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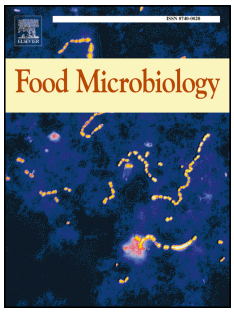

High prevalence of *Clostridium botulinum* in vegetarian sausages

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Short communication

Title: High prevalence of *Clostridium botulinum* in vegetarian sausages

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1. Introduction

In line with sustainable development goals, plant-based foods and meat substitutes are becoming a preferred source of protein among increasing number of consumers. A popular alternative for meat products are vegetarian sausages, with a range of products being available on the market. Most products are vacuum-packaged chilled foods, but also frozen and canned vegetarian sausages are retailed. Ingredients include typically a plant or fungal protein source (soy, wheat protein, chickpea, pea protein, mycoprotein), other vegetables (corn, potato, pepper, tomato, onion, garlic etc.), herbs, spices, salt, vegetable oil, and additives (thickening agents, stabilizers, pH regulators, antioxidants).

A major food safety concern in vacuum-packaged chilled foods are psychrotrophic, botulinum neurotoxin (BoNT) producing clostridia, particularly *Clostridium botulinum* Group II (Lindström et al., 2006a; Peck, 2006). These bacteria produce resistant endospores, grow in anaerobic conditions, and produce the highly potent BoNTs during growth. Once ingested, BoNTs inhibit the release of acetylcholine at the neuromuscular junction and cause a potentially lethal flaccid paralysis, botulism. *C. botulinum* spores exist widely in environment and can contaminate food raw materials. The spores can survive pasteurization and, under favorable conditions, may germinate and outgrow into toxic cultures.

Both home-canned and commercially processed vegetables are common sources of foodborne botulism (Sobel et al., 2004; Anniballi *et al.,* 2017; Hellmich *et al.,* 2018). A range of vegetables have been implicated in outbreaks, including onions, potatoes, corn, peppers, asparagus, carrots, beans, olives, and garlic (MacDonald et al., 1985; Morse et al., 1990; Angulo et al., 1998; Sobel et al., 2004; Zanon et al., 2006; Date et al., 2011; Jalava et al., 2011; Lindström et al., 2011; Hill et al., 2013). In addition, tofu has caused botulism, introducing soy into the list of implicated vegetables (Chai et al., 2013). However, screening studies on the prevalence of *C. botulinum* spores in non-outbreak-related vegetables are scarce. Negligible positive findings (sample size ranging between 10-200 g) suggest a very low prevalence and spore contamination levels below 1–10 spores/kg (Insalata et al., 1969; Lilly et al., 1995; Braconnier et al., 2001;

Sevenier et al., 2012; Barker et al., 2016). The few positive screening samples and most outbreak investigations revealed *C. botulinum* types A and B. While type A strains are exclusively of the mesophilic Group I, which do not possess a risk of growth under refrigeration, BoNT B-producing strains may belong to either Group I or Group II and can be distinguished by metabolic features (Dahlsten et al., 2008) and by the neurotoxin gene sequence (Hill et al., 2007). Unfortunately, information on the physiological group or toxin gene sequence for early *C. botulinum* type B findings in vegetables are not available, but a recent report on a subtype B4 neurotoxin gene present in two out of three studied samples of porcini mushrooms (Barker et al., 2016) confirms that the psychrotrophic Group II strains may be of concern. This is in line with our previous finding of Group II *C. botulinum* type E in a sample of vegetarian sausage (Lindström et al., 2001).

Vegetarian sausages can be categorized as refrigerated processed foods of extended durability (REPFED). Typically, REPFEDs are processed at mild pasteurization temperatures, cooled rapidly after processing, and stored refrigerated over extended periods of time (Gorris and Peck, 1998). The safety of REPFEDs relies on hurdle technology combining multiple preservation factors to control microbial growth (Gorris and Peck, 1998). The applied heat treatments, prevailing storage temperatures, and the use of preservatives define the product shelf-life and safety.

While there are no reported cases of botulism due to vegetarian sausages, the possibility of raw material contamination with *C. botulinum* Groups I and II spores, mild pasteurization, vacuum-packaging, and long shelf-lives contribute to an apparently high risk of *C. botulinum* growth and BoNT production related to vacuum-packaged chilled vegetarian sausages. Here we show a high overall prevalence of 32% of *C. botulinum* in 74 vegetarian sausage products.

2. Material and methods

2.1 Vegetarian sausages.

A total of 74 samples of frozen (8) or chilled (66) packaged vegetarian sausages from seven producers were purchased in Finland and Germany. The pH of such products is above 5.7 and added NaCl concentrations in the range of 1.2–1.9%. Assuming dry-matter concentrations of 27–68% (Havlik et al., 2010), the corresponding water-phase NaCl concentrations are mainly in the range of 2–4%, and exceed 5% only in the rare occasions when added NaCl concentration exceeds 1.9% and dry-matter content exceeds 64%. The shelf-lives of the investigated vacuum-packaged products remaining at the time of purchase varied from less than 2 weeks to 6 months. Some of the investigated products contained detailed instructions for cooking, including heating temperature and time, some products contained just a suggestion of heating method without time indications, and some products were advised to be served either heated or cold. The main ingredients of the vegetarian sausages were soy (soy protein or tofu), wheat protein, vegetable oil, sugar, spices, salt, and corn, wheat, or potato starch. Additives such as pH regulators, emulsifiers, stabilizers, and antioxidants were commonly included but were not identified in more detail. Some of the products contained also oat, rice, egg white, apple, onion and/or garlic.

2.2 Microbial analyses.

Before laboratory analysis, the vegetarian sausage samples were stored at temperatures instructed by the manufacturers, either frozen or at refrigeration. The quantity of *C. botulinum* was determined from non-heat-shocked samples using the most probable number (MPN) method (Cochran, 1950), using PCR detection of *C. botulinum* growth and the formula of Thomas (1942) for MPN estimation based on the number of PCR-positive tubes (Hielm et al., 1996). A sample size of 20 to 111 g was inoculated into a set of tubes containing tryptone–peptone–glucose–yeast extract (TPGY) broth (1:10) and incubated under anaerobic conditions at 30°C (Group II) or at 37°C (Group I) for 72–96 hours, followed by overnight cultures in fresh TPGY (1:10) under identical conditions. Cells from 1-ml aliquots were prepared for PCR templates as described (Lindström et al., 2001). The presence of genes encoding BoNT types A, B, E, and F was determined using multiplex PCR (Lindström et al., 2001). Attempts to isolate *C. botulinum* from all PCR-positive samples were made on egg yolk agar plates (Hauschild and Hilsheimer, 1977), and amplified

fragment length polymorphism (AFLP) method was used to genotype the *C. botulinum* isolates (Keto-Timonen *et al.,* 2006).

3. Results

We show a strikingly high overall prevalence of 32% for *C. botulinum* in vacuum-packaged vegetarian sausages (Table 1). Apart from one positive frozen sample (13%), all other positive samples were detected among chilled products (23 samples, 35%) with advised maximum storage temperatures of 6–10°C. Genes for BoNT types A, B, E, and F were detected, with types B (33 %), A (33%), and E (25%) detected frequently, and types B and F together, and types B and E together, once (4%, Table 1). The highest MPN counts were detected for Group II type E, up to 1200 cells/kg. At the time of purchase, eight samples had remaining shelf-lives of 3 to 6 months, and six of them were PCR-positive for *C. botulinum*.

Eight isolates were recovered from eight PCR-positive samples (MPN counts in these samples were 30-110 cells/kg): two type A, five type B, and one type E isolate. AFLP typing showed all the A and B isolates to be of *C. botulinum* Group I and the one E isolate to be of Group II (Fig. 1.). Successful isolation of *C. botulinum* validated the positive PCR findings. With successful recovery of a BoNT gene-carrying isolate as the sole criterion of a positive sample, the overall prevalence of *C. botulinum* in the vegetarian sausages appeared to be 11%.

4. Discussion

The MPN counts of *C. botulinum* in the vegetarian sausage samples varied between 20 to 1200 cells or spores/kg (average/median cell or spore count of 176/110 MPN/kg). Such counts are up to 3 log-units higher than the predicted counts of 1–10 spores/kg in raw materials (Barker et al., 2016), and one log-unit higher than counts found in vacuum-packaged hot-smoked fish products (Hyytiä et al., 1998) relatively frequently linked with botulism outbreaks (Kautter, 1964; Korkeala et al., 1998; Lindström et al., 2006b; King et al., 2009). We found the highest *C. botulinum* counts (10^3 cells or spores/kg) for *C. botulinum* Group

II type E, which are psychrotrophic and can grow and produce BoNT at temperatures as low as 3°C within 8 weeks (Graham et al., 1997). The high counts were detected in products with remaining shelf-lives at 6–10°C of up to 6 months. Thus, the safety risk related to these vacuum-packaged vegetarian sausages appears to be high.

The origin of *C. botulinum* in vegetarian sausages remains partially unclear. While vegetables are a common source for Group I *C. botulinum* types A and B, Group II type B strains are associated with pork meat preparations and occasionally to fish and seafood (Galazka and Przybylska, 1999; Lindström et al., 2006a; Mazuet et al., 2018). Type E is mostly associated with fish and seafood (Lindström et al., 2006a). It remains to be discussed how *C. botulinum* types less frequently associated with vegetables contaminate vegetarian sausages. A possible source for type E could be sea-salt as *C. botulinum* type E strains prevail in aquatic ecosystems (Hielm et al. 1998) and their spores have been detected in sea-salt (Fenicia et al., 2002).

While BoNT/A is exclusively produced by *C. botulinum* Group I strains and BoNT/E by Group II, BoNT/B and BoNT/F toxins can be produced by both *C. botulinum* Group I and II strains. The applied PCR methodology does not distinguish between BoNT/B or BoNT/F genes from Group I and II strains, and Group identification was obtained only when isolation of *C. botulinum* was successful. Indeed, AFLP typing showed all the five *bont/B*-positive isolates to belong to *C. botulinum* Group I. It remains unclear if the *bont/B*-positive and *bont/F*-positive samples that did not yield *C. botulinum* isolates were due to Group I or Group II. However, the similar prevalence and counts of *bont/A* and *bont/E* findings demonstrate that both Group I and Group II strains are prevalent in these products, and the applied heat treatments did not eliminate any of the two Groups. How the vegetarian sausages support the growth of Group I and II *C. botulinum* needs to be established.

Since the applied PCR assay (Lindström et al., 2001) fails to detect the genes for BoNT subtypes A2, A3 and A4 (De Medici et al., 2009), we may have underestimated the prevalence of *C. botulinum* type A. The only

type F-positive sample was also positive for type B, either due to the presence of two distinct strains each representing one of the two toxinotypes, or caused by a single bivalent type BF strain (Gimenez and Gimenez, 1993; Barash and Arnon, 2004). Another dual-positive sample yielded PCR signals for toxin types B and E. No bivalent type BE strains have been described in literature, thus this result is likely caused by the presence of two strains. Unfortunately, these two samples did not yield positive isolates for further analysis.

While *C. botulinum* Group I strains are of food safety concern due to the high heat resistance of their spores, their growth and toxin production can be controlled by refrigeration. However, Group II strains, whose spores are of moderate heat resistance, are of concern due to their growth and toxinogenic potential at low temperature (Graham et al., 1997; Derman et al., 2011). The time to toxinogenesis varies greatly by multiple factors, including the number of spores present, efficiency of the heat treatment applied, storage temperature, and intrinsic factors like water activity and pH (Segner et al., 1966; Hauschild and Hilsheimer, 1979; Meng and Genigeorgis, 1993; Peck et al., 1995). In previous inoculation studies, puréed mushrooms supported visible growth and toxin production in 20 days at 5°C (Carlin and Peck, 1996). At 3°C, growth and toxinogenesis were observed in a laboratory medium within 35 days (Graham et al., 1997). In the scenario of product shelf-life of 6 months (180 days) or longer, maintaining the storage temperature of vegetarian sausages strictly below 2.5°C (Graham et al., 1997) throughout the entire product lifespan appears imperative. Inoculation studies are needed to establish the growth potential from *C. botulinum* Group II spores in vegetarian sausages during storage at the temperatures of 6–8°C commonly applied for chilled foods. There is no information available on the growth and toxinogenic potential of *C. botulinum* Group II at 0–3°C for extended time periods of 6–9 months. This information appears pivotal for estimating the safety of extremely long shelf-lives for chilled products with obvious risk of *C. botulinum* growth.

To control the safety of vacuum-packaged chilled foods with shelf-lives over 10 days, it is advised that the products are given a 6D heat treatment, a process that will reduce the risk of *C. botulinum* Group II spores by a factor of 10^6 (ACMSF, 1992; ECFF, 2006). A temperature-time combination of 90°C 10 min has been proposed to provide a 6-log reduction (ACMSF, 1992; ECFF, 2006). Detection of 10^2–10^3 *C. botulinum* Group II (type E) cells or spores/kg in the vacuum-packaged vegetarian sausages suggests that the pasteurization processes used to prepare these products are substantially less efficient than a 6D kill. Moreover, matrices containing lysozyme or other lytic enzymes might assist sublethally injured spores to germinate (Peck et al., 1993), thus heat treatments exceeding 90°C 10 min might be required to achieve a 6D kill (Peck and Fernandez, 1995). Vegetarian sausages may contain hen egg white, which is a rich source of lysozyme (Lund and Peck, 1994). Also plant-based lysozyme activity has been measured in wheat and maize (Lund and Peck, 1994). Whether these lytic activities challenge the heat treatments used in the production of vegetarian sausages, needs to be established. It is recommended that additional factors like chilled storage below 3°C, water activity below 0.97, corresponding to water-phase NaCl concentration of 5% or higher, or pH below 5.0 be applied to control spore germination and outgrowth (ACMSF, 1992). Since the cold chain cannot be controlled in the consumer domain, and the product pH is usually above 5.0 and the water-phase NaCl concentrations are mainly below 4%, the use of preservatives appears as an important means to control product safety.

Nitrite is a well-established antibotulinal agent used to cure meats (Kim and Foegeding, 1992, Keto-Timonen et al., 2012). However, its use is restricted in most non-meat products. While some leafy vegetables like lettuce and spinach and some root vegetables can contain significant amounts of nitrates that are reduced to nitrite during storage, soybean and soy products contain only 0.1 mg/kg nitrite (Kalaycıoğlu and Erim, 2019). Thus natural nitrite levels in vegetarian sausages are supposed to be low (Kalaycıoğlu and Erim, 2019).

Salts of organic acids have potential in controlling *C. botulinum* Group I and/or II growth. Potassium sorbate, sodium lactate, and sodium acetate at concentrations of 2–6% have growth-inhibitory potential (Seward et al., 1982; Kim and Foegeding, 1992; Miller et al., 1993; Meng and Genigeorgis, 1994) and are used in some vegetarian sausage products on the market. With the maximum concentration of 1000 mg/kg permitted in foods, sorbic acid also controlled *C. botulinum* Group I growth at pH below 5.5 (Lund et al. 1987). The effects of preservatives targeted to tackle with the product pH need to be properly established for soy-based products: in the presence of soy, *C. botulinum* Group I has been shown to grow and produce toxin at a pH as low as 4.1 (Young-Perkins and Merson, 1987) as opposed to the generally referred growth-inhibitory pH of 4.6 (Hauschild et al., 1975; Peck, 2006).

If measures fail to control *C. botulinum* growth and toxin production, BoNT can be destroyed by heating at 85°C for 5 minutes or at 80°C for 20 minutes (Woodburn et al., 1979). Vegetarian sausages are likely heated prior to consumption; however, not all products contain instructions for cooking. Moreover, some products are advised for consumption as cold, and light heating regimes might not destroy all preformed BoNT. Thus the safety of vegetarian sausage products must rely on multiple hurdles controlling growth and toxin production, and never merely on toxin inactivation during cooking.

In conclusion, a high prevalence of *C. botulinum* in vegetarian sausages suggests that these products could be a potential source of botulism. Mild heat treatments enable survival of *C. botulinum* Group I and Group II spores, and long shelf-lives may support spore germination, outgrowth and toxinogenesis. Inoculated pack studies and shelf-life tests are required to determine the growth and toxic potential of *C. botulinum* in vegetarian sausages and the length of safe shelf-lives.

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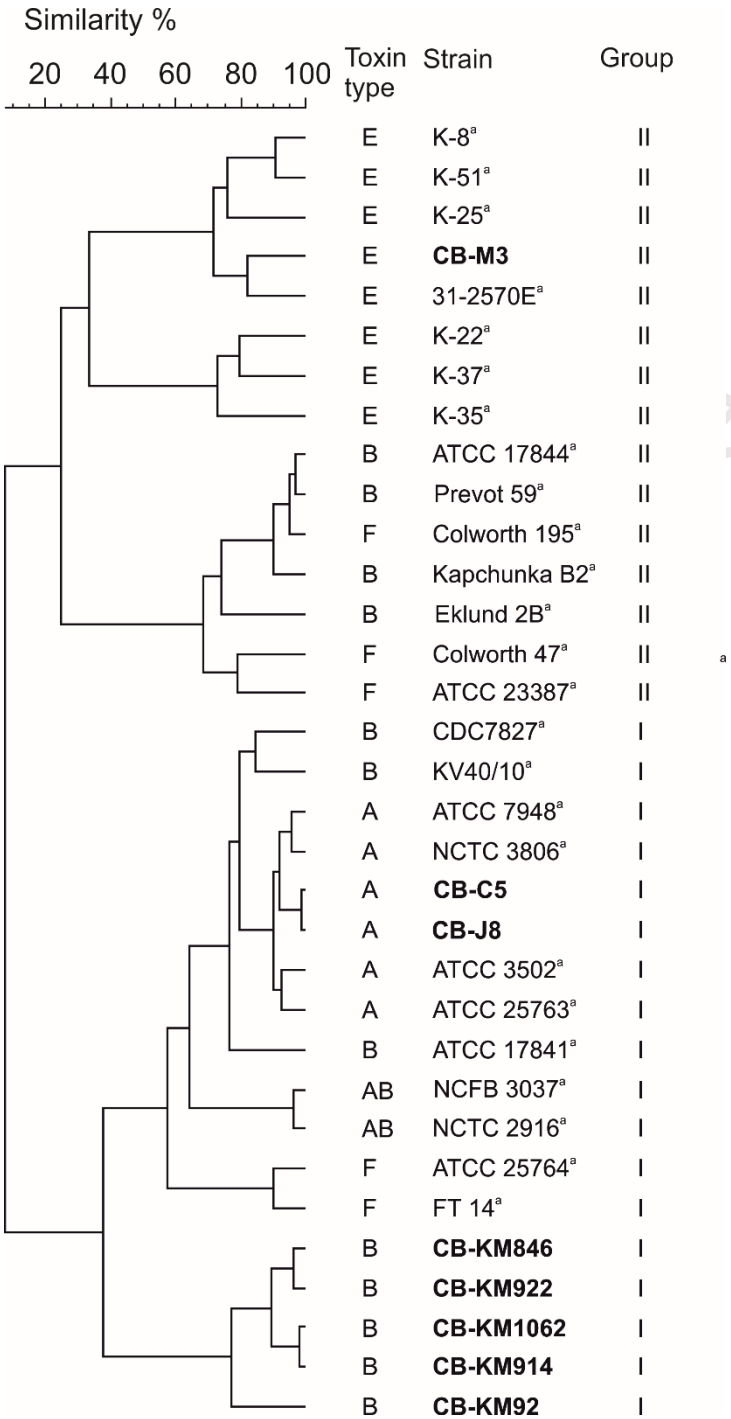

Similarity %
20 40 60 80 100
Toxin type
Strain
Group
E K-8[a] II
E K-51[a] II
E K-25[a] II
E CB-M3 II
E 31-2570E[a] II
E K-22[a] II
E K-37[a] II
E K-35[a] II
B ATCC 17844[a] II
B Prevot 59[a] II
F Colworth 195[a] II
B Kapchunka B2[a] II
B Eklund 2B[a] II
F Colworth 47[a] II
F ATCC 23387[a] II
B CDC7827[a] I
B KV40/10[a] I
A ATCC 7948[a] I
A NCTC 3806[a] I
A CB-C5 I
A CB-J8 I
A ATCC 3502[a] I
A ATCC 25763[a] I
B ATCC 17841[a] I
AB NCFB 3037[a] I
AB NCTC 2916[a] I
F ATCC 25764[a] I
F FT 14[a] I
B CB-KM846 I
B CB-KM922 I
B CB-KM1062 I
B CB-KM914 I
B CB-KM92 I

459 FIGURE LEGEND

460 Fig.1. Dendrogram of eight *Clostridium botulinum* isolates originating from vegetarian sausages and 25 *C.*
461 *botulinum* strains included in Keto-Timonen et al. (2006) based on AFLP analysis. A similarity analysis was
462 performed using the Pearson product-moment correlation coefficient, and clustering was performed by
463 using the unweighted pair-group method with arithmetic averages. Isolates originating from vegetarian
464 sausages are written in bold font. [a]Previously published by Keto-Timonen et al. (2006).

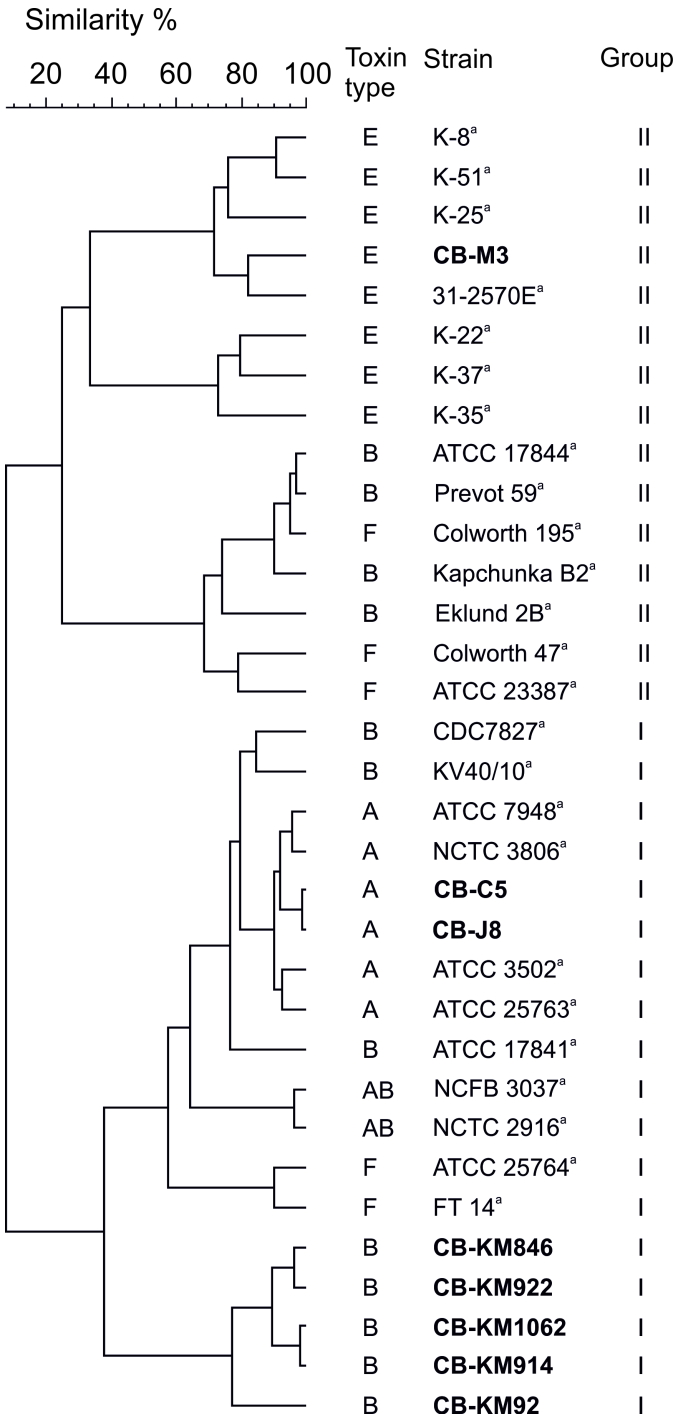
465 Table 1. The prevalence of *Clostridium botulinum* and BoNT genes in vegetarian sausages.

Product type	No. of samples examined	No. of positive samples (%)	MPN estimate of *C. botulinum* cell count (cells/kg)	No. of samples positive for one or two BoNT genes (% of positive samples)					
				Type A	Type B	Type E	Type F	Types B and E[a]	Types B and F[a]
Vacuum-packaged	66	23 (35%)	20–1200	8 (35%)	7 (30%)	6 (26%)	ND	1 (4%)	1 (4%)
Frozen	8	1 (13%)	110	ND	1 (100%)	ND	ND	ND	ND
Total	74	24 (32%)	20–1200	8 (33%)	8 (33%)	6 (25%)	ND	1 (4%)	1 (4%)

466 [a]Both types detected in the same sample.

467 ND, not detected.

468


Similarity %
20 40 60 80 100
Toxin type
Strain
Group
E K-8[a] II
E K-51[a] II
E K-25[a] II
E CB-M3 II
E 31-2570E[a] II
E K-22[a] II
E K-37[a] II
E K-35[a] II
B ATCC 17844[a] II
B Prevot 59[a] II
F Colworth 195[a] II
B Kapchunka B2[a] II
B Eklund 2B[a] II
F Colworth 47[a] II
F ATCC 23387[a] II
B CDC7827[a] I
B KV40/10[a] I
A ATCC 7948[a] I
A NCTC 3806[a] I
A CB-C5 I
A CB-J8 I
A ATCC 3502[a] I
A ATCC 25763[a] I
B ATCC 17841[a] I
AB NCFB 3037[a] I
AB NCTC 2916[a] I
F ATCC 25764[a] I
F FT 14[a] I
B CB-KM846 I
B CB-KM922 I
B CB-KM1062 I
B CB-KM914 I
B CB-KM92 I

- High prevalence of *Clostridium botulinum* found in vegetarian sausage products
- *C. botulinum* Groups I and II, and genes for neurotoxins A, B, E, and F were found
- *C. botulinum* Group II is the main food safety concern in chilled packaged foods

Declarations of interest: none.