of 8°C wide, while temperature range did not exceed 2°C for the other either strict mesophilic or thermophilic species.

The literature reports z values comprised between 6 and 12°C whatever the species (Stumbo, 1973; Jenson and Jensen, 2001; Tucker and Featherstone, 2011). In our study, z values were in this range and all values were below 10°C. Note that strict anaerobic thermophiles exhibited the lowest values as observed by Jenson and Jensen (2001).

As expected, the canned-food F_0-value observed in industry was strongly related to heat resistance of the strains isolated. The most heat-resistant species, *M. thermoacetica/thermoautotrophica*, was isolated from canned products treated at $F_0 > 20$ min. *G. stearothermophilus* was regularly isolated from products treated at moderate or high heat levels (F_0 between 5 to 20 min and F_0 above 20 min). The isolation conditions of *B. coagulans* strains proved the most significant, yielding a strict correlation between heat resistance level and F_0-value applied to the canned products. The less heat-resistant strain (3105 044) was isolated from a pasteurization process, the next-least-heat-resistant strain (3105 018) from a canned

foie gras (Fo <5 min) and the third least-heat-resistant strain (3105 018) from a cassoulet treated at 5 < Fo <20 min.

However, our data do not explain why *M. thermoacetica*/*thermoautotrophica* was not isolated from spoiled products that were treated at F_0-values too low to inactivate its spores. The most realistic hypothesis is that the low growth rate of this species favors competiting bacteria that were faster to spoil the product as they were not destroyed by the low-level heat treatment.

Conclusion

This paper reports results from a long-term ten-year survey of the causes of food spoilage in high-temperate heat-treated canned foods in France. With these 462 isolates from spoiled canned food of 122 canneries, the study enriches previous data on species isolated from spoiled LACF products (Landry et al., 2001; Tucker and Featherstone, 2011). The spoilage bacteria involved are highly heat-resistant, thermophilic and non-pathogenic with 2 species representing 69% of spoilage cases. Our results confirmed that thermophilic bacteria are good indicators of food hygiene, as highlighted by Burgess (Burgess et al., 2010). Adapted bacterial indicator groups for canned food spoilage were proposed. These data and more specifically the heat resistance parameters determined for strains isolated from industrial food processes, bring valuable insights for the management of processing line contamination and for the calculation of scheduled sterilization heat treatments. Screening for these indicators in raw materials, ingredients and samples from processing lines could help single out several contamination locations in canneries for a better hygiene control.

Better control of thermophilic spores by proper cleaning of processing lines and higher standards of quality for raw materials and the determination of the elevated-temperature stability of LACF should help improve food safety monitoring: any increase in 55°C-non-stability frequency should therefore be considered as a warning sign that hygiene conditions on processing lines are degraded for either thermophilic spoilage and mesophilic pathogen spore forming bacteria. In this purpose, it should be necessary to determine precisely the process steps where these species proliferated and to know the genetic and physiologic diversity of strains to link raw material, multiplication area and spoiled products.

Acknowledgments

This work was supported by FranceAgriMer. We thank the French canned food manufacturers that kindly collected the spoiled samples.

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Figure 1 Phylogenic tree of the thermophilic anaerobes isolated from this study, and type strains calculated from >1-kb 16S rRNA gene sequence using percent identity

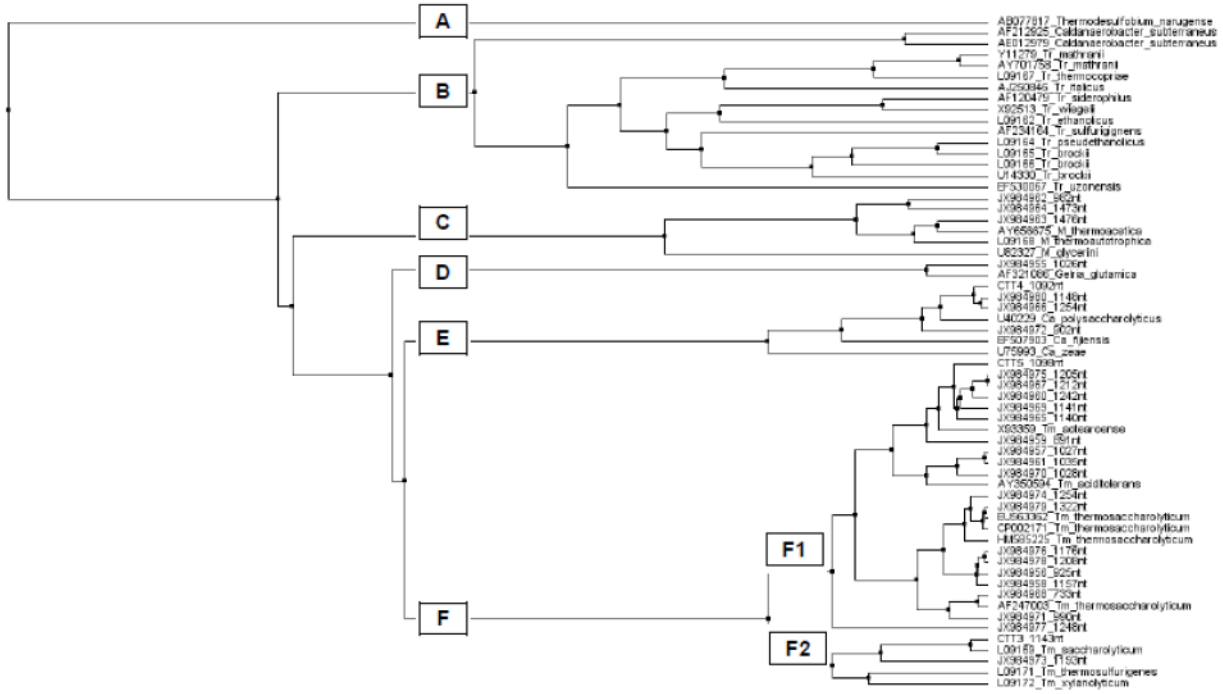

Table 1 Number of different species identified in isolates recovered from spoiled canned food samples

Number of spoiled samples	Number of isolates identified from each sample	Number of different species from each sample
76	2	1
8	3	1
5	4	1
3	5	1
7	2	2

Table 2 Species and number of each species of different isolates identified in the three global food categories

Species	Number of isolates	% of each genus in the food category	% of each species in the food category
Vegetables			
M. thermoacetica/thermoautotrophica	106	41.1	41.1
G. stearothermophilus	90		34.9
G. caldoxylosilyticus	4	37.2	1.6
Geobacillus sp	1		<0.4
G. thermoglucosidasius	1		<0.4
Tm. thermosaccharolyticum	6		2.2
Thermoanaerobacterium sp	4	7.0	1.6
Tm. aciditolerans	6		2.2
Tm. aciditolerans	2		0.8
B. licheniformis	5		1.9
B. smithii	4		1.6
B. coagulans	3	5.8	1.2
B. amyloliquefaciens	2		0.8
B. thermoamylovorans	1		<0.4
Tr. mathranii/thermocopriae	4	2.7	1.6
Thermoanaerobacter sp	3		1.2
Ca. zeae/fijiensis/polysaccharolyticus	7	2.7	2.7
A. contaminans	5	1.9	1.9
P. macerans	2	0.8	0.8
C. thermopalmarium/thermobutyricum	1	<0.4	<0.4
Ge. glutamica	1	<0.4	<0.4
Total	258		

Species	Number of isolates	% of each genus in the food category	% of each species in the food category
Ready-made meals			
M. thermoacetica/thermoautotrophica	60	34.7	34.7
G. stearothermophilus	61		35.3
G. debilis	1	36.4	<0.6
Geobacillus sp	1		<0.6
Tm. thermosaccharolyticum	8		4.6
Thermoanaerobacterium sp	6	9.2	3.5
Tm. aciditolerans	1		<0.6
Tm. aotearoense	1		<0.6
B. coagulans	6		3.5
B. licheniformis	3		1.7
B. thermoamylovorans	3	9.2	1.7
B. smithii	2		1.2
B. amyloliquefaciens	1		<0.6

Bacillus sp	1		<0.6
Tr. thermohydrosulfuricus	4		2.3
Tr. mathranii/thermocopriae	3	6.4	1.7
Thermoanaerobacter sp	4		2.3
Ca. zeae/fijiensis/polysaccharolyticus	2	1.2	1.2
A. flavothermus	3	1.7	1.7
P. macerans	1	1.2	<0.6
Paenibacillus. sp	1		<0.6
Total	173		

Species	Number of isolates	% of each genus in the food category	% of each species in the food category
Products containing fatty duck			
M. thermoacetica/thermoautotrophica	0	0	0
G. stearothermophilus	1	6.4	3.2
G. pallidus	1		3.2
Tm. aotearoense	5	38.7	16.1
Tm. thermosaccharolyticum	5		16.1
Thermoanaerobactrium sp	2		6.4
B. coagulans	4	16.1	12.9
B. smithii	1		3.2
C. thermopalmarium / thermobutyricum	11	35.5	35.5
Thermoactinomyces sp	1	3.2	3.2
Total	31		

Abbreviations: *A.: Anoxybacillus, B.: Bacillus, Ca.: Caldanaerobius, C.: Clostridium, Ge.: Gelria, G.: Geobacillus, M.: Moorella, P.: Paenibacillus, Tr.: Thermoanaerobacter, Tm.: Thermoanaerobacterium*

Table 3 Spoiled food samples examined by category and occurrence of the main bacterial groups identified by canned food category, recipe and corresponding routine average Fo[a] used in canneries

Product	Number of samples	Routine F_o (min)	*M. thermoacetica / thermoautotrophica*	*G. stearothermophilus*	*Thermoanaerobacterium spp*	*B. coagulans*	*C. thermopalmarium*	*Anoxybacillus spp*
Vegetables								
Peas	65	>20	43	11	2			
Mixed vegetables	31	>20	7	19	2			
Peas - carrots	30	>20	23	4	1		1	
Green beans	27	5-20	8	11	2			
Mushrooms	21	>20	8	5	3			
Vegetable soup	14	>20	2	5	1			5
Sweetcorn	11	>20	2	9				
Spinach	7	>20	3		1			
Flageolet beans	5	5-20		4				
White beans	4	nc[c]		4				
Other recipes[b]	4 or less each	nc	0 or 1	3 or less each	0 or 1	3		
Subtotal	258							
Ready-made meals								
- Meat-based meals								
Red meat	21	5-20	3	9	2	1		
Cassoulet	17	5-20	3	6	2	3		
Poultry	11	5-20	8	3				
Cottage pie	10	>20	7	1	1			
Sausages-lentils	8	>20	7					
Snails	6	>20	2	1				
Couscous	4	nc	4					
Other recipes[d]	<4	nc	2 or less each	0 or 1	2 or less each	2		
Subtotal	112							
- Other types								
Ravioli	11	<20	5	6				
Quenelles	10	5-20		9				
Dairy products	9	5-20	1	6				
Sauce	6	variable	1	1	3			

Other recipes[e]	<5	nc	2 or less each	1 or 2	0				
Subtotal	46								
- Seafood-based ready-made meals									
Fish soup	4	nc							3
Other recipes[f]	<3	nc	1 or 0	3 or less each	1 or 0				
Subtotal	15								
Products containing fatty duck									
Foie gras	24	<5			7	4	11		
Fat-preserved duck	7	<5	1		5				
Subtotal	31								
Total	**462**		**152**	**166**	**46**	**13**	**12**	**8**	

[a] F_o: Sterilization value achieved at cold point of Low-Acid Canned Foods calculated with reference temperature 121,1°C and z = 10°C; values edited only for products analyzed more than 5 times per recipe; [b] lettuce, lentils, potatoes, artichokes, chestnuts, leeks, carrots, red kidney beans, coral lentils, soy, miscellaneous;;[c] not communicated; [d] duck, terrine, meatballs, stuffed cabbage, paella, paté, cocktail sausages, miscellaneous; [e] garnish, rice, miscellaneous; [f] bisque, hake, fish pie, cod, miscellaneous; Abbreviations: *B.*: *Bacillus*, *C.*: *Clostridium*, *G.*: *Geobacillus*, *M.*: *Moorella*

Table 4 Heat resistance parameters of species isolated in spoiled canned food

Species	Strain	Growth and sporulation temperature [a] (C°)	Calculated temperature (°C) for D = 10 min	$Z_{loglinear}$ values (°C)	$Z_{Weibull}$ values (°C)
M. thermoacetica	1901 058	55	125.9	7.9	8.0
M. thermoacetica	1901 053	55	125.6	8.2	8.6
M. thermoacetica	1901 020	55	125.3	6.1	6.5
G. stearothermophilus	2804 168	55	115.6	7.4	7.2
G. stearothermophilus	2804 138	55	114.8	7.6	7.8
G. stearothermophilus	2804 173	55	113.6	9.4	8.7
Ca. zeae/figensis/ polysaccharolyticus	2503 005	55	118.1	6.9	7.0
Tm. aotearoense	2503 020	37-55	116.5	7.1	7.7
Tm. aotearoense	2501 001	37-55	116.3	7.4	7.5
Tm. thermosaccharolyticum	2506 011	55	116.4	5.8	5.8
Tr. pseudothanolicus	2510 001	55	109.1	6.9	6.9
B. smithii	3108 003	37-55	113.1	6.1	6.3
		37-55	110.9	6.7	6.9
B. smithii	3108 010	37-55	109.6	7.5	7.2
B. smithii	3108 021	37-55	105.0	7.5	7.8
C. thermopalmarium/ thermobutyricum	3216 001	55	108.3	6.4	6.2
Paenibacillus sp	2911 002	37	108.0	6.2	6.3
Paenibacillus sp	2901 020	37	102.3	8.0	8.2
Paenibacillus sp	2901 002	37	101.4	7.5	7.7
B. subtilis	3111 037	37	108.0	8.9	9.0
B. subtilis	3111 002	37	103.2	10.1	10.0
B. coagulans	3105 032	37-55	106.8	6.2	6.4
B. coagulans	3105 018	37-55	99.3	7.5	7.8
B. coagulans	3105 044	37-55	98.6	8.5	8.4
B. licheniformis	3107 028	37-55	102.4	8.5	9.2
B. licheniformis	3107 043	37-55	97.7	8.0	6.7
		37-55	97.1	6.7	6.9
B. licheniformis	3107 017	37-55	96.7	8.7	8.6
B. licheniformis	3107 022	37-55	94.9	8.1	8.1
C. haemolyticum	3207 002	37	100.6	8.2	9.2
C. sporogenes	3222 002	37	100.1	7.8	7.7
C. sporogenes	3222 001	37	99.2	7.4	7.5
C. sporogenes	3213 005	37	98.3	6.6	6.6
P. macerans	2903 017	37	99.4	7.8	7.6
P. macerans	2903 006	37	99.2	7.6	7.2
P. polymyxa	2904 003	37	90.3	7.8	8.0
P. polymyxa	2904 005	37	89.0	7.9	7.9
C. novii	3210 016	37	84.7	7.9	8.3

[a] underlined : sporulation temperature; Abbreviations: *A.*: *Anoxybacillus*, *B.*: *Bacillus*, *Ca.*: *Caldanaerobius*, *C.*: *Clostridium*, *G.*: *Geobacillus*, *M.*: *Moorella*, *P.*: *Paenibacillus*, *Tr.*: *Thermoanaerobacter*, *Tm.*: *Thermoanaerobacterium*

Highlights:

- 455 samples of non-stable Low-Acid Canned Food (LACF) thah showed signs of spoilage after storage at 55°C from 122 canneries were collected over 10 years.
- From 93% of samples only one species was isolated.
- Two species, *Moorella thermoacetica/thermoautotrophica* and *Geobacillus stearothermophilus*, represented 69% of spoilage cases.
- Different hygienic indicators are proposed for different food categories.