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Potential risk of botulinum neurotoxin -producing clostridia occurrence in canned fish

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Abstract

Introduction: Heat treatment is indispensable in fish canning to provide an acceptable shelf life. Its optimisation reduces the risk of the presence of *Clostridium botulinum* spores, which could potentially cause botulism cases. This study evaluated canned fish samples for botulism neurotoxin (BoNT)-producing clostridia contamination and can bulging through microbiological contaminant growth. A new analytical approach was developed for detection of such clostridia and phenotypically similar species. **Material and Methods:** A total of 70 canned fish samples suspected of exhibiting bulging features were analysed. Culture methods were used to detect clostridia. The isolates obtained were evaluated on the basis of the exhibited phenotypic characteristics. Also, PCRs were used for the detection of genes determining BoNT production (non-toxic non-haemagglutinin (*ntnh*) genes) and the amplification of conservative 16S rDNA genes, which were Sanger sequenced. The obtained sequences were analysed using the Basic Local Alignment Search Tool. **Results:** *Clostridium* genus species were isolated from 17 (24%) bulging and organoleptically changed samples. No *ntnh* genes were present in these isolates; however, sequencing confirmed the presence of *C. sporogenes*, a species with close affinity to *C. botulinum.* **Conclusion:** To eliminate the threat of foodborne botulism, laboratory diagnostic techniques must detect species of the *Clostridium* genus and elucidate their ability to produce BoNTs. Although *Clostridium botulinum* is the most common cause of botulism, the possibility may not be ignored that non-pathogenic *Clostridium* species may acquire botulinum toxigenicity. The similarity between the isolated strains of *C. sporogenes* and *C. botulinum* should be incorporated in the optimisation of heat treatment to guarantee a sterilised, microbiologically safe product.

Keywords: *Clostridium sporogenes*, *Clostridium botulinum*, canned fish, microbiological contamination.

Introduction

The susceptibility of raw fish to biophysical, chemical and microbiological changes makes necessary the use of effective thermal preservation methods such as pasteurisation and sterilisation in order for a retail product to be offered with acceptable spoilage time. Correctly conducted sterilisation of canned fish should inactivate microflora and ensure sterility, and consequently, consumer safety (12). As a result of heat treatment, in addition to achieving an appropriate shelf life of the product, organoleptic and other quality characteristics are changed (1, 2). Optimisation of the temperature and time parameters of the process preserves the desired nutritional values and reduces the risk of underheating failing to prevent the growth of resistant microflora, especially pathogenic or potentially pathogenic strains of *Clostridium* spp. (12). The risk exists of the occurrence of botulinum neurotoxin (BoNT)-producing clostridia, particularly *C. botulinum* – the cause of foodborne botulism. Based on physiological differences and 16S rRNA gene sequences, strains of this pathogen have been divided into four metabolic groups: group I includes all type-A strains and proteolytic type-B and -F strains, group II all type-E strains and non-proteolytic type-B and -F strains, group III consists of type-C and -D strains and group IV of type-G strains. These groups are related to other species considered non-toxigenic: *C. sporogenes* and the recently described *C. tepidum*

are considered related to group I; *C. beijerinckii*, *C. butyricum* and *C. taeniosporum* to group II; *C. novyi* to group III; and *C. subterminale* and *C. schirmacherense* to group IV (10, 11, 27, 37). These neurotoxins produced by *Clostridium* species are the most potent natural toxins yet identified. Classically, the toxic neurological syndrome is characterised in all forms by acute symmetrical descending flaccid paralysis. When botulism is described as a clinical syndrome, the typical description refers to the foodborne form. The laboratory diagnosis of foodborne botulism is based on the detection of BoNTs or BoNT-producing clostridia in clinical specimens or food samples and the obligation to identify the BoNT involved is usually attached to the laboratory's task. Foodborne botulinum intoxication is often underdiagnosed; the initial symptoms can be confused with more common clinical conditions (*e.g.* stroke, myasthenia gravis, the Miller–Fisher variant of Guillain–Barré syndrome, Lambert–Eaton syndrome, tick paralysis or shellfish or tetrodotoxin poisoning (15). Moreover, effective inactivation of bacterial spores through the use of heat treatment can be challenging because of the high heat resistance of the endospores of some species, such as *C. sporogenes*. This species shows close affinity with proteolytic *C. botulinum* strains belonging to group I and is usually not able to produce BoNTs. The proteolytic nature of *C. sporogenes* and its ability to produce spores that show greater resistance to high temperatures than those of *C. botulinum* may cause the faster spoilage of food products contaminated with this species, and this poses a potential threat to public health (6, 7). Clostridia naturally inhabit the marine and freshwater sediments of wetlands, rivers, and lakes; therefore, the possibility of its occurrence in unprocessed fish seems to be relatively high. Consequently, the risk of primary or secondary contamination of canned products is also relatively high. Compliance on the manufacturer's part with the appropriate hygiene requirements and maintenance of adequate sterilisation parameters in line processing are essential. Inadequate sanitary and hygiene conditions may fail to prevent contamination of canned fish products by pathogens or extremely resistant *Clostridium* spp. bacteria. The anaerobic conditions of the canning process create a favourable environment for the germination of *Clostridium* spores, causing their transition into the vegetative form and subsequent toxin production (8, 14).

The first aim of this study was to determine the risk associated with the possibility of BoNT-producing clostridia in canned fish samples which had previously been submitted to a sterilisation process. The second aim was to evaluate the impact of these clostridia on the safety of the product intended to be provided by production line processing. In order to recognise this problem, an advanced molecular biology method was used for detection and characterisation of *Clostridium* spp. anaerobes.

Material and Methods

Canned fish samples. The study was carried out on 70 samples of canned fish suspected of showing features of bulging, taken from warehouse to check the microbiological sterility of the questioned products and sent to the National Veterinary Research Institute (NVRI) in an official animal-origin food safety monitoring programme conducted by the Veterinary Inspectorate in Poland. The following 14 production batches were used: 5 batches comprising 25 samples of herring in tomato sauce, designated P1–P2, P6–P7, and P13; and 9 batches comprising 45 samples of sprat in tomato sauce, designated P3–P5, P8–P12, and P14. All canned products, as declared by the manufacturer, were subjected to heat treatment at 117°C with an sterilisation value $F_0>10$. In the five batches of canned products P6–P10, can bulging was observed. Organoleptic changes were also noted, *i.e.* a disagreeable odour and atypical consistency of the can contents. The distribution of substandard organoleptic quality among the batches is presented in Fig. 1.

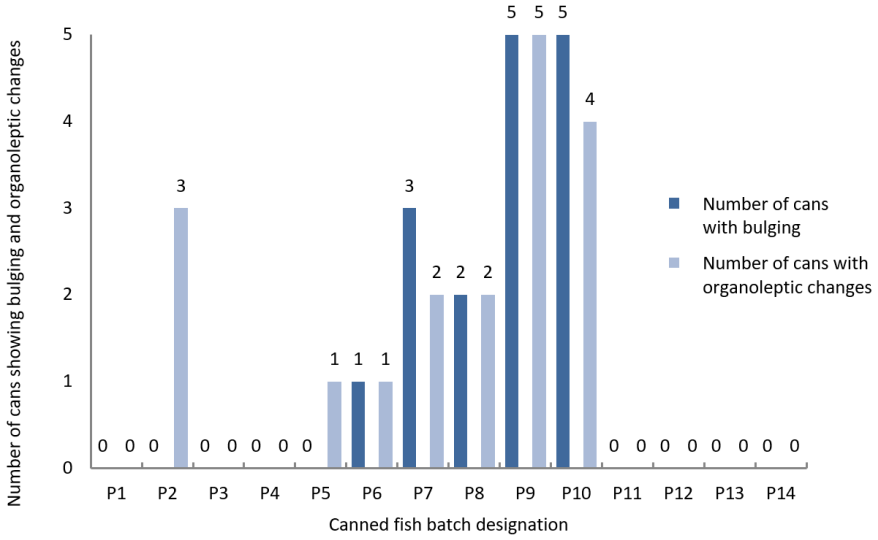

Fig. 1. Canned fish by batch exhibiting bulging and organoleptic changes

Culture. A 10g mass was taken from each canned fish sample and inoculated into 90 mL of trypticase-peptone-glucose-yeast extract (50 g/L casein enzymic hydrolysate, 5 g/L peptic digest of animal tissue, 20 g/L yeast extract, 4g/L dextrose, 1g/L sodium thioglycolate, pH 7.0 ± 0.2) at 25°C. In addition, 1 g of material from each sample was inoculated into medium according to Wrzosek (40): 1 L liver extract, 10 g/L peptone, 5 g/L sodium chloride, 5 g/L glucose, 3–4 beef liver cubes. The inoculum was prepared in duplicate, one of which was heat treated at 70°C for 15 min (in order to inactivate the vegetative microflora and stimulate potential germination). Subsequently, the inocula prepared in this way were subjected to incubation at 37℃ for 48 h under anaerobic conditions, which were obtained using anaerobic jars, anaerobic atmosphere–generating sachets (AnaeroGen; Thermo Scientific, Waltham, MA, USA) and test strips to detect an anaerobic atmosphere (Merck Millipore, Darmstadt, Germany). At the end of this period, the growth of the tested microflora was assessed by visual evaluation of the turbidity and gas production.

In the next step, approximately 10 µL of the macroscopically evaluated liquid cultures (with visible turbidity changes and gas production) was spread on two varieties of agar. One was Willis–Hobbs agar: 10 g of peptic digest of animal tissue, 10 g/L meat extract, 5 g/L sodium chloride, 12 g/L lactose, 0.032 g/L neutral red, 10 g/L skim milk powder, 2 g of yolk powder, 10 g/L agar pH 7.0 ± 0.2 at 25°C. The second was fastidious anaerobe agar (FAA): 23 g/L peptone, 5 g/L sodium chloride, 1 g/L soluble starch, 0.4 g/L sodium bicarbonate, 0.5 g/L L-cysteine hydrochloride hydrate, 0.25 g/L sodium pyrophosphate, 1 g/L L-arginine, 0.5 g/L sodium succinate, 0.01 g/L haemin, 0.001 g/L vitamin K, 2g of egg yolk powder, 12 g/L agar pH 7.2 ± 0.2 at 25°C (28). The prepared plates were incubated anaerobically at 37°C for 48 h. The obtained colonies were evaluated for their size, shape, and lipolytic, lecithinolytic, and proteolytic features.

Bacterioscopic preparations of the colonies stained with the Gram method were examined under a BX53 biological microscope, and visualised in cellSens Standard software (Olympus, Tokyo, Japan) (3).

DNA isolation. DNA was isolated from 1 mL of liquid cultures and selected colonies obtained from agar plates. The extraction of the genetic material was carried out using a Genomic Mini AX Bacteria kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instructions. The amount of DNA used in the PCR reaction varied between 1 and 25 ng. The extracted DNA was directly analysed or frozen at −20°C to be preserved for further studies.

Detection of the *ntnh* gene by real-time PCR. A method based on a real-time PCR was used, which amplified a fragment of the *ntnh* gene. This gene determines the production of the non-toxic non-haemagglutinin component (NTNH), which is common to all types of toxic *Clostridium* strains capable of producing botulinum toxins. A set of seven primers and a TaqMan probe were used for detection of the *ntnh* gene as described by Raphael and Andreadis (25). The reaction mixtures constituted of 5 µL of DNA, 4 µL of LightCycler TaqMan Master (Roche, Basel, Switzerland), 0.7 µM of each primer and 0.24 µM of NTNH410 TaqMan probe. The real-time PCR was performed on a LightCycler 2.0 thermocycler (Roche, Basel, Switzerland) and the following temperature profile was used: initial denaturation at 95°C for 10 min and 40 cycles of denaturation at 95°C for 15 s, annealing at 42°C for 15 s and elongation at 55°C for 1 min. The fluorescence signal was captured in each cycle at the elongation step. As the control samples, DNA isolated from the following reference strains was used: *C. botulinum* National Collection of Type Cultures (NCTC) 887 (type A), *C. botulinum* NCTC 3815 (type B), *C. botulinum* NCTC 8266 (type E) and *C. botulinum* NCTC 10281 (type F).

Detection of *Clostridium* strains by 16S rDNA amplification and Sanger sequencing. Undetermined *Clostridium* isolates were subjected to identification at the species level using a PCR method amplifying the conserved 16S rDNA genes according to the methodology described by Vaneechoutte *et al.* (36). Each reaction mixture was 25 µL in volume and was constituted of 5 µL of DNA from a *Clostridium* sp. isolate, 2.5 µL of 10× Taq Buffer with KCl (Thermo Fisher Scientific, Waltham, MA, USA), 4mM of $MgCl_2$, 200 µM of dNTP, 0.3 µM of each primer and 1.25 U/25 µL of Taq polymerase. Each reaction was carried out on a Biometra T1 thermocycler (Biometra, Gottingen, Germany) and proceeded through these steps: an initial denaturation at 95°C for 5 min was followed by 35 cycles including denaturation at 95°C for 45 s, annealing at 55°C for 1 min, and extension at 72°C for 2 min. The cycling step was followed by final strand elongation at 72°C for 10 min. The length of the obtained amplicons (about 1,500 bp) was verified on agarose gel, and these amplicons were Sanger sequenced by Genomed (Warsaw, Poland). The results provided in FASTA files (a text-based format for representing either nucleotide sequences) were analysed using the BLAST (Basic Local Alignment Search Tool) algorithm available in the National Center for Biotechnology Information (NCBI) bioinformatics tools. The aim of the analysis was to determine the similarity between the obtained amplicons and the 16S rDNA sequences available in the NCBI database.

Electrophoresis of PCR products. Agarose gels were prepared at a concentration of 2% in 1× Tris-acetate-ethylenediaminetetraacetic acid and rendered visible with SimplySafe nucleic acid stain (EURx, Gdansk, Poland). Electrophoretic separation was carried out for 1.5 h at 100 V. The length of obtained products expressed in base pairs (bp) was evaluated using GeneRulerTM100 bp DNA Ladder Plus (Thermo Fisher Scientific).

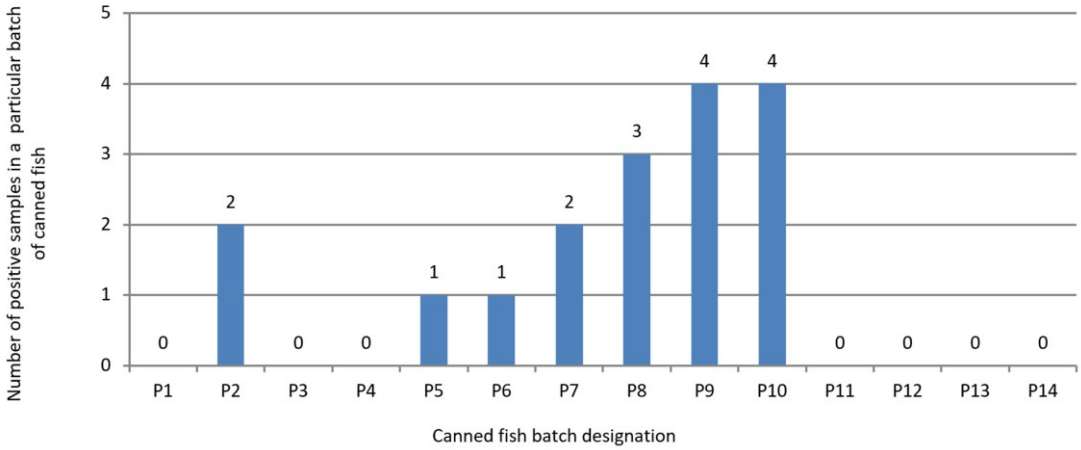

Fig. 2. Canned fish samples by batch in which the presence of anaerobes was revealed

Table 1. Results of the 16S rDNA gene identification of isolates from canned fish

Amplicon sample	Sequence ID	% Similarity	Similar sequence from the GenBank database
S1	MT356160.1	98.40	*Clostridium sporogenes* strain SM5 16S rRNA
S2	MT356160.1	99.25	*Clostridium sporogenes* strain SM5 16S rRNA
S3	MT356160.1	99.15	*Clostridium sporogenes* strain SM5 16S rRNA
S4	MT356160.1	99.43	*Clostridium sporogenes* strain SM5 16S rRNA
S5	MT356160.1	99.32	*Clostridium sporogenes* strain SM5 16S rRNA
S6	MT356160.1	99.51	*Clostridium sporogenes* strain SM5 16S rRNA

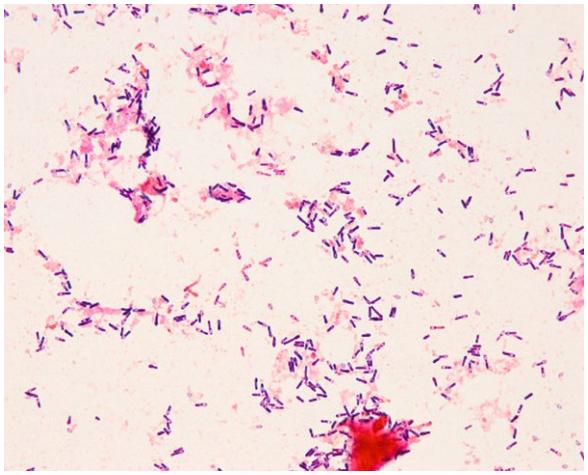

Fig. 3. Isolated Gram-positive *Clostridium* bacteria with subterminal spores

Results

Identification of isolated strains. Anaerobic *Clostridium* spore-forming rods were found in 17 samples (24%). The growth of round colonies with regular shapes exhibiting lipolytic properties was observed on Willis–Hobbs and FAA agars. Gram-stained bacterioscopic slides showed the presence of Gram-positive spore-forming bacilli typical of the genus *Clostridium* (Fig. 3). In most cases, strains were isolated from canned food samples in which bulging and organoleptic changes were observed (Fig. 2).

Real-time PCR analysis results. Analysis of DNA extracted from isolated strains from liquid culture and agar media did not reveal the presence of the *ntnh* gene in canned fish samples. Positive analysis results were obtained only for the *C. botulinum* NCTC 887, *C. botulinum* NCTC 3815, *C. botulinum* NCTC 8266 and *C. botulinum* NCTC 10281 reference strains.

Results of 16S rDNA gene sequencing of *Clostridium* genus isolates. Analysis of the amplicon sequencing results using the BLAST bioinformatics tool revealed the presence of bacteria in the canned fish samples which were genetically similar to *C. sporogenes*. The percentage of similarity between the obtained amplicon sequences and those deposited in the NCBI database ranged from 98.4% to 99.5% (Tab. 1).

Discussion

Thermal sterilisation is one of the most commonly used methods of heat treatment, and it guarantees the commercial sterility of food products at a high level of microbiological safety. Optimisation of this technological process requires consideration of a number of key factors, such as the specific microflora liable to be present and their thermal resistance, the physical properties of the raw materials used in the production process, the intended physical properties of the end product after packaging, and the pH value during the process, on which the development of microorganisms

largely depends (35). Despite optimising the sterilisation process, there are still incidences of thermoresistant microflora of the *Clostridium* genus in canned products, resulting in particular from improperly conducted thermal processing and failure to achieve the required minimum sterilisation value $F_0 \geq 3$ (24, 26). Determination of the F_0 value enables the temperature and time of the canned food heat treatment to be specified so as to eliminate microbiological hazards in the shortest possible exposure of the product to high temperature (9).

The obtained isolates showed morphological features characteristic of strains of *C. botulinum*, although the results of 16S rDNA analysis indicated the presence of sequences characteristic of *C. sporogenes*. The absence of the *ntnh* gene determining toxinogenicity was noted. Literature reports point to the occurrence of microbiological contamination in food caused by the presence of highly heat resistant species of *Clostridium*, including *C. sporogenes* (32, 33, 34). The ability to produce BoNTs is primarily inherent in pathogenic species such as *C. botulinum*; several species are to be regarded as botulinum toxigenic and considering BoNT-producing clostridia as a single species is now only a historical notion. Significant progress has been made in understanding the structure and function of clostridial neurotoxins. As demonstrated by the scattered phyletic distribution of neurotoxin-producing clostridia and the sequence similarities between different neurotoxin gene clusters, genes appear to have undergone significant horizontal transfers between different species of *Clostridium*.

Active botulinum toxin forms a component of the protoxin complex, which also contains haemagglutinin and NTNH. The conversion of inactive BoNT to the toxic form occurs by the release of active toxin from the protoxin complex due to the activity of proteolytic enzymes produced by bacterial strains and host-derived enzymes (4, 20, 27). The genes encoding botulinum neurotoxin are a component of a gene cluster that also includes non-toxic components that ensure BoNT stability, among them the gene encoding NTNH, the presence of which is associated only with toxic species (13, 21, 39). The *bont* genes are primarily located in chromosomes and can be carried by mobile elements such as plasmids, whereas these genes are transported by bacteriophages in species belonging to group III, a difference serving to distinguish this group (30, 31).

Research carried out in recent years has shown that *C. sporogenes* not only shows phenotypic similarity to the toxic *C. botulinum*, but also may produce botulinum neurotoxin by itself, being an epidemic agent responsible for botulism in humans (6, 18). This threat emanates from the possibility of horizontal transfer of genes determining the production of botulinum neurotoxin between species of *C. botulinum* group I and *C. sporogenes* (6, 17, 38). Strains of *C. botulinum* in group I (and to a lesser extent *C. sporogenes*) are a major cause of the three most frequent types of botulism in humans (foodborne, infant and wound botulism) and are also responsible for botulism in animals. Foodborne botulism is a severe and often lethal neuroparalytic intoxication that can be caused by the consumption of as little as 50 ng of botulinum neurotoxin. Destruction of the spores of *C. botulinum* group I is the aim of the botulinum cook (121°C / ≥ 3 min) given to low-acid canned foods. Foodborne botulism outbreaks have been associated with a failure to apply the botulinum cook to canned or bottled foods, and also with the temperature abuse of products intended to be stored chilled. The commercial implications of foodborne botulism outbreaks can be significant, and continued extreme vigilance is important to ensure that the incidence of the disease is minimised (6).

Despite the possibility of *C. sporogenes* strains acquiring toxigenic features, most cases of botulism occurring due to food contamination are associated with the presence of *C. botulinum* strains. The pathogenic nature of *C. sporogenes* may cause serious problems such as can bulging and organoleptic changes. Its similarity to *C. botulinum* makes it an excellent model organism for optimisation of sterilisation processes for adequate reduction of the risk of food contamination with *C. botulinum* spores (16, 23). One measure for ensuring food safety is using appropriate prognostic models of potential *C. botulinum* growth and toxin production during processing. However, these models are subject to a margin of error, which is why conducting microbiological challenge tests also plays an important role in minimising the danger of canned food botulinal contamination. Given the threat posed by the pathogenicity of *C. botulinum,* it is recommended to perform challenge tests using surrogates with properties identical to those exhibited by the pathogen except for the ability to produce botulinum toxins. The use of surrogate organisms makes it possible to determine the optimal conditions for preventing the growth of *C. botulinum*, thus providing a more reliable way of manufacturing products free of microbiological hazards (22).

Laboratory detection and identification of *C. botulinum* and phenotypically similar strains of other *Clostridium* species should be integral to ensuring the microbiological safety of food products. Coordination of the analytical process and selection of appropriate testing methods is a major challenge on account of the heterogeneity of species of the *Clostridium* genus and the possibility of loss of toxigenic properties that may occur during analysis. Classification of a species and strain suspected of belonging to the BoNT-producing clostridia based on biochemical characterisation of the microorganism may prove unreliable, but the molecular biology methods implemented in the present study appear to be a suitable and effective tool for detecting *Clostridium* to strain level and identifying genes that determine neurotoxin production. In addition, PCR methods based on detection of the *ntnh* and *bont* genes

are an innovative approach to the development of new techniques that enable the identification of BoNT-producing clostridia and would be beneficial to adopt in routine monitoring of potential food contamination. The introduction of a monitoring system, the implementation of line process control and the observation of appropriate hygienic standards in the production process are of fundamental importance in ensuring product safety. The food technological issues described above and the potential epidemiological effect of failure to achieve sterility require that particular attention be paid to evaluating the effectiveness of the production processes, including heat treatment, in preventing the occurrence of spore-forming bacteria of the *Clostridium* genus. The non-toxic species *Clostridium sporogenes* shares nearly identical metabolic properties with group I *C. botulinum*, including the formation of lipase-positive colonies when grown on egg-yolk agar. Because of these similarities and the comparable heat resistance of its spores, *C. sporogenes* has been used as a surrogate organism for *C. botulinum* in the study of thermal processing of foods (29). It seems reasonable to subject each product to challenge tests using model microorganisms such as *C. sporogenes* before it is introduced to the Polish market, in order not to permit canned food to be retailed in which the growth of and potential toxin production by *C. botulinum* and genetically similar strains is possible.

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References

1. Al-Baali A.A.G., Farid M.M.: Chapter 5, Thermal Sterilization of Food in Cans. In: *Sterilization of Food in Retort Pouches*, Food Engineering Series, Springer Science+Business Media, New York, NY, 2006, pp. 45–62, doi: 10.1007/0-387-31129-7_5.
2. Banga J.R., Balsa-Canto E., Moles C.G., Alonso A.A.: Improving food processing using modern optimization methods. Trends Food Sci Technol 2003, 14, 131–144, doi: 10.1016/S0924-2244(03)00048-7.
3. Barra–Carrasco J., Olguín–Araneda V., Plaza–Garrido A., Miranda–Cárdenas C., Cofré–Araneda G., Pizarro–Guajardo M., Sarker M.R., Paredes–Sabja D.: The *Clostridium difficile* exosporium cysteine (CdeC)-rich protein is required for exosporium morphogenesis and coat assembly. J Bacteriol 2013, 195, 3863–3875, doi: 10.1128/JB.00369-13.
4. Benoit R.M.: Botulinum neurotoxin diversity from a gene-centered view. Toxins 2018, 10, 310, doi: 10.3390/toxins 10080310.
5. Brown J.L., Tran-Dinh N., Chapman B.: *Clostridium sporogenes* PA 3679 and its uses in the derivation of thermal processing schedules for low-acid shelf-stable foods and as a research model for proteolytic *Clostridium botulinum*. J Food Prot 2012, 75, 779–792, doi: 10.4315/0362-028X.JFP-11-391.
6. Brunt J., Van Vliet A.H.M., Carter A.T., Stringer S.C., Amar C., Grant K.A., Godbole G., Peck M.W.: Diversity of the genomes and neurotoxins of strains of *Clostridium botulinum* group I and *Clostridium sporogenes* associated with foodborne, infant and wound botulism. Toxins 2020, 12, 586, doi: 10.3390/toxins12090586.
7. Brunt J., Plowman J., Gaskin D.J.H., Itchner M., Carter A.T., Peck M.W.: Functional characterisation of germinant receptors in *Clostridium botulinum* and *Clostridium sporogenes* presents novel insights into spore germination systems.PLOS Pathog 2014, 10, doi: 10.1371/journal.ppat.1004382.
8. Cortés-Sánchez A.: About *Clostridium botulinum*, Fish and Tilapa, Mod Appl Sci 2021, 15, doi: 10.5539/mas.v15n3p1
9. Doornmalen J.P.C.M., Kopinga K.: Temperature dependence of F-, D- and z-values used in steam sterilization processes. J Appl Microbiol 2009, 107, 1054–1060, doi: 10.1111/j.1365-2672.2009.04290.x.
10. Grenda T., Kwiatek K., Goldsztejn M., Sapała M., Kozieł N., Domaradzki P.: Clostridia in insect processed animal proteins–is an epidemiological problem possible? Agriculture 2021, 11, 270, doi:10.2290/agriculture11030270.
11. Hill K.K., Xie G., Foley B.T., Smith T.J., Munk A.C., Bruce D., Smith L.A., Brettin T.S., Detter J.C.: Recombination and insertion events involving the botulinum neurotoxin complex genes in *Clostridium botulinum* types A, B, E and F and *Clostridium butyricum* type E strains. BMC Biol 2009, 7, doi: 10.1186/1741-7007-7-66.
12. Ismail I.M., Fahmy A., Azab A., Abadir M., Fateen S.E.,: Optimizing the sterilization process of canned food using temperature distribution studiem. IOSR-JAVS 2013, 6, 26–33, doi: 10.9790/2380-0642633.
13. Johnson E.A., Bradshaw M.: *Clostridium botulinum* and its neurotoxins; a metabolic and cellular perspective. Toxicon 2001, 39, 1703–1722, doi: 10.1016/s0041-0101(01)00157-x.
14. Lalitha K.V., Gopakumar K.: Growth and toxin production by *Clostridium botulinum* in fish (*Mugil cephalus*) and shrimp (*Penaeus indicus*) tissue homogenates stored under vacuum. Food Microbiol 2001, 18, 651–657, doi: 10.1006/fmic.2001.0433.
15. Lonati D., Schicchi A., Crevani M., Buscaglia E., Scaravaggi G., Maida F., Cirronis M., Petrolini V.M., Locatelli C.A.: Foodborne botulism: clinical diagnosis and medical treatment. Toxins 2020, 12, 509, doi: 10.3390/toxins12080509.
16. Mah J.H., Kang D.H., Tang J.: Comparison of viability and heat resistance of *Clostridium sporogenes* stored at different temperatures. J Food Sci 2009, 74, 23–27, doi: 10.1111/j.1750-3841.2008.00984.x.
17. Maikanov B., Mustafina R., Auteleyeva L., Wiśniewski J., Anusz K., Grenda T., Kwiatek K., Goldsztejn M., Grabczak M.: *Clostridium botulinum* and *Clostridium perfringens* occurrence in Kazakh honey samples. Toxins 2019, 11, 472, doi: 10.3390/toxins11080472.
18. Nawrocki E.M., Bradshaw M., Johnson E.A.: Botulinum neurotoxin–encoding plasmids can be conjugatively transferred to diverse clostridial strains. Sci Rep 2018, 8, doi: 10.1038/s41598-018-21342-9.
19. Num S.M., Useh N.M.: *Clostridium:* pathogenic roles, industrial uses and medicinal prospects of natural products as ameliorative agents against pathogenic species. JJBS 2014, 7, 81–94, doi: 10.12816/0008220.
20. Peck M., Smith T.J., Anniballi F., Austin J.W., Bano L., Bradshaw M., Cuervo P., Cheng L.W., Derman Y., Dorner B.G., Fisher A., Hill K.K., Kalb S.R., Korkeala H., Lindström M., Lista F., Lúquez C., Mazuet C., Pirazzini M., Popoff M.R., Rossetto O., Rummel A., Sesardic D., Singh B.R., Stringer S.C.: Historical perspectives and guidelines for botulinum neurotoxin

subtype nomenclature. Toxins 2017, 18, doi: 10.3390/toxins9010038.
21. Peck M.W., Van Vliet A.H.M.: Impact of *Clostridium botulinum* genomic diversity on food safety. Curr Opin Food Sci 2016, 10, 52–59, doi: 10.1016/j.cofs.2016.09.006.
22. Poortmans M., Vanoirbeek K., Dorner M.B., Michiels C.W.: Selection and development of nontoxic nonproteolytic *Clostridium botulinum* surrogate strains for food challenge testing. Foods 2022, 11, doi: 10.3390/foods11111577.
23. Poulain B., Popoff M.R.: Why are botulinum neurotoxin-producing bacteria so diverse and botulinum neurotoxins so toxic? Toxins 2019, 11, 34, doi: 10.3390/toxins11010034.
24. Rahman M.S.: Canning and Sterilization of Foods. In: *Handbook of Food Preservation*. CRC Press Taylor&Francis Group, Danvers, 2007, pp. 585-625.
25. Raphael B.H., Andreadis J.D.: Real-time PCR detection of the nontoxic nonhemagglutinin gene as a rapid screening method for bacterial isolates harboring the botulinum neurotoxin (A–G) gene complex. J. Microbiol Methods 2007, 71, 343–346, doi: 10.1016/j.mimet.2007.09.016.
26. Raseta M., Brankovic-Lazic I., Mrdovic B., Baltic B., Zsolt B., Djordjevic V.: Optimization of liver pate sterilization from the aspect of preserving nutritional value and ensuring food safety. Meat Technology 2019, 60, 97–105, doi: 10.18485/meattech.2019.60.2.4.
27. Reddy N.R., Skinner G.E., Oh S.: *Clostridium botulinum* and its control in low-acid canned foods. Food Sci Biotechnol 2006, 15, 499–505.
28. Saeed E.M.A.: Studies on Isolation and Identification of Clostridium botulinum Investigating Field Samples Specially from Equine Grass Sickness Cases, Doctoral dissertation, Faculty of Agriculture, Georg-August-University Göttingen, Göttingen, 2004
29. Simpson L.: The life history of a botulinum toxin molecule. Toxicon 2013, 68, 40–59, doi: 10.1016/j.toxicon.2013.02.014.
30. Smith T.J., Hill K.K., Raphael B.H.: Historical and current perspectives on *Clostridium botulinum* diversity. Res Microbiol 2015, 166, 290–302, doi: 10.1016/j.resmic.2014.09.007.
31. Smith T.J., Tian R., Imanian B., Williamson Ch.H.D., Johnson S.L., Daligault H.E., Schill K.M.: Integration of complete plasmids containing bont genes into chromosomes of *Clostridium parabotulinum, Clostridium sporogenes*, and *Clostridium argentinense.* Toxins 2021, 13, 473, doi: 10.3390/toxins13070473.
32. Stersky A., Todd E., Pivnick H.: Food poisoning associated with post-process leakage (PPL) in canned foods. J Food Prot 1980, 43, 465–476, doi: 10.4315/0362-028X-43.6.465.
33. Sumner J., Ross T.: A semi-quantitative seafood safety risk assessment. Int J Food Microbiol 2002, 25, 55–59, doi: 10.1016/s0168-1605(02)00062-4.
34. Taylor R.H., Dunn M.L., Ogden L.V., Jefferies L.K., Eggett D.L., Steele F.M.: Conditions associated with *Clostridium sporogenes* growth as a surrogate for *Clostridium botulinum* in nonthermally processed canned butter. J Dairy Sci 2013, 96, 2754–2764, doi: 10.3168/jds.2012-6209.
35. Valentas K.J., Rotstein E., Singh R.P (red).: Chapter 2, Sterilization Process Engineering. In: *Handbook of Food Engineering Practice*. CRC Press LLC, Salem, 1997, pp. 46–78, doi: 10.1201/9780367802110.
36. Vaneechoutte M., Cartwright C.P., Williams E.C., Jäger B., Tichy H.V., de Baere T., de Rouck A., Verschraegen G.: Evaluation of 16S rRNA gene restriction analysis for the identification of cultured organisms of clinically important *Clostridium* species. Anaerobe 1996, 4, 249–256, doi: 0.1128/JCM.42.5.2197–2202.2004.
37. Weigand MR., Pena-Gonzales A., Shirey T.B., Broeker R.G., Ishaq M.K., Konstantinidis K.T., Raphael B.H.: Implications of genome-based discrimination between *Clostridium botulinum* group I and *Clostridium sporogenes* strains for bacterial taxonomy. Appl Environ Microbiol 2015, 81, 5420–5429, doi: 10.1128/AEM.01159-15.
38. Wentz T.G., Tremblay B.J.M., Bradshaw M., Doxey A.C., Sharma S.K., Sauer J.D., Pellett S.: Endogenous CRISPR-Cas systems in group I *Clostridium botulinum* and *Clostridium sporogenes* do not directly target the botulinum neurotoxin gene cluster. Front Microbiol 2022, 12, doi: 10.3389/fmicb.2021.787726.
39. Williamson Ch.H.D.,Vazquez A.J., Hill K., Smith T.J., Nottingham R., Stone N.E., Sobek C.J., Cocking J.H., Fernández R.A., Caballero P.A., Leiser O.P., Kelm P., Sahl J.W: Differentiating botulinum neurotoxin- producing clostridia with a simple, multiplex PCR Assay. Appl Environ Microbiol 2017, 83, doi: 10.1128/AEM.00806-17.
40. Wrzosek A.: Weitere Untersuchungen uiber die Zuchtung von obli-gatorischen Anaeroben in a&rober Weise. Cent. f. Bakt. Abt. I. Orig. 1907, xliv, 607–616.