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DIAGNOSTICS OF *CLOSTRIDIUM BOTULINUM* AND THERMAL CONTROL OF NONPROTEOLYTIC *C. BOTULINUM* IN REFRIGERATED PROCESSED FOODS

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ACADEMIC DISSERTATION

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Cover illustration: Heat-resistant spores surviving moist heat treatment in a Finnish sauna. Miia Lindström, acrylics and pencil, 2003.

Marja-Liisalle

Ei unelmointi riitä,
*   uni suuri tarvitaan.*
Ei etäinen maali riitä,
*   tie maaliin tarvitaan.*
Tien löytäminen ei riitä,
*   on mentävä kulkemaan.*
Yksinkin edeltä, myös ensimmäisenä.
Eikä tahtominen riitä,
*   tehtävä, tehtävä on.*

Mihaly Vacy

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ABBREVIATIONS

a_w, water activity

BoNT, botulinum neurotoxin

CFU, colony-forming unit

D_T**-value**, decimal reduction time (min), i.e. the time required to eliminate a bacterial population by 90% at temperature T

dNTP, deoxynucleotide triphosphate

ELCA, enzyme-linked coagulation assay

ELISA, enzyme-linked immunosorbent assay

EMG, electromyography

EYA, egg yolk agar

FMM, Food Micro Model

HA, haemagglutinin component

MA, modified atmosphere

MPN, most probable number

NSF, *N*-ethylmaleimide-sensitive fusion protein

NTNH, non-toxic non-haemagglutinin component

PCR, polymerase chain reaction

PFGE, pulsed-field gel electrophoresis

PHA, passive haemagglutination assay

PMP, Pathogen Modelling Program

RAPD, randomly amplified polymorphic DNA

REPFED, refrigerated processed food of extended durability

RH, relative humidity (%)

RT-PCR, reverse transcription PCR

RIA, radioimmunoassay

SNAP-25, synaptosomal associated protein-25

SNARE, soluble NSF-attachment protein receptors

TDT, thermal death time curve

TPGY, tryptone-peptone-glucose-yeast extract broth

VAMP, vesicle associated membrane protein

ABSTRACT

Evaluation of the applicability of three commercially available biochemical test systems (API 20 A, Rapid ID 32 A, and RapID ANA II) in the identification of *Clostridium botulinum* revealed that none of the tests could identify both group I (nonproteolytic) and group II (proteolytic) *Clostridium botulinum*. Neither were they capable of distinguishing between *C. botulinum* group I and II from their nontoxigenic counterparts. These test systems are therefore not suitable for the identification of *C. botulinum*.

A multiplex PCR assay was developed for the simultaneous detection of *Clostridium botulinum* types A, B, E, and F in food and faecal material. The method was specific for *C. botulinum*, and was 10-fold more sensitive to *C. botulinum* type B than to the other serotypes. Following two-step enrichment the assay was very sensitive, its detection limit in food and faecal samples being 10^{-1}-10^{-2} spore/g. Five out of 72 (7%) naturally contaminated food samples were positive for *C. botulinum* types A, B, or E. The multiplex PCR assay markedly improves the diagnostics of *C. botulinum*.

The heat resistance of nonproteolytic *C. botulinum* type E spores was greater in rainbow trout medium than in whitefish medium. When the spores were heated in the presence of lysozyme, biphasic thermal destruction curves were observed in both fish media, indicating that 0.1% of the spore population was more heat resistant than the rest of the spores. The decimal reduction times (D-values) of the heat-resistant spore fraction were observed to be greater than those previously reported for type E spores in fish media.

Safety evaluation of thermal processes employed in the Finnish fish and sous vide food industry showed that a number of vacuum-packaged hot-smoked fish products and sous vide products are grossly under-processed with respect to the elimination of nonproteolytic *C. botulinum* spores. As the storage temperatures at the retail and consumer level frequently exceed 3ºC, a great botulism hazard is associated with these products. Therefore, heat treatments which controlled the growth and toxin formation from $10^{5.3}$-10^6 spores of nonproteolytic *C. botulinum* in vacuum-packaged hot-smoked fish products and in sous vide processed meat products stored at 4-8ºC were identified. Moist heat treatments at 85ºC for 34 min and 42 min combined with a high relative humidity of >70% controlled *C. botulinum* type E in vacuum-packaged smoked rainbow trout and whitefish stored at 8ºC for 35 d. With sous vide foods, heating at 85ºC for 515 and 67 min controlled the growth and toxigenesis of nonproteolytic type B in pork cubes stored at 8ºC for 14 d and in ground beef stored at 4ºC for 28 d, respectively. Heating at 85ºC for 15 min or less resulted in toxin formation in smoked fish and sous vide products stored at 8ºC.

The sensory quality of all sous vide foods remained acceptable during the entire storage period at 8ºC. The intensified heat processes shown to control nonproteolytic *C. botulinum* enhanced the sensory attributes of sous vide meat products and did not markedly affect those of hot-smoked whitefish, but they slightly decreased those of hot-smoked rainbow trout. In order to control the risk presented by nonproteolytic *C. botulinum* in refrigerated processed foods of extended durability, the use of the intensified processes described in the present work in combination with proper refrigeration is strongly recommended for improving the safety of these food products.

LIST OF ORIGINAL PUBLICATIONS

The present thesis is based on the following original articles referred to in the text by the Roman numerals I to V:

I   Lindström, M., Jankola, H., Hielm, S., Hyytiä, E. and Korkeala, H. 1999. Identification of *Clostridium botulinum* with API 20 A, Rapid ID 32 A and RapID ANA II. FEMS Immunol. Med. Microbiol. 24, 267-274.

II   Lindström, M., Keto, R., Markkula, A., Nevas, M., Hielm, S., and Korkeala, H. 2001. Multiplex PCR assay for detection and identification of *Clostridium botulinum* types A, B, E, and F in food and fecal material. 2001. Appl. Environ. Microbiol. 67, 5694-5699.

III   Lindström, M., Nevas, M., Hielm, S., Lähteenmäki, L., Peck, M.W., and Korkeala, H. Thermal inactivation of nonproteolytic *Clostridium botulinum* type E in model fish media and in vacuum-packaged hot-smoked vacuum-packaged fish products. Appl. Environ. Microbiol. In press.

IV   Hyytiä, E., Skyttä, E., Mokkila, M., Kinnunen, A., Lindström, M., Ahvenainen, R. and Korkeala, H. 2000. Safety evaluation of sous vide processed products with respect to nonproteolytic *Clostridium botulinum* using challenge studies and predictive microbiological models. Appl. Environ. Microbiol. 66, 223-229.

V   Lindström, M., Mokkila, M., Hyytiä, E., Lähteenmäki, L., Hielm, S., Ahvenainen, R. and Korkeala, H. 2001. Inhibition of growth of nonproteolytic *Clostridium botulinum* type B in sous vide cooked meat products is achieved by using thermal processing but not nisin. J. Food Protect. 64, 838-844.

1. INTRODUCTION

Consumers' demands for fresh-like high-nutrition foods and easy cooking – heat up and eat – has generated an entire branch of the food industry over the last decades. New packaging technologies ensuring extended shelf lives in combination with minimal heat processing and a limited use of preservatives are a prerequisite in today's food processing. Unfortunately the revolutionary processing methodologies do not only promise convenience and health, but pose serious hazards due to dangerous micro-organisms, the most important of these being the spore-forming *Clostridium botulinum*.

Since the first reported incidents of human botulism in the early 19th century (Kerner, 1820), *Clostridium botulinum* has frightened food processors in the canning industry, including the numerous innocent home-canners with their tiny little leaking jars, in the smoked fish industry, and among various ethnic cultures with their original food preparation habits. As soon as the major hazards related to the canning industry were overcome by introducing the 12-D concept (botulinum cook), a heat process reducing the probability of growth from a single spore by a factor of 10^{12}, concerns over the less heat-resistant, but psychrotrophic, strains of nonproteolytic *C. botulinum* were raised. In the 1960's in the USA, large type E human botulism outbreaks due to vacuum-packaged hot-smoked fish led to extensive research efforts concerning the prevalence, growth, and toxin formation of nonproteolytic *C. botulinum* in fish – this work is the base for today's scientific activities around botulism.

The recognition of the great hazard presented by nonproteolytic *C. botulinum* in refrigerated processed foods of extended durability (REPFED) produced in Europe, led to recommendations by the Advisory Committee on the Microbiological Safety of Foods (ACMSF) (1992) and by the European Chilled Food Federation (ECFF) (1996) for safe processing and manufacturing of these foods. Analogously to the 12-D process introduced in the canning industry, a 6-D process was proposed. Based on large *in vitro* test series, several time temperature combinations ensuring a 10^6 reduction in nonproteolytic spore numbers were proposed, but they have since been shown to be inadequate (Fernández and Peck, 1999). Therefore, the importance of subjecting all new products to challenge testing by inoculated pack studies or predictive modelling is emphasized.

In view of the severity of botulism and the great hazard it poses to the food industry, the diagnostics of *C. botulinum* and its toxin is still poorly developed (Robinson and Nahata, 2003). Though rapid methods for the detection and identification of the organism in food laboratories would facilitate the identification of risk products, the diagnostics of *C. botulinum* is still mainly based on toxigenicity detection by the mouse bioassay. While being the only standard method for toxin detection available (Nordic Committee on Food Analysis, 1991a), the assay, apart from being expensive and time-consuming, is a source of great ethical concern. In addition to conventional culturing with toxicity testing, molecular detection methods such as PCR have been developed. However, these protocols are only able to detect a single serotype of *C. botulinum* at a time, and their use in extensive screening surveys for the presence of *C. botulinum* spores in foods and in the environment is laborious. Therefore there is a great demand for more sophisticated rapid methods for the diagnostics of *C. botulinum*.

2. REVIEW OF THE LITERATURE

2.1 *Clostridium botulinum* and human botulism

2.1.1 Classification of *Clostridium botulinum*

As all clostridia, *Clostridium botulinum* is an anaerobic Gram-positive rod-shaped bacterium that forms resistant spores (Cato *et al.*, 1986). The taxonomic denominator for *C. botulinum* is the production of botulinum neurotoxin (BoNT). Based on the serological properties of the toxin they produce, *C. botulinum* strains are divided into seven types A to G. Generally, *C. botulinum* strains of types A, B, E, and F are pathogenic to humans, whereas those of types C and D are animal pathogens. *C. botulinum* type G has not been associated with disease. Due to the great differences in the metabolic, phenotypic and genotypic properties between *C. botulinum* strains, the species is divided into four groups I to IV (Lee and Riemann, 1970b; Wu *et al.*, 1972; Johnson and Francis, 1975; Smith and Sugiyama, 1988). Group I includes the proteolytic strains of *C. botulinum* types A, B, and F, while group II consists of the nonproteolytic strains of *C. botulinum* types B, E, and F. Group III includes all *C. botulinum* type C and D strains. The earlier group IV *C. botulinum* includes type G toxin producing strains, but due to the distinct phenotypic and genotypic features of the group IV organisms (Giménez and Ciccarelli, 1970), a species name of *Clostridium argentinense* has been adopted (Suen *et al.*, 1988). In addition to *C. botulinum*, some strains of its close relatives *Clostridium butyricum* and *Clostridium baratii* are known to produce botulinum neurotoxin types E and F, respectively (Hatheway, 1993).

2.1.2 Phenotypical characteristics and the microbial ecology of *Clostridium botulinum*

C. botulinum is a Gram-positive, rod-shaped, anaerobic bacterium that forms heat-resistant spores. The phenotypic characteristics of *C. botulinum* strains vary greatly between groups I to III.

The organisms of group I are proteolytic and capable of utilizing amino acids as an energy source. These strains readily ferment glucose and fructose, but their use of other sugars is limited (Smith and Sugiyama, 1988). As their main metabolic end products the group I strains produce isobutyric, isovaleric, and beta-phenylpropionic acids (Smith and Sugiyama, 1988; Hatheway, 1993). The minimum growth temperature of group I strains is 10°C (Lynt *et al.*, 1982) with the optimum being 35 to 40°C (Smith and Sugiyama, 1988). Under otherwise optimal conditions, their growth is typically inhibited by a water activity (a_w) of 0.94, corresponding to approximately 10% of NaCl (w:v) in brine. Growth may occur at pHs as low as 4.3 (Smelt *et al.*, 1982) to 4.5. The spores of group I *C. botulinum* possess a very high heat resistance (Stumbo *et al.*, 1975). Their nontoxigenic counterpart, *Clostridium sporogenes*, is phenotypically similar and genotypically related to group I *C. botulinum* (Lee and Riemann, 1970a, b; Nakamura *et al.*, 1977).

The strains of group II *C. botulinum* are nonproteolytic and saccharolytic. Basically they do not metabolize amino acids but ferment a number of carbohydrates as their main energy source (Cato *et al.*, 1986). Their minimum growth temperature is 3.0°C (Schmidt *et al.*, 1961; Eklund *et al.*, 1967a, b; Graham *et al.*, 1997), with the optimum being generally between 26 and 30°C (Smith and Sugiyama, 1988; Hatheway, 1993). The inhibitory a_w for group II organisms is typically 0.97, corresponding to 5% of NaCl in brine. Growth may occur at pH 5 and above (Segner *et al.*, 1966). The spores of the nonproteolytic group II organisms are less heat-resistant than those of group I. Nontoxigenic type E-like counterparts of nonproteolytic *C. botulinum* have been identified, with some of these strains having been reported to inhibit the nonproteolytic *C. botulinum* by producing boticins (Kautter *et al.*, 1966; Lynt *et al.*, 1982). A high genetic relatedness between the toxigenic and nontoxigenic organisms has been observed (Lee and Riemann, 1970a).

Group III *C. botulinum* strains are mainly nonproteolytic and their main fermentation products include propionic and butyric acids (Smith and Sugiyama, 1988). These organisms grow generally at temperatures above 15°C, with the optimum being at approximately 40°C (Segner *et al.*, 1971). The growth of group III *C. botulinum* is inhibited by pH of 5.1 to 5.6 and a NaCl brine content of 2.5% (w:v). The group III organisms have an intermediate heat resistance as compared to those of groups I and II (Hatheway, 1993). A reversible conversion of group III *C. botulinum* to its nontoxic variant, *Clostridium novyi*, has been reported to occur as a consequence of bacteriophage transmission (Nakamura *et al.*, 1983).

2.1.3 Human botulism

With a few exceptions of type F botulism, the majority of human botulism cases worldwide are due to types A, B, and E toxins. All forms of human botulism develop as a consequence of BoNT entering the blood circulation and blocking neurotransmitter release in the peripheral nerve endings. Therefore, independent of the form of botulism the clinical manifestation of all forms of botulism is similar. This typically includes a descending flaccid paralysis with dysphagia, a dry mouth, double vision, difficulty in swallowing, dilated pupils, dizziness, and muscle weakness. These are accompanied by the paralysis of the more peripheral parts of the body, and finally by the respiratory muscle paralysis which may lead to death. In addition, non-specific symptoms related to different forms of botulism may precede the actual paralysis.

The classical foodborne botulism is an intoxication that follows when food containing botulinum neurotoxin is eaten. Therefore, the first indications of illness before the paralytic condition are typically gastrointestinal, mainly nausea, vomiting, and abdominal cramps. The typical incubation period is 18-72 h, tending to be the shorter when higher amounts of toxin are ingested. The treatment of foodborne botulism includes the administration of a therapeutic trivalent antitoxin and intensive symptomatic treatment, particularly respiratory support (Robinson and Nahata, 2003). As the regeneration of new nerve-endings is a prerequisite for recovery, the treatment period is typically weeks to months. The most common differential diagnoses include Guillain-Barré syndrome, chemical intoxication, stroke, or staphylococcal food poisoning (Centers for Disease Control, 1979; Hughes *et al.*, 1981). The estimated case-

fatality rate of foodborne botulism outbreaks worldwide is 20% (Hatheway, 1995). During the last decades, a worldwide average of 450 outbreaks of foodborne botulism with 930 cases has been reported annually (Hatheway, 1995). More than half of the cases (52%) were due to BoNT type B, whereas 34% and 12% were due to types A and E, respectively. On rare occasions type F toxin has been associated with human botulism (Harvey *et al.*, 2002).

The majority (72%) of botulism outbreaks have occurred in Poland; other countries with a high incidence include China, the former Soviet Union, Germany, Italy, the United States, France, and Yugoslavia. The geographical distribution of botulism due to different toxin types follows the distribution of respective spore types found in the environment (Hauschild, 1989). Group I *C. botulinum* prevails in the temperate areas including southern Europe, the United States, Central and South America, China and Southern Asia with the majority of outbreaks being associated with vegetables. Group II predominates in the colder regions of the northern hemisphere including northern Europe and Alaska, with meat being the main source of type B botulism and fish and marine mammals being the main source of type E botulism (Hauschild, 1993). Home-prepared foods (Roblot *et al.*, 1994; Vukovic, 2000) as well as commercial products (Anonymous, 1964, 1991, 1998; Townes *et al.*, 1996; Korkeala *et al.*, 1998) have been reported to serve as vehicles for human botulism. The mishandling of food products which might cause human botulism frequently occurs in homes (Genigeorgis, 1986).

Unlike the classical foodborne botulism, the other forms of human botulism are originally infections where the toxigenesis occurs *in vivo*. Infectious botulism is thus mainly considered to be caused by strains of group I *C. botulinum* that have an optimum growth temperature close to the body temperature of 37°C, whereas the growth of group II organisms at the same temperatures is limited (Smith and Sugiyama, 1988). As for infant botulism (Pickett *et al.*, 1976; Midura and Arnon, 1976), however, in addition to *C. botulinum* types A, B (Hatheway *et al.*, 1981; Hatheway and McCroskey, 1987), and F (Hoffman *et al.*, 1982), types E and F botulism cases due to toxigenic *Clostridium butyricum* (Aureli *et al.*, 1986; McCroskey *et al.*, 1986; Hatheway and McCroskey, 1987) and *Clostridium baratii* (Hall *et al.*, 1985), respectively, have been reported. Infant botulism affects small children under 1 year of age, and the condition typically develops as a consequence of ingesting spores of BoNT-producing clostridia (Arnon, 1986). As the intestinal microflora of small babies is poorly developed, *C. botulinum* spores may germinate and form a vegetative culture in the intestine with subsequent toxin production. Infant botulism typically starts with constipation that may last for several days, followed by the distinctive flaccid paralysis that is manifested by impaired feeding due to difficulties in sucking and swallowing, facial muscle paralysis, ptosis, and general weakness (Arnon, 1989). Infant botulism has been suggested to be a causative agent of sudden infant death syndrome, and is occasionally misdiagnosed as cot death (Nevas *et al.*, 2002b, c). The treatment concentrates on high quality supportive care with special attention to the patient's nutrition and respiratory functions (Arnon *et al.*, 1977; Johnson *et al.*, 1979). The use of antitoxin is usually not required (Arnon *et al.*, 1979), and the case-fatality rate is less than 2% (Centers for Disease Control and Prevention, 1998). The only foodstuff that has been associated with infant botulism is honey (Aureli *et al.*, 2002) that carries high numbers of *C. botulinum* spores (Arnon, 1992; Dodds, 1993; Nevas, 2002a). Dust

and other materials in the environment seem to be important sources of spores (Arnon, 1992; Dodds, 1993).

Wound botulism is a rare form of botulism, although it is increasingly found among injecting drug abusers who use contaminated needles or impure heroin (Passaro *et al*., 1998; Athwal *et al*., 2000, 2001; Werner *et al*., 2000; Mulleague *et al*., 2002). Wound botulism develops when *C. botulinum* spores germinate and grow in profound wounds or abscesses that provide *C. botulinum* with anaerobic conditions. The clinical picture is similar to foodborne botulism with the absence of the gastrointestinal signs. The median incubation period is 7 d. Apart from respiratory support, the treatment of wound botulism includes surgical debridement, antibiotics, and the administration of antitoxin. The estimated case-fatality rate is 15% (Hatheway, 1995).

The adult form of infectious botulism is rare and resembles infant botulism in its pathogenesis and clinical status, as a result of the colonization of the intestinal tract by BoNT-producing clostridia (Chia *et al*., 1986; McCroskey and Hatheway, 1988; McCroskey *et al*., 1991; Fenicia *et al*., 1999). People with altered intestinal flora due to for example abdominal surgery (Isacsohn *et al*., 1985; Freedman *et al*., 1986), prolonged antimicrobial treatment or gastrointestinal wounds and abscesses are usually affected (Chia *et al*., 1986). Since a patient history of the ingestion of toxic foods has typically not been found (McCroskey and Hatheway, 1988), the diagnosis of classical foodborne intoxication may be excluded.

Inhalation botulism may result from aerosolization of BoNT, accidentally or intentionally when attempting to weaponize it. A few human cases have been reported (Holzer, 1962). Iatrogenic botulism with local or generalized weakness is rare and has been reported to develop as a consequence of therapeutic injection of BoNT (Mezaki *et al*., 1996; Bakheit *et al*., 1997).

2.2 Botulinum neurotoxin (BoNT)

2.2.1 Structure

C. botulinum strains produce seven immunologically distinct BoNTs, types A to G. The BoNTs are synthesized as single-chain polypeptides of approximately 150 kDa. These polypeptides are nicked by proteases to yield an active dichain form, with the resulting heavy chain (100 kDa) and light chain (50 kDa) being linked to each other by a single disulphide bond (DasGupta and Sugiyama, 1972; Yokosawa *et al*., 1986; Oguma *et al*., 1995). Generally, the proteolytic organisms belonging to group I produce the proteases required to yield the dichain toxin form, whereas the nonproteolytic strains belonging to group II require external proteolytic activity, e.g. by trypsin in the gastrointestinal tract. The BoNTs are metalloendopeptidases containing a zinc atom associated with the light subunit (Schiavo *et al*., 1992), which possesses protease activity (Oguma *et al*., 1997). In culture fluids and foods, the toxin molecules appear as progenitor toxins, larger complexes with the single-chain polypeptide being accompanied by nontoxic components of various molecular masses (Kitamura *et al*., 1968; Sugii and Sakaguchi, 1975). These include the nontoxic-non-haemagglutinin component (NTNH) and haemagglutinin components (HA) of various amino

acid composition and molecular mass (Oguma *et al*., 1997). Depending on the *C. botulinum* type, various combinations of progenitor toxins are produced.

2.2.2 *BoNT* gene cluster

The gene cluster regulating the production of BoNT by groups I and II *C. botulinum* is located in the bacterial chromosome, whereas in group III *C. botulinum* toxigenesis is mediated by a bacteriophage (Nakamura *et al*., 1983) and in group IV by a plasmid (Zhou *et al*., 1995). The complete nucleotide sequences of the seven distinct *BoNT* genes (*BoNT/A* to *BoNT/G*) have been published (Binz *et al*., 1990; Thompson *et al*., 1990; East *et al*., 1992; Poulet *et al*., 1992; Whelan *et al*., 1992a; Whelan *et al*., 1992b; Elmore *et al*., 1995). The amino acid sequences of different BoNTs have regions of high similarity, particularly those associated with the metalloprotease activity and the disulphide bonding between the light and heavy subunits of the dichain toxin molecule. The BoNTs of the same serotype within a physiological group are identical (Henderson *et al*., 1997). The complete nucleotide sequences of the genes regulating the nontoxic components have been published (Minton, 1995; Oguma *et al*., 1997; Oguma *et al*., 1999). These genes form a cluster with the *BoNT* gene and are located immediately upstream of the *BoNT* gene (Somers and DasGupta, 1991; Hauser *et al*., 1994; Henderson *et al*., 1996; Henderson *et al*., 1997). The amino acid sequences of the NTNH and various HA components are highly conserved and show greater overall similarity between different serotypes than the neurotoxin sequences (Henderson *et al*., 1997). The gene expression at the *BoNT* gene cluster is a consequence of complex regulatory cascades. The factors affecting the regulatory process are still not well understood, but at least include exogenous nitrogen levels (Bowers and Williams, 1963; Patterson-Curtis *et al*., 1989; Malizio *et al*.,1993) that play a communicative role in bacterial signalling (Parkinson and Kofoid, 1992).

2.2.3 Mode of action

BoNT is the most potent naturally occurring toxin to man (Lamanna, 1959). Generally, BoNT blocks neurotransmitter release in the peripheral neuromuscular junctions and causes a descending paralysis that may lead to death as the respiratory musculature fails.

The role of the nontoxic components of progenitor toxin is to protect the neurotoxin from the acidity and proteases of environmental factors, such as foods and the stomach (Oguma *et al*., 1995). Therefore, the larger is the progenitor toxin complex, the more potent is the toxin. In the small intestine, the HA component is involved with the adhesion of the progenitor toxin to the intestinal epithelium, leading to efficient absorption of the toxin (Oguma *et al*., 1995; Fujinaga *et al*., 1997, 2000). Unsialylated oligosaccharides on the surface of the small intestine have been suggested to be the receptors for type A progenitor toxin, but not for the toxin itself (Inoue *et al*., 2001). The non-acidic conditions in the small intestine cause the toxin molecule to dissociate from the NTNH-HA complex and the nontoxic components are absorbed into the lymphatic system (Sugii *et al*., 1977). The single-chain toxin molecule dissociates into the active dichain form as a result of the action of

proteolytic enzymes. The mechanism by which the toxin enters the lymphatic vessels is still unclear.

The mechanism by which BoNT affects a nerve cell consists of four steps: cell binding, internalisation, membrane translocation, and target modification in the cytosol (Montecucco and Schiavo, 1994). The binding of BoNT to the presynaptic membrane is mediated by the C-terminal of the heavy chain through type-specific receptors with a high affinity (Nishiki *et al.*, 1996). After binding, the BoNT is internalised in membrane vesicles into the nerve cell through an energy-dependent process (Black and Dolly, 1986). After this, the toxin can no longer be inactivated by a specific antitoxin. Inside the cell, the light chain of the BoNT molecule is transferred to the cytosol by membrane translocation. The light chain acts through its zinc-endopeptidase activity (Montecucco and Schiavo, 1993) and specifically cleaves the SNARE complex proteins (soluble NSF-attachment protein receptors [NSF, *N*-ethylmaleimide-sensitive fusion protein]), such as vesicle-associated membrane protein (VAMP)/synaprobrevin (Schiavo *et al.*, 1992), synaptosomal protein (SNAP-25) (Blasi *et al.*, 1993; Schiavo *et al.*, 1993), and syntaxin (Schiavo *et al.*, 1995) that are involved with neurotransmitter release from synaptic vesicles. This is seen as a blocked neuronal impulse and the paralysis of the muscle.

2.3 Diagnostics of botulism

The diagnosis of botulism is primarily based on the history of eating suspected foods as well as detecting BoNT in patients and in suspected food samples (Kautter and Solomon, 1977; Centers for Disease Control and Prevention, 1998; Nordic Committee on Food Analysis, 1991a). The detection of *C. botulinum* cells in clinical and food specimens strongly supports the diagnosis (Nordic Committee on Food Analysis, 1991b). Electromyography (EMG) may be used to distinguish botulism from similar neurological diseases (Centers for Disease Control and Prevention, 1998).

The complexity of the diagnostics is due to the fact that sensitive and specific *in vitro* methods for the detection of BoNTs have not been validated, and the only standard method is the mouse bioassay, which leads to ethical concern due to the use of laboratory animals. The culture method is complicated by the fact that no growth media selective for both proteolytic and nonproteolytic *C. botulinum* are available. Moreover, the presence of nontoxigenic strains, closely resembling *C. botulinum*, in foods and environmental samples greatly complicates the conventional diagnostics of the organism (Broda *et al.*, 1998).

2.3.1 Botulinum neurotoxin

Mouse assay

The mouse assay is the only standard method for the detection of BoNTs. Apart from toxin detection in clinical and food samples, the assay may be used to show toxigenesis in cultures providing the identification of *C. botulinum* strains (Kautter and Solomon, 1977). The toxin in a sample is eluted in a phosphate buffer and injected intraperitoneally into two mice. Trypsin

activation of the eluate is generally required when strains from group II *C. botulinum* are concerned (Duff *et al.*, 1956), as those strains lack the required proteolytic activity. If the sample is toxic, the mice show typical signs of botulism, including fuzzy hair, muscle weakness, and respiratory failure that is indicated by a wasp-like narrowed waist, usually within four days post-injection. Due to differences in the potencies of BoNTs, the time it takes to obtain a positive test result varies with the toxin type, with types A and B being more potent that type E toxin. The toxin type is determined by seroneutralization of the toxin with specific antitoxins (Centers for Disease Control, 1987). Basically, mice injected with the neutralizing antitoxin survive while the others develop botulism. Although the method is very sensitive, with one intraperitoneal mouse lethal dose (MLD) corresponding to 10 pg/ml (Smith and Sugiyama, 1988), it is expensive and in terms of clinical use it may require too much time to make a diagnosis. Moreover, false-positive test results due to the presence of a high number (10^7) of *C. botulinum* spores (Mitamura *et al.*, 1982) or endotoxins from gram-negative bacteria have been reported (Solberg *et al.*, 1985).

Another type of mouse assay with a more humane end point of local muscle paralysis as a consequence of a subcutaneous injection of BoNT type A has been explored (Sesardic *et al.*, 1996). The non-lethal mouse assay is equal to the conventional bioassay as far as sensitivity and specificity are concerned, but it does not cause signs of distress or impaired movements in the animals (Sesardic *et al.*, 1996).

Immunological methods

A number of immunoassay formats have been reported for the detection of botulinal neurotoxins. The production of detection antibodies against the BoNTs is relatively easy and most of the immunoassays are technically simple and rapid to perform (Ekong, 2000). However, many of these assays, such as radioimmunoassay (RIA) (Ashton *et al.*, 1985), gel diffusion assay (Vermilyea *et al.*, 1968; Ferreira *et al.*, 1981), and passive haemagglutination assay (PHA) (Johnson *et al.*, 1966) have poor sensitivities or specificities, which decreases the diagnostic value of the methods. The most widely used assay format is enzyme-linked immunosorbent assay (ELISA) (Notermans *et al.*, 1978) with a variety of modifications (Doellgast *et al.*, 1993; Roman *et al.*, 1994). ELISA-based formats may reach sensitivities similar to the mouse bioassay (Dezfulian and Bartlett, 1984; Shone *et al.*, 1985; Ekong *et al.*, 1995; Ferreira *et al.*, 2003). ELISA procedures for the detection of BoNTs in clinical specimens (Poli *et al.*, 2002) as well as in foods have been reported (Shone *et al.*, 1985; Potter *et al.*, 1993; Rodriguez and Dezfulian, 1997; Ferreira *et al.*, 2001).

Endopeptidase assay

The fact that botulinum neurotoxin possesses a highly specific zinc-endopeptidase activity with selected targets in the synaptic vesicle/synaptic membrane docking system has inspired the development of an *in vitro* assay for its detection. The endopeptidase assay is based on specific cleavage of the SNARE proteins by BoNTs. Methods for the detection of BoNT types A (Ekong *et al.*, 1997; Hallis *et al.*, 1996), B (Hallis *et al.*, 1996; Wictome *et al.*, 1999a, b),

and E (Ekong *et al*., 1999) have been described. The rapid method provides a sensitivity similar to or even better than that of the mouse assay (Wictome *et al*., 1999b). No cross-reactivity between different toxin types has been reported. However, with the type B toxin assay, an increased likelihood of false-negative results has been reported due to the serotypical differences between BoNT type B produced by the nonproteolytic and proteolytic *C. botulinum* (Wictome *et al*., 1999b).

2.3.2 *Clostridium botulinum*

Culture methods

The conventional detection and isolation of *C. botulinum* is based on culturing and the subsequent detection of culture toxicity by the mouse assay (Kautter and Solomon, 1977). In suspect cases of human botulism, the samples are cultivated as such, as well as treated with ethanol in order to eliminate vegetative bacteria but not bacterial spores (Nordic Committee on Food Analysis, 1991b). Strict anaerobic techniques, including deoxygenation of culture media and anaerobic incubation, are required for the successful cultivation of *C. botulinum*. The routine media include chopped meat-glucose-starch (CMGS) medium (Centers for Disease Control and Prevention, 1998), cooked meat medium (Robertson, 1916; Quagliaro, 1977), tryptone-peptone-glucose-yeast extract (TPGY) broth, sometimes supplemented with trypsin (TPGYT) (Lilly *et al*., 1971), and reinforced clostridial medium (RCM) (Gibbs and Hirsch, 1956). Blood agar and egg yolk agar (EYA) (Hauschild and Hilsheimer, 1977) serve as the most common plating media, with EYA enabling the lipase reaction typical for *C. botulinum* and certain other clostridia. A few selective media have been developed for *C. botulinum* (Dezfulian *et al*., 1981; Mills *et al*., 1985; Silas *et al*., 1985). These media improve the isolation of some *C. botulinum* strains (Glasby and Hatheway, 1985) but, however, they do not allow the growth of all *C. botulinum* strains (Whitmer and Johnson, 1988). The identification of botulinum toxin in and around *C. botulinum* colonies grown on agar plates by immunoblotting and immunodiffusion procedures facilitates the identification of the organism, but it may lack specificity like other immunological techniques (Ferreira *et al*., 1981; Dezfulian and Batrlett, 1985; Goodnough *et al*., 1993). The isolation of *C. botulinum* from various sources is frequently complicated by the presence of nontoxigenic *C. botulinum*-like cultures (Lee and Riemann, 1970a; Broda *et al*., 1998).

Biochemical test systems

Rapid test systems based on various growth-dependent and non-growth-dependent biochemical reactions have been developed for the identification of anaerobic bacteria. Contradictory reports on the ability of the tests to identify *Clostridium* spp. have been published: various tests have been able to correctly identify from not more than 54% to as much as 96% of the clostridial strains studied to the species level (Gresser *et al*., 1984; Burlage and Ellner, 1985; Head and Ratnam, 1988; Marler *et al*., 1991). *C. botulinum* was reported to be correctly identified by the Rapid ID 32 A system to the genus level but not to

the species level (Brett, 1998). Factors such as the incubation environment (Peiffer and Cox, 1993), incubation time (Gresser *et al*., 1984), and the concentration of cell suspension (Brett, 1998) have been reported to drastically affect the success of identification, thus reducing the reliability of the tests.

Molecular detection methods

The molecular detection of *C. botulinum* typically involves the detection of the *BoNT* gene, indicating the presence of the organism in a sample. The molecular approaches include the sensitive and specific polymerase chain reaction (PCR) and the use of molecular probes (Campbell *et al*., 1993; Franciosa *et al*., 1994). In PCR, a gene locus determined by specific oligonucleotide primers is amplified by a thermotolerant polymerase enzyme. The amplification product is then visualized in agarose gels. A labelled molecular probe may be further hybridised to a homologous DNA sequence and visualized immunologically.

The reported sensitivities of PCR and gene probe assays for different *C. botulinum* types in various sample materials vary from 1-2.5 pg of DNA (Szabo *et al*., 1994; Takeshi *et al*., 1996) to 0.3 ng of DNA (Craven *et al*., 2002), $0.1 - 10^3$ cfu or spores/g of food (Fach *et al*., 1993; Fach *et al*., 1995; Sciacchitano and Hirshfield, 1996; Aranda *et al*., 1997; Braconnier *et al*., 2001; Córdoba *et al*., 2001), $10\text{-}10^3$ cfu/g of faeces (Dahlenborg *et al*., 2001, 2003), or $10\text{-}10^3$ cells or spores in environmental samples (Franciosa *et al*., 1996; Williamson *et al*., 1999). Nested PCR protocols involve several subsequent amplifications, thus increasing the assay sensitivity in e.g. faecal samples (Kakinuma *et al*., 1997; Dahlenborg *et al*., 2001). The disadvantage of PCR detection directly from a sample is the possible detection of dead cells due to intact DNA after cell lysis. This problem is overcome by combining enrichment procedures with the PCR protocol (Hielm *et al*., 1996). Alternatively, reverse transcription-PCR (RT-PCR) in which gene expression is detected rather than the gene itself, may be employed to distinguish viable and dead bacterial cells. A quantitative RT-PCR protocol for *C. botulinum* has been described (McGrath *et al*., 2000).

Molecular typing methods

Molecular typing methods enable the genomic analysis of bacterial strains, and they have been applied to study the genetic diversity of *C. botulinum* (Lin and Johnson, 1995; Hielm *et al*., 1998a; Hielm *et al*., 1998b; Hyytiä *et al*., 1999a) and in tracing the causative agents in botulism outbreaks (Korkeala *et al*., 1998; Austin, 2001). Pulsed-field gel electrophoresis (PFGE) has an excellent discriminatory power and reproducibility, while a PCR-based method, randomly amplified polymorphic DNA assay (RAPD) is less reproducible but can be quickly performed. The application of rRNA gene restriction pattern analysis (ribotyping) has been used to identify bacterial species yielding distinct patterns for group I and II *C. botulinum* (Hielm *et al*., 1999).

Quantification techniques

Conventional plating on anaerobic media and most probable number (MPN) technique combined with either visual or PCR detection of growth (Hielm *et al*., 1996), are commonly employed in order to quantify *C. botulinum* in a sample. Plating may be complicated by the presence of oxygen or NaCl in the plating medium (Montville, 1984), and to obtain an optimal quantification of *C. botulinum*, a heat-shock may be required (Montville, 1981). A more sophisticated approach is real-time PCR, based on the quantification of amplified DNA. The method has been applied for nonproteolytic *C. botulinum* type E to be monitored in fish (Kimura *et al*., 2001). Competitive RT-PCR is based on the rate of *BoNT* gene expression, and it has been applied in the quantification of nonproteolytic *C. botulinum* type E (McGrath *et al*. 2000).

2.4 Prevalence of nonproteolytic *Clostridium botulinum* in foods

Nonproteolytic *C. botulinum* is widely spread in the environment predominating in mild aquatic environments in the Northern hemisphere, including Northern Europe (Johannsen, 1962, 1963; Cann *et al*., 1965; Kravchenko and Shishulina, 1967; Huss *et al*., 1974; Ala-Huikku *et al*., 1977; Huss, 1980; Hielm *et al*., 1996; Hielm *et al*., 1998b, c), Alaska and Northern parts of the United States (Eklund and Poysky, 1965, 1967; Craig and Pilcher, 1967; Nickerson *et al*., 1967; Bott *et al*., 1968; Cockey and Tatro, 1974; Miller, 1975; Smith, 1975, 1978; Sayler *et al*., 1976), Canada (Laycock and Loring, 1972), Japan (Yamamoto *et al*., 1970; Yamakawa *et al*., 1988; Yamakawa and Nakamura, 1992), and Western Asia (Tanasugarn, 1979; Haq and Suhadi, 1981; Dhaked *et al*., 2002). The nonproteolytic *C. botulinum* types B and E are generally more prevalent in nature than type F. The prevalence and spore counts of type E in the environment seem to be somewhat higher in the Nordic countries (Johannsen, 1962, 1963; Cann *et al*., 1967; Cann *et al*., 1968; Huss *et al*., 1974; Ala-Huikku *et al*., 1977; Huss, 1980; Hielm *et al*., 1996; Hielm *et al*., 1998b; Hielm *et al*., 1998c) than in other European countries, in which type B seems to predominate (Zaleski *et al*., 1973; Haagsma, 1974; Burns and Williams, 1975; Smith and Moryson, 1975, 1977; Borland *et al*., 1977; Smith *et al*., 1977, 1978, 1987; Notermans *et al*., 1979; Smith and Milligan, 1979; Smith and Young, 1980; Sonnabend *et al*., 1987; Klarmann, 1989; Notermans *et al*., 1989; Ortiz and Smith, 1994). The Baltic Sea has been suggested to be one of the most highly contaminated areas in the world with respect to nonproteolytic *C. botulinum* type E (Hielm *et al*., 1998c).

2.4.1 Unprocessed foods

As a consequence of the high prevalence of nonproteolytic *C. botulinum* in the environment the spores may contaminate raw foods, particularly fish (Table 1). Only a few reports on the presence of nonproteolytic *C. botulinum* in raw meats (Klarmann, 1989) and vegetables (Johannsen, 1963; Hauschild *et al*., 1975) have been published (Table 1). Compared to environmental contamination, the prevalence and the average counts of nonproteolytic

C. botulinum in raw foods are generally lower (Table 1). In fish (Johannsen, 1963; Huss *et al*., 1974; Miller, 1975; Rouhbakhsh-Khaleghdoust, 1975; Hielm *et al*., 1998b; Hyytiä *et al*., 1998; Hyytiä-Trees *et al*., 1999), the prevalence and spore counts are higher than in meats (Klarmann, 1989), in which nonproteolytic *C. botulinum* seems to be a rather infrequent contaminant (Simunovic *et al*., 1985) despite the high reported prevalence of spores in faeces of pigs and cattle (Dahlenborg *et al*., 2001, 2003). The majority of the earlier reports on the prevalence of *C. botulinum* in the environment concern *C. botulinum* type E spores. Most of the previous studies concerning types B and F do not report the physiological group of *C. botulinum*. However, the low incubation temperature of 28-30ºC and trypsin activation required in the detection and isolation of *C. botulinum* in these studies suggest that these organisms belong to group II (Table 1).

2.4.2 Processed foods

A limited number of reports on the prevalence of nonproteolytic *C. botulinum* in processed foods have been published. In comparison with unprocessed foods (Table 1), the mean spore counts in the processed foods are as expected lower (Table 2). The prevalence in fish products has been most intensively studied (Cann *et al*., 1966; Pace *et al*., 1967a; Hayes *et al*., 1970; Rouhbakhsh-Khaleghdoust, 1975; Hyytiä *et al*., 1998). There are only a few reports on the prevalence of nonproteolytic *C. botulinum* type E and probably nonproteolytic types B and F in other products, such as vacuum-packaged meats and cheese (Insalata *et al*., 1969), packaged ready-to-eat foods (Taclindo *et al*., 1967), and smoked turkey products (Abrahamsson and Riemann, 1971) (Table 2). Though these papers do not report the physiological group of *C. botulinum* types B and F, the relatively low incubation temperature of 28-30ºC used suggests that these strains belong to group II (Table 2).

2.5 Thermal resistance of nonproteolytic *Clostridium botulinum* spores

Bacterial spores are generally much more heat-resistant than vegetative bacteria. The spores of nonproteolytic *C. botulinum* strains possess a moderate heat resistance as opposed to group I *C. botulinum*. Heating medium and physiological variations between bacterial strains affect the heat resistance of bacterial spores. A great variation in the D-values of nonproteolytic *C. botulinum* heated in various foods has been shown (Table 3). As for seafood, generally shorter D-values were measured in oyster homogenate (Bucknavage *et al*., 1990; Chai and Liang, 1992) than in cod homogenate (Gaze and Brown, 1990), crawfish (De Pantoja, 1986), and crabmeat (Lynt *et al*., 1977, 1983; Cockey and Tatro, 1974; Peterson *et al*., 1997). In crabmeat, the D-values measured by Peterson *et al*. (1997) were generally greater than those measured by other authors. This might be a methodological difference, but more probably it may be due to the presence of lysozyme or other lytic enzymes in the crabmeat.

When present in the recovery medium of heat-injured spores, lysozyme and other enzymes with similar activities have been reported to increase the apparent heat resistance of

Table 1. Prevalence of nonproteolytic *Clostridium botulinum* in raw foods.

Country	Sample type and size	Positive samples (%)	Mean spore count (spores/kg)[a]	Group II *C. botulinum* type	Reference
Canada	Mushrooms, 450 g	NR[b]	28[c]	B[d]	Hauschild *et al.*, 1975
Denmark	Fish, NR	65	2.0[c]	E	Huss *et al.*, 1974
Finland	Fish, 5 g	7.1	15	E	Ala-Huikku *et al.*, 1977
Finland	Fish, 33 g	19	180	E	Hyytiä *et al.*, 1998
Finland	Fish skin and intestines, 33 g	10	238	E	Hielm *et al.*, 1998b
Finland	Fish roe, 33 g	7.7	58[c]	E	Hyytiä *et al.*, 1998
Germany	Fish, intestines, gills, skin, 5 g	30	80[c]	E	Hyytiä-Trees *et al.*, 1999
Germany	Meat, NR	36	NE[e]	E	Klarmann, 1989
Indonesia	Fish, 10 g	5.1	5.3	B[d], E, F[d]	Haq and Suhadi, 1981
Italy	Vegetables, NR	4.3	NE	B[d]	Quarto *et al.*, 1983
Japan	Fish, NR	4.5	NE	E, F[d]	Yamamoto *et al.*, 1970
Nordic countries	Fish intestines, NR	15	NE	E	Huss and Pedersen, 1979
Nordic countries	Shellfish, NR	14	NE	E	Huss and Pedersen, 1979
Norway	Fish, NR	11	NE	E	Tjaberg and Håstein, 1975
Poland	Fish, herring intestines, NR	18	NE	E	Zaleski *et al.*, 1978
Russia	Fish, 6 g	35	73	E	Rouhbakhsh-Khaleghdoust, 1975
Sweden	Fish, NR[b]	46	NE	E	Johannsen, 1963
Sweden, Norway	Fish , 2 g	4.8	25	E	Cann *et al.*, 1966; Cann *et al.*, 1967
Sweden	Peels of potato, 6 g	68	197	E	Johannsen, 1963
Thailand	Fish intestines, 100 g	2.3	0.02	E	Tanasugarn, 1979
UK	Fish, trout, NR	10	NE	B, E, F	Cann *et al.*, 1975
UK	Fish, NR	1.4	NE	B	Burns and Williams, 1975
USA	Fish, NR	6.3	NE	E	Chapman and Naylor, 1966
USA	Fish and seafood, 70g	43	8.0	B, E, F	Baker *et al.*, 1990a
USA	Fish and seafood, 100 g	3.6	0.4	B, E, F	Baker *et al.*, 1990a
USA, Alaska	Fish, salmon gills and viscera, NR	1.2	NE	E	Houghtby and Kaysner, 1969
USA, Alaska	Fish, salmon viscera, roe, and flesh, 1 g	4.9	50	E	Miller, 1975
USA, East cost	Fish intestines, NR	4.5	< 43 c	E	Nickerson *et al.*, 1967
USA, Great Lakes	Fish, intestinal contents, NR	17	NE	E	Bott *et al.*, 1966

Table 1 continues.

Country	Sample type and size	Positive samples (%)	Mean spore count (spores/kg)[a]	Group II *C. botulinum* type	Reference
USA, Great Lakes	Fish intestinal contents, NR	11	NE	E	Bott *et al.*, 1967
USA, Milwaukee	Fish, fresh and frozen, > 10 g	8.7	< 9.1	B[d], E	Pace *et al.*, 1967a; Pace *et al.*, 1967b
USA, West coast	Fish, gills and viscera, NR	9.5	NE	B[d], E	Craig and Pilcher, 1967
USA, West coast	Crab, NR	53	NE	B[d], E	Eklund and Poysky, 1967
USA, West coast	Shellfish, NR	23	NE	B[d], E	Craig *et al.*, 1968

[a] Mean spore count extrapolated from all data reported, using the MPN technique.

[b] NR, not reported.

[c] Mean spore count calculated from the actual numbers reported by the authors.

[d] The physiological group of *C. botulinum* types B and F was not indicated, but an incubation temperature of 28-30ºC was used and/or trypsin activation was required in the detection of types B and F toxins.

[e] NE, not estimated.

Table 2. Prevalence of nonproteolytic *Clostridium botulinum* in processed foods.

Country	Sample type and size	Positive Samples (%)	Group II *C. botulinum* type	Mean spore count (spores/kg)[a]	Reference
Finland	Vacuum-packaged hot-smoked fish, 33 g	7.0	E	35[b]	Hyytiä *et al.*, 1998
Finland	Vacuum-packaged cold-smoked fish, 33 g	3.0	E	160[b]	Hyytiä *et al.*, 1998
Finland	Air-packaged hot-smoked fish, 33 g	3.3	E	45[b]	Hyytiä *et al.*, 1998
Japan	Honey, 20 g	8.3	F[c]	30-60	Nakano and Sakaguchi, 1991
Russia	Salted and smoked fish, 6 g	11	B[c], E	19.6	Rouhbakhsh-Khaleghdoust, 1975
UK	Vacuum-packaged fish, 2 g	0.8	E	3.9	Cann *et al.*, 1966
USA	Vacuum-packaged meats and cheese, NR[d]	1.0	B[c]	NR	Insalata *et al.*, 1969
USA, West coast	Air-packaged smoked fish, 5-10 g	4.6	E	<9.4	Hayes *et al.*, 1970
USA, Milwaukee	Smoked fish, > 10 g	1.3	B[c], E	<1.3	Pace *et al.*, 1967a
USA	Packaged ready-to-eat foods, 24 g	1.8	B[c], E	0.8	Taclindo *et al.*, 1967
USA	Smoked turkey, 30 g	2.4	B[c]	0.8	Abrahamsson and Riemann, 1971
USA	Venison jerky, NR	NR	F	NR	Midura *et al.*, 1972

[a] Mean spore count extrapolated from all data reported, using the MPN technique.

[b] Mean spore count calculated from the actual spore numbers reported by the authors.

[c] The physiological group of *C. botulinum* types B was not indicated, but an incubation temperature of 28-30ºC was used in the detection of types B and F toxins.

[d] NR, not reported.

nonproteolytic *C. botulinum* spores (Alderton *et al*., 1974; Peck *et al*., 1992a, b; Peck *et al*., 1993). It has been suggested that these enzymes are able to permeate the heat-injured spore coat and induce germination by hydrolyzing peptidoglycan in the spore cortex (Gould, 1989). A total of 0.1% to as much as 20% of a nonproteolytic *C. botulinum* spore population have been reported to be naturally permeable to lysozyme possessing a higher measured heat resistance than spores that are not permeable to lysozyme (Peck *et al*., 1992a, b; Peck *et al*., 1993). This explains the biphasic thermal destruction curve, where spores non-permeable to lysozyme are destroyed more rapidly than those permeable to lysozyme (Peck *et al*., 1992a, b). Concerns regarding the safety of minimally heat-treated foods have arisen in the food industry, as lysozyme and other lytic enzymes are present in a number of foods (Proctor and Cunningham, 1988; Scott and Bernard, 1985; Lie *et al*., 1989; Peck and Stringer, 1996; Stringer and Peck, 1996; Stringer *et al*., 1999). The impact of lysozyme on the heat resistance of spores heated in various media is clearly demonstrated in Table 4. *C. botulinum* type E spores were shown to possess a higher heat resistance when heated in raw fish mince than when heated in autoclaved fish (Alderman *et al*., 1972), probably indicating a higher activity of lytic enzymes in raw foods than in processed foods (Lund and Peck, 1994).

A tailed thermal destruction curve without using lysozyme in the recovery medium was reported by Smelt (1980). The biphasic curve was explained by a mere genetic heterogeneity among the spore population, with a small subpopulation being more heat resistant than the rest of the spores. Differences in thermal inactivation kinetics within a bacterial spore population have also been suggested to explain nonlinear thermal inactivation curves (Whiting, 1995; Peleg and Cole, 2000).

The a_w of the heating medium seems to have a considerable effect on the thermal destruction of *C. botulinum* spores. The greatest heat resistance at 110ºC was observed for *C. botulinum* type E spores at a_w 0.8 to 0.9 and only a slight decrease in the heat resistance followed when the a_w was decreased to 0.2 (Murrell and Scott, 1957). However, when the a_w was increased to 0.998, the heat resistance of the type E spores decreased drastically by a factor of 30 000 (Murrell and Scott, 1957). The effect of 'moist' heat has thereafter been used to facilitate the hot-smoking of fish products. Processing at 82ºC for 30 min, the heat treatment officially recommended for the commercial hot-smoking of fish in the US in the 1960's (City of Milwaukee, 1964; Anonymous, 1964), combined with an ambient relative humidity (RH) of 70% inside the smoking chamber was sufficient to eliminate *C. botulinum* type E spores in whitefish chubs by a factor of 10^5. When the same heat treatment was employed in the presence of a lower RH, growth and toxin production from 10^5 to 10^6 type E spores was observed (Christiansen *et al*., 1968; Alderman *et al*., 1972; Pace *et al*., 1972).

The z-values (the temperature gradient [ºC] yielding a 10-fold change in the D-value) reported for nonproteolytic *C. botulinum* spores heated in various media are typically around 6-9ºC, but z-values around 4 to 5ºC (Lynt *et al*., 1983; Chai and Liang, 1992) to as high as 16.5ºC have been reported (Scott and Bernard, 1982) (Table 3).

Table 3. Heat resistance of spores of nonproteolytic *Clostridium botulinum* heated in various media.

Heating medium	Group II *C. botulinum* type	Temperature (°C) for D-value	D-value (min)	Temperature range (°C) for z-value	z-value (°C)	Reference
Phosphate buffer	B	77.5	4.0-103			Smelt, 1980
Phosphate buffer	B	80.0	0.6-2.3[a]			Juneja *et al.*, 1995
Phosphate buffer	B	85.0	2.5-51.0	85.0-90.0	6.3[a]	Smelt, 1980
Phosphate buffer	B	87.5	1.5-24.0			Smelt, 1980
Phosphate buffer	B	90.0	0.4-8.3			Smelt, 1980
Phosphate buffer	E	77.5	1.5-38.0			Smelt, 1980
Phosphate buffer	E	80.0	0.4-3.9[a]			Juneja *et al.*, 1995
Phosphate buffer	E	80.0	1.2-36.0	80.0-87.5	8.3-9.4[a]	Smelt, 1980
Phosphate buffer	E	82.5	0.5-23.6			Smelt, 1980
Phosphate buffer	E	85.0	0.3-10.4			Smelt, 1980
Phosphate buffer	E	87.5	0.2-6.1			Smelt, 1980
Phosphate buffer	F	73.9	9.1-12.7	71.1-85.0	5.2-6.7[a]	Lynt *et al.*, 1983
Phosphate buffer	F	76.6	1.7-6.6			Lynt *et al.*, 1983
Phosphate buffer	F	79.4	0.9-2.1			Lynt *et al.*, 1983
Phosphate buffer	F	82.2	0.3-0.8			Lynt *et al.*, 1983
Phosphate buffer	F	85.0	0.4			Lynt *et al.*, 1983
Pork and pea broth	B	82.2	1.5-32.3	75.0-100.0	6.5-16.5	Scott and Bernard, 1982
Pork and pea broth	E	82.2	0.3	75.0-100.0	8.7	Scott and Bernard, 1982
Phosphate buffer, L[b]	B	75.0	283			Peck *et al.*, 1993
Phosphate buffer, L	B	80.0	2.5-4.3a			Juneja *et al.*, 1995
Phosphate buffer, L	B	85.0	73.6-90	85.0-95.0	7.6	Peck *et al.*, 1993
Phosphate buffer, L	B	90.0	18.1			Peck *et al.*, 1993
Phosphate buffer, L	B	95.0	4.6			Peck *et al.*, 1993
Phosphate buffer, L	E	80.0	1.0-4.5[a]			Juneja *et al.*, 1995
Phosphate buffer, L	E	85.0	48.3	85.0-95.0	8.3	Peck *et al.*, 1993
Phosphate buffer, L	E	90.0	12.6			Peck *et al.*, 1993
Phosphate buffer, L	E	90.6	13.5			Alderton *et al.*, 1974
Phosphate buffer, L	E	95.0	3.2			Peck *et al.*, 1993
Phosphate buffer, L	E	93.3	3.8			Alderton *et al.*, 1974

Table 3 continues.

Heating medium	Group II *C. botulinum* type	Temperature (°C) for D-value	D-value (min)	Temperature range (°C) for z-value	z-value (°C)	Reference
Distilled water, L	E	90.6	5.0			Alderton *et al.*, 1974
Pork and pea broth, L	B	82.2	28.2-2224			Scott and Bernard, 1985
Pork and pea broth, L	E	82.2	24.2			Scott and Bernard, 1985
Crabmeat	B	88.9	12.9	88.9-94.4	8.5	Peterson *et al.*, 1997
Crabmeat	B	90.6	8.2			Peterson *et al.*, 1997
Crabmeat	B	92.2	5.3			Peterson *et al.*, 1997
Crabmeat	B	94.4	2.9			Peterson *et al.*, 1997
Crabmeat	E	73.9	6.2-13	73.9-85.0	6.4-8.1[a]	Lynt *et al.*, 1977, 1983
Crabmeat	E	76.6	1.7-4.1			Lynt *et al.*, 1977, 1983
Crabmeat	E	79.4	1.1-1.7			Lynt *et al.*, 1977, 1983
Crabmeat	E	82.2	0.5-0.7			Lynt *et al.*, 1977
Crabmeat	E	82.2	0.5-0.8			Lynt *et al.*, 1983
Crabmeat	E	85.0	0.2[a]			Cockey and Tatro, 1974
Crabmeat	E	85.0	0.3			Lynt *et al.*, 1977, 1983
Crabmeat	F	76.6	9.5	76.6-85.0	6.6[a]	Lynt *et al.*, 1983
Crabmeat	F	79.4	3.6			Lynt *et al.*, 1983
Crabmeat	F	82.2	1.2			Lynt *et al.*, 1983
Crabmeat	F	85.0	0.5			Lynt *et al.*, 1983
Crawfish	E	80.0	4.9-7.0	80.0-95.0	8.0-14.5	DePantoja, 1986
Crawfish	E	85.0	6.7-8.8			DePantoja, 1986
Crawfish	E	90.0	2.5-3.1			DePantoja, 1986
Oyster homogenate	E	73.9	2.0-9.0	73.9-82.2	4.2-6.2	Chai and Liang, 1992
Oyster homogenate	E	75.0	1.3-5.3			Chai and Liang, 1992
Oyster homogenate	E	76.7	0.7-2.7			Chai and Liang, 1992
Oyster homogenate	E	79.4	0.3-1.0			Chai and Liang, 1992
Oyster homogenate	E	80.0	0.8	70.0-80.0	7.6	Bucknavage *et al.*, 1990
Oyster homogenate	E	82.2	0.1-0.4			Chai and Liang, 1992
Cod homogenate	B	75.0	53.9	75.0-92.0	8.6	Gaze and Brown, 1990
Cod homogenate	B	80.0	18.3			Gaze and Brown, 1990

Table 3 continues.

Heating medium	Group II *C. botulinum* type	Temperature (°C) for D-value	D-value (min)	Temperature range (°C) for z-value	z-value (°C)	Reference
Cod homogenate	B	85.0	4.0			Gaze and Brown, 1990
Cod homogenate	B	90.0	1.1			Gaze and Brown, 1990
Cod homogenate	B	92.0	0.6			Gaze and Brown, 1990
Cod homogenate	E	75.0	58.5	75.0-92.0	8.3	Gaze and Brown, 1990
Cod homogenate	E	80.0	15.1			Gaze and Brown, 1990
Cod homogenate	E	85.0	4.8			Gaze and Brown, 1990
Cod homogenate	E	90.0	0.8			Gaze and Brown, 1990
Cod homogenate	E	92.0	0.6			Gaze and Brown, 1990
Whitefish chubs	E	80.0	1.6-4.3	80.0-90.0	5.7-7.6[a]	Crisley *et al.*, 1968
Turkey slurry, L	B	75.0	32.5	70.0-90.0	9.4	Juneja *et al.*, 1995; Juneja, 1998
Turkey slurry, L	B	80.0	15.2			Juneja *et al.*, 1995; Juneja, 1998
Turkey slurry, L	B	85.0	4.9			Juneja *et al.*, 1995; Juneja, 1998
Turkey slurry, L	B	85.0	7.8	80.0-90.0	10.8	Juneja and Eblen, 1995
Turkey slurry, L	B	90.0	0.8			Juneja *et al.*, 1995; Juneja, 1998
Turkey slurry, L	B	90.0	1.1			Juneja and Eblen, 1995
Turkey slurry, L	E	75.0	18.1	70.0-90.0	9.9	Juneja *et al.*, 1995; Juneja, 1998
Turkey slurry, L	E	80.0	13.4			Juneja *et al.*, 1995; Juneja, 1998
Turkey slurry, L	E	85.0	1.2			Juneja *et al.*, 1995; Juneja, 1998
Carrot homogenate	B	75.0	19.4	75.0-92.0	9.8	Gaze and Brown, 1990
Carrot homogenate	B	80.0	4.2			Gaze and Brown, 1990
Carrot homogenate	B	85.0	1.6			Gaze and Brown, 1990
Carrot homogenate	B	90.0	0.4			Gaze and Brown, 1990
Carrot homogenate	B	92.0	0.4			Gaze and Brown, 1990
Carrot homogenate	E	75.0	18.1	70.0-90.0	9.8	Gaze and Brown, 1990
Carrot homogenate	E	80.0	4.3			Gaze and Brown, 1990
Carrot homogenate	E	85.0	0.7			Gaze and Brown, 1990
Carrot homogenate	E	90.0	0.5			Gaze and Brown, 1990

[a] Extrapolated from thermal destruction data reported by authors.
[b] L, heating medium containing added lysozyme.

Table 4. Time to growth or toxin production from 10^4 to 10^6 spores of nonproteolytic *C. botulinum* types B, E, and F processed in laboratory media and vacuum-packaged seafood stored at 10°C or below.

Medium	Heat process		Storage temperature (°C)	Time to toxicity (d)	pH	NaCl content (% v:v)	Type and number of spores	Reference
	Process temperature (°C)	Process time (min)						
Meat medium	85	11.4	5	58	6.5	NR[a]	BEF 10^6	Fernández and Peck, 1997
Meat medium	85	18.1	5	104	6.5	0.6	BEF 10^6	Graham *et al.*, 1996a
Meat medium	85	11.4	8	24	6.5	NR	BEF 10^6	Fernández and Peck, 1997
Meat medium	85	19.2	8	53	6.1-6.3	NR	BEF 10^6	Peck *et al.*, 1995
Meat medium	85	17.3	8	>60	6.1-6.3	NR	BEF 10^6	Peck *et al.*, 1995
Meat medium	85	23.3	8	>90	6.5	NR	BEF 10^6	Fernández and Peck, 1997
Meat medium	85	17.5	8	>91	6.5	0.6	BEF 10^6	Graham *et al.*, 1996a
Meat medium, L[b]	85	23.3	5	>90	6.4-6.6	NR	BEF 10^6	Fernández and Peck, 1999
Meat medium, L	85	35.7	5	>90	6.4-6.6	NR	BEF 10^6	Fernández and Peck, 1999
Meat medium, L	90	10.3	5	>90	6.4-6.6	NR	BEF 10^6	Fernández and Peck, 1999
PYGS medium, L	90	1.0	5	>161	6.8	NR	BEF 10^6	Stringer *et al.*, 1997
Meat medium, L	85	18.1	8	43	6.5	2.5	BEF 10^6	Graham *et al.*, 1996a
Meat medium, L	85	35.7	8	48	6.4-6.6	NR	BEF 10^6	Fernández and Peck, 1999
Meat medium, L	90	10.3	8	54	6.4-6.6	NR	BEF 10^6	Fernández and Peck, 1999
Meat medium, L	90	10.9	8	58	6.4-6.6	NR	BEF 10^6	Fernández and Peck, 1999
Meat medium, L	85	23.3	8	61	6.4-6.6	NR	BEF 10^6	Fernández and Peck, 1999
Meat medium, L	85	18.1	8	64	6.5	0.6	BEF 10^6	Graham *et al.*, 1996a
Meat medium, L	85	52.0	8	>90	6.4-6.6	NR	BEF 10^6	Fernández and Peck, 1999
Meat medium, L	90	15.3	8	68	6.4-6.6	NR	BEF 10^6	Fernández and Peck, 1999
PYGS medium, L	90	15.0	10	7	6.8	1.5	B $10^{5.1}$	Stringer and Peck, 1997
PYGS medium, L	90	15.0	10	14	6.8	3.0	B $10^{5.1}$	Stringer and Peck, 1997
PYGS medium, L	90	60.0	10	>161	6.8	NR	BEF 10^6	Stringer *et al.*, 1997
Crab analog	85	15.0[c]	10	>120	7.2	2.1	B 10^4	Peterson *et al.*, 2002
Hot-smoked salmon	92.2	45.0[d]	10	>120	7.2-7.4	1.0-2.0	BE 10^6	Eklund *et al.*, 1988

[a] NR, not reported.
[b] L, lysozyme was added in the recovery medium of heat-damaged spores.
[c] Process time does not include the effect of the come-up time of 12 min to the target temperature of 85°C.
[d] Process time includes a come-up time of 27.7 min to the target temperature of 92.2°C.

2.6 Risk of nonproteolytic *Clostridium botulinum* in REPFED products

2.6.1 REPFED products

The increased demand among consumers for convenient and fresh-like foods with minimal thermal processing and preservation has led to a tremendous increase in the sales of REPFED food products in Europe. These foods are given mild heat treatments with maximum process temperatures typically reaching 65-95ºC, which may allow for the survival of bacterial spores. The heat treatment is followed by rapid cooling and chilled storage at 1 to 8ºC. The microbiological quality of REPFED foods thus relies mainly upon the mild heat treatment and a refrigerated storage temperature. As REPFED products are typically packaged under vacuum or in MA ensuring anaerobic conditions, the shelf lives established for these foods may be as long as 42 d. This has raised concern in regard to food safety due to the hazard caused by spore-forming bacteria (Del Torre *et al*., 1998, 2001), particularly anaerobic nonproteolytic *C. botulinum* (Genigeorgis, 1985; Baker and Genigeorgis, 1989). Three main types of REPFED products are being produced: (1) Foods that are first packaged and then processed, such as sous vide foods; (2) foods that are processed and then packaged, such as vacuum-packaged hot-smoked fish products; and (3) foods that are first cooked, then packaged, and finally pasteurized. All types of ingredients are used in the production of REPFED foods (Gorris and Peck, 1998).

2.6.2 Factors predisposing to risk caused by nonproteolytic *Clostridium botulinum* in REPFED products

Several factors increase the risk associated with nonproteolytic *C. botulinum* in REPFED foods (Peck, 1997). (1) As an environmental organism, nonproteolytic *C. botulinum* may contaminate the raw materials applied in REPFED technology. (2) The heat treatments employed in the production of REPFED products are generally too mild to eliminate bacterial spores, whereas they readily destroy the vegetative bacterial flora and thus enable the spore-formers to germinate and grow in the absence of competitive microflora. (3) Vacuum and MA-packaging delay the oxygen-related spoilage resulting in extended shelf lives, and thus allow the multiplication of facultatively anaerobic and anaerobic bacteria. (4) Nonproteolytic *C. botulinum* may grow at temperatures as low as 3.0-3.3ºC (Schmidt *et al*., 1961; Eklund *et al*., 1967a, b; Graham *et al*., 1997), while the typical storage temperatures measured at the retail and consumer level may exceed 10ºC (Evans, 1998). (5) The use of NaCl and particularly other preservatives in REPFED foods is limited. (6) Pre-cooked products are not always further heated, and toxin production by nonproteolytic *C. botulinum* may precede the sensory spoilage of the product (Post *et al*., 1985; Garcia *et al*., 1987; Ikawa and Genigeorgis, 1987; Gorris and Peck, 1998; Reddy *et al*., 1999; Lawlor *et al*., 2000).

2.6.3 Control of nonproteolytic *Clostridium botulinum* in REPFED products

The control of risk caused by nonproteolytic *C. botulinum* in REPFED products includes the elimination of spores present in the raw materials and/or the inhibition of germination and growth from spores surviving heat treatment. According to the ACMSF (1992) and ECFF (1996) guidelines, the safety of REPFED foods with respect to nonproteolytic *C. botulinum* should be ensured by a 6D heat treatment, reducing the initial number of nonproteolytic *C. botulinum* spores by a factor of 10^6. A time-temperature combination of 10 min at 90ºC or one with equivalent lethality was proposed to ensure a 6D reduction (ACMSF, 1992; ECFF, 1996).

If the 6D process can not be guaranteed, the germination and outgrowth of spores should be inhibited by other factors (ACMSF, 1992; ECFF, 1996) such as a NaCl content of 5% (w:v), a_w of 0.97 (Ohye and Christian, 1966; Baird-Parker and Freame, 1967; Emodi and Lechowich, 1969), or pH below 5.0 (Segner *et al.*, 1966; Lund *et al.*, 1990). Alternatively, a storage temperature of <3.0ºC throughout the entire storage period would effectively control the risk of growth and toxin formation from nonproteolytic *C. botulinum*. However, as the germination of nonproteolytic *C. botulinum* occurs at a wider temperature range than growth, 1 to 50ºC with the optimum being 9 to 25ºC (Strasdine, 1967; Ando and Iida, 1970; Grecz and Arvay, 1982; Evans *et al.*, 1997; Plowman and Peck, 2002), even a slight fluctuation in the storage temperature may be harmful.

The application of various gas atmospheres in the MA-packages has been used to control botulinal growth in REPFED foods. As opposed to vacuum packaging, air-packaging and MA-packaging with 75% CO_2 delayed toxin formation at 8ºC from 10^2 nonproteolytic spores in catfish by 3 and 12 d, respectively (Reddy *et al.*, 1997). The inhibitory effect of CO_2 was also demonstrated by the time to toxin production from 10^4 type E spores in vacuum and MA (100% CO_2) packaged salmon to be 9 and 12 - >21 d, respectively, at 8ºC (Lindroth and Genigeorgis, 1986; Baker *et al.*, 1990b). Moreover, the time to toxin production at 4ºC from $10^{1.7}$ nonproteolytic spores was extended from 14 to 28 d by replacing 100% N_2 with a mixture of 30% CO_2 and 70% N_2 in MA-packaged turkey (Lawlor *et al.*, 2000). The CO_2 levels of 65, 90, and 100%, combined with O_2 at level of 4, 1, and 0%, respectively, allowed botulinal growth or toxigenesis at 8ºC in 5, 8, and 20 d, respectively (Post *et al.*, 1985).

Many preservatives, although rarely used in REFPED technology, have been reported to effectively control the growth of nonproteolytic *C. botulinum*. Sodium lactate at the levels of 2.4% and 1.8% in sous vide beef and chicken, respectively, stored at 12ºC, was shown to delay toxigenesis from 10^4 spores of nonproteolytic *C. botulinum* by at least 40 d, whereas in sous vide processed salmon a lactate level of 4.8% was required (Meng and Genigeorgis, 1994). In cooked turkey with 1% NaCl in brine, a sodium lactate level of 3% prevented toxigenesis for 32 d at 12ºC (Meng and Genigeorgis, 1993). Bacteriocins inhibiting the growth of *C. botulinum* in foods (Okereke and Montville, 1991) include nisin (Scott and Taylor, 1981a, b; Taylor *et al.*, 1985; Somers and Taylor, 1987) and subtilin (LeBlanc *et al.*, 1953). In addition to its growth-inhibitory action, nisin has also been reported to decrease the heat resistance of bacterial spores (Penna and Moraes, 2002). Lactic acid bacteria have also

been shown to inhibit the growth of *C. botulinum* (Lyver *et al.*, 1998a, b; Skinner *et al.*, 1999).

While the inhibition of proteolytic *C. botulinum* by sodium nitrite has been extensively studied (Roberts and Ingram, 1973; Roberts, 1975; Christiansen *et al.*, 1974; Tompkin *et al.*, 1978; Sofos *et al.*, 1979) reports on its effects on nonproteolytic strains are scarce (Cuppett *et al.*, 1987; Hyytiä *et al.*, 1997; Keto-Timonen *et al.*, 2002). In heat processed foods nitrite forms the Perigo factor that is inhibitory to *C. botulinum* (Perigo *et al.*, 1967; Christiansen *et al.*, 1973). The use of nitrite, however, is limited in European countries due to its possible adverse health effects. In fish products produced in the EU countries the use of nitrite is prohibited (European Parliament and the Commission of the European Communities, 1995). A large number of other compounds inhibiting nonproteolytic *C. botulinum* in laboratory media or in foods have been widely reviewed (Roberts and Gibson, 1982; Rhodehamel *et al.*, 1992). However, as the intrinsic preservative factors in REPFED foods are limited, the protection from nonproteolytic *C. botulinum* in REPFED products relies mainly upon heat treatment and refrigerated storage (Conner *et al.*, 1989; Gorris and Peck, 1998) (Table 4). Tables 4 and 5 list challenge studies with nonproteolytic *C. botulinum* spores grown in various media with or without preceding heat processing, followed by incubation at refrigerated or slightly abused temperatures.

Mathematical models predicting the lag time to growth and time to toxin production from unheated and heated spores of nonproteolytic *C. botulinum* in foods have been developed based on large data series obtained in laboratory media and model food media (Lindroth and Genigeorgis, 1986; Jensen *et al.*, 1987; Baker and Genigeorgis, 1990; Baker *et al.*, 1990b; Genigeorgis *et al.*, 1991; Meng and Genigeorgis, 1993; Graham *et al.*, 1996b; Fernández and Peck, 1997; Skinner and Larkin, 1998; Fernández and Peck, 1999, Fernández *et al.*, 2001) (Tables 4 and 5). The models typically describe the estimated reduction in spore numbers and/or the probability of growth from a single spore when various spore loads are treated by different time-temperature combinations and subsequently incubated under a range of conditions. However, the commercially available microbiological models do not take into account the possible effect of lysozyme present in the foods, or the effect of other process parameters such as RH. Moreover, as the data employed in the development of the models are often derived from studies using media providing optimal conditions for growth, the models frequently generate 'fail safe' predictions (Meng and Genigeorgis, 1993; Gould, 1999; Hyytiä *et al.*, 1999b). Similarly, the reports on the models predicting a product to be safer than it is, suggest an inconsistency between the models and real foods, emphasizing the importance of safety evaluations by inoculated pack studies (Hyytiä, 1999; Hyytiä *et al.*, 1999b).

Table 5. Time to growth or toxin production from 1 to 10^6 spores of nonproteolytic *C. botulinum* types B, E, and F grown in laboratory media and various foods stored at temperatures below 10°C.

Medium	Storage temperature (°C)	Time to growth or toxigenesis (d)	Type and number of spores	pH	NaCl in brine (%)	Reference
Laboratory media						
PYGS medium	3	35-49	BEF 10^5	6.2	1.0	Graham *et al.*, 1997
PYGS medium	4	21-28	BEF 10^5	6.0-7.0	1.0	Graham *et al.*, 1997
PYGS medium	5	14-28	BEF 10^5	5.7-7.0	1.0	Graham *et al.*, 1997
PYGS medium	5	12-13	BEF 10^6	6.0-6.1	0.1-2.0	Graham *et al.*, 1996b
PYGS medium	5	14	BEF 10^6	6.5	1.5	Fernández *et al.*, 2001
PYGS medium with 90% CO_2	5	18	BEF 10^6	6.5	1.5	Fernández *et al.*, 2001
PYGS medium	7	8	BEF 10^6	5.9	2.0	Graham *et al.*, 1996b
PYGS medium	8	1-3	BEF 10^5	5.5-6.0	1.0	Graham *et al.*, 1997
PYGS medium with 90% CO_2	8	8	BEF 10^6	6.5	1.5	Fernández *et al.*, 2001
Meat medium	8	5-7	BEF 10^6	6.5	0.6-1.5	Graham *et al.*, 1996a; Fernández *et al.*, 2001
PYGS medium	8	25	BEF 10^6	5.3	1.0	Graham *et al.*, 1996b
Meats						
Cooked turkey	4	80	BE 10^3	6.3	1.5	Genigeorgis *et al.*, 1991
Cooked turkey	8	7	BE 10^4	6.2-6.3	0.5	Meng and Genigeorgis, 1993
Cooked beef	8	8	BE 10^4	5.8	NR[a]	Meng and Genigeorgis, 1994
Cooked turkey	8	8	BE 10^4	6.3	1.5	Genigeorgis *et al.*, 1991
Cooked chicken	8	16	BE 10^4	5.9	NR	Meng and Genigeorgis, 1993, 1994
Fish						
Vacuum-packaged salmon	4	15	BEF 10^4	6.3	NR	Garcia and Genigeorgis, 1987
Vacuum-packaged rockfish	4	21	BEF 10^3	6.7	NR	Lindroth and Genigeorgis, 1986
Vacuum-packaged shrimps	4	>21	E 10^3-10^4	NR	NR	Garren *et al.*, 1994
Vacuum-packaged cod and whiting	4	18	E $10^{1.7}$	NR	NR	Post *et al.*, 1985
Vacuum-packaged catfish	4	46	E 10^2	6.6	NR	Reddy *et al.*, 1997
Air packaged tilapia	4	>47	E 10^2	6.4	NR	Reddy *et al.*, 1996
Air-packaged catfish	4	>54	E 102	6.6	NR	Reddy et al., 1997

Table 5 continues.

Medium	Storage temperature (ºC)	Time to growth or toxigenesis (d)	Type and number of spores	pH	NaCl in brine (%)	Reference
Vacuum-packaged tilapia	4	>90	E 102	6.4	NR	Reddy *et al.*, 1996
Sous vide processed salmon	8	8	BE 104	6.4	6.4	Meng and Genigeorgis, 1994
Vacuum-packaged salmon	8	3-12	BEF 1-10	6.2-6.4	NR	Garcia and Genigeorgis, 1987; Garcia *et al.*, 1987; Baker *et al.*, 1990b
Vacuum-packaged salmon and rockfish	8	9	E 10^4	6.4	NR	Baker *et al.*, 1990b
MA-packaged salmon, 100% CO2	8	9-15	BEF 10-10^3	6.2-6.3	NR	Garcia and Genigeorgis, 1987; Garcia *et al.*, 1987; Baker *et al.*, 1990b
Sous vide processed salmon with 2.4 % Na- lactate	8	12	BE 10^4	6.4	NR	Meng and Genigeorgis, 1994
Vacuum-packaged sole	8	12	E 10^4	6.5	NR	Baker *et al.*, 1990b
MA-packaged rockfish, 100% CO_2	8	12-15	BEF 10^1-10^3	6.7	NR	Lindroth and Genigeorgis, 1986; Ikawa and Genigeorgis, 1987
Vacuum-packaged rockfish, salmon, sole and red snapper	8	12-21	BEF 10^2-10^3	6.4-6.7	NR	Lindroth and Genigeorgis, 1986; Ikawa and Genigeorgis, 1987; Baker *et al.*, 1990b
Vacuum-packaged smoked trout	8	14	E 10^2	6.2-6.5	1.7-2.1	Dufresne *et al.*, 2000
Vacuum-packaged tilapia	8	17	E 10^2	6.4	NR	Reddy *et al.*, 1996
Vacuum-packaged cod and whiting	8	17	E $10^{1.7}$	NR	NR	Post *et al.*, 1985
Air packaged tilapia	8	20	E 10^2	6.4	NR	Reddy *et al.*, 1996
Sous vide processed salmon with 4.8 % Na-lactate	8	>90	BE 10^4	6.4	NR	Meng and Genigeorgis, 1994
Vegetables						
Mushroom	10	5	BEF 10^3	6.4	NR	Carlin and Peck, 1996
Asparagus	10	8	BEF 10^3	5.3	NR	Carlin and Peck, 1996
Broccoli	10	19	BEF 10^3	5.5	NR	Carlin and Peck, 1996

[a] NR, not reported.

3. AIMS OF STUDY

The aims of the present thesis were to develop the diagnostics of *C. botulinum* and to improve the safety of REPFED foods with respect to nonproteolytic *C. botulinum* by intensified thermal processing. The specific aims were as follows:

1. to evaluate the applicability of commercially available biochemical test systems in the identification of *Clostridium botulinum* (I)

2. to develop a multiplex PCR assay for the detection of *Clostridium botulinum* types A, B, E, and F in pure cultures, food and faecal material (II)

3. to determine the thermal destruction parameters of spores of nonproteolytic *Clostridium botulinum* type E heated in model fish media (III)

4. to evaluate the safety of the thermal processes employed in the production of commercial REPFED foods with respect to nonproteolytic *C. botulinum* (III, IV)

5. to develop thermal processes controlling the growth and toxin production from $10^{5.3}$-10^6 spores of nonproteolytic *Clostridium botulinum* in REPFED products (III-V), and

6. to evaluate the sensory quality of REPFED products shown to control the growth and toxin production from $10^{5.3}$-10^6 spores of nonproteolytic *Clostridium botulinum* (III-V).

4. MATERIALS AND METHODS

4.1 Bacterial strains and culturing (I-V)

A total of 95 strains of proteolytic and nonproteolytic *C. botulinum* and 37 strains of other bacterial species were used in the evaluation and development of diagnostic methods in studies I and II (I and II, Table 1). The strain mixtures used in studies III to V included nonproteolytic *C. botulinum* types B (706B [studies III, IV]; 2B [IV]; 17B [IV]), E (31-2570E [III]; 4062E [III]; C-60E [III]; Beluga E [V]; 211E [V]; 250E [V]), and F (FT10F [III]) strains. All *Clostridium* spp. spore suspensions were prepared according to Food and Agricultural Organization (1991) and enumerated as described (Doyle, 1991), and stored at 4ºC until use.

All *C. botulinum* strains were cultivated in TPGY broth (Food and Agricultural Organization, 1991) at 30 (group II organisms) to 37ºC (group I organisms) for 2 to 3 d. Thereafter, in study I the cultures were grown on blood agar plates at respective temperatures for 2 to 3 d prior to use in the biochemical test systems. In studies II-V, the broth cultures were further transferred to fresh TPGY medium for 16 to 18 h at respective temperatures in order to synchronize the logarithmic growth of the organisms. In study II, the food and faecal samples inoculated with spores of *C. botulinum* were initially diluted in peptone water (1:9 w:v) prior to the two-step culturing.

4.2 Evaluation of biochemical test systems and development of multiplex PCR assay (I, II)

4.2.1 Biochemical tests (I)

The biochemical test systems evaluated in study I included API 20 A (bioMérieux SA, Marcy-l'Etoile, France), Rapid ID 32 A (bioMérieux SA), and RapID ANA II (Innovative Diagnostic Systems, Inc., Norcross, GA, USA). All tests were performed according to the manufacturers' instructions.

4.2.2 Multiplex PCR detection of *Clostridium botulinum* (II)

The multiplex PCR developed in study II for the detection of *C. botulinum* types A, B, E, and F was preceded by a cell wash and lysis procedure (Franciosa *et al.*, 1994; Hielm *et al.*, 1996) to release the bacterial DNA. Four primer pairs with equal melting temperatures, each pair being specific for *BoNT* type A, B, E, or F (study II, Table 2), were designed by Primer 3 software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) based on published *BoNT* gene sequences (Bintz *et al.*, 1990; Thompson *et al.*, 1990; East *et al.*, 1992; Poulet *et al.*, 1992; Whelan *et al.*, 1992a, b; Elmore *et al.*, 1995). The 50-µl reaction mixture consisted of 1 µl of cell lysate as template, 0.3 µM of each primer (Sigma-Genosys Ltd., Cambridgeshire, UK), 220 nM of each deoxynucleotide triphosphate (dNTP; Finnzymes, Espoo, Finland), 32 mM Tris-HCl, 80 mM KCl, 4.8 mM MgCl, and 2U of DNA polymerase

(DynaZyme, Finnzymes). Each of 28 PCR cycles included denaturation at 95ºC for 30 s, annealing at 60ºC for 25 s, and extension at 72ºC for 1 min 25 s, followed by a final extension at 72ºC for 3 min. The PCR amplicons were detected in 2% agarose gels (iD.N.A Agarose, BioWhittaker Molecular Applications, Rockland, ME, USA) stained with ethidium bromide with comparison to standard-weight molecular markers.

The detection limit of the assay was tested with minced meat, fish, and faecal samples inoculated with 10^{-2} to 10^3 spores/g of sample material. A total of 72 natural food samples consisted of meat, fish, and vegetables were investigated for the presence of *C. botulinum* types A, B, E, and F by the multiplex PCR assay.

4.3 Determination of heat-resistance parameters for nonproteolytic *Clostridium botulinum* spores heated in model fish media (III)

Model media (pH 6.4) consisting of rainbow trout or whitefish were prepared by modification of a method for meat medium (Peck *et al.*, 1995) (study III). Tubes containing 5-ml aliquots of the media were inoculated with 10^6 nonproteolytic *C. botulinum* type E spores and exposed to heat treatments of 0.5 to 450 min at various target temperatures in the range of 75 to 93ºC, followed by a rapid cooling. Uninoculated test tubes were used to monitor the temperature data (Envic DP-158, Envic, Turku, Finland) of the thermal treatments. These data were converted into equivalent process times at the target temperature by the formula $10^{(T-Tref)/z}$, where T is the actual temperature and T_{ref} is the target temperature. A z-value of 7ºC was used (ECFF, 1996). The tubes were cultured with TPGY medium supplemented with hen egg white lysozyme (625 IU/ml, Sigma Chemical Co., St. Louis, MO, USA) in order to detect the heat-resistant spore fraction. The tubes were stored at 30ºC for 90 d and observed for growth at regular intervals. In addition, the numbers of spores surviving each heat treatment were determined by MPN with the detection limit being 2 spores. Growth was observed visually. The survival data were fitted to biphasic thermal destruction curves by linear regression analysis, yielding D-values and the percentage of heat-resistant spores at various temperatures. The z-values were extrapolated from the thermal death time (TDT) curves.

4.4 Safety evaluation and development of thermal processes employed in the REPFED industry (III-V)

The safety of five fish hot-smoking processes (III) and 16 sous vide processes (IV) employed in the Finnish REPFED food industry were evaluated with respect to their lethality to spores of nonproteolytic *C. botulinum* types B and E. The safety evaluation included the estimation of a pasteurization ($P_{85ºC}$) value for each process, describing the equivalent process time at 85ºC, and inoculated pack studies with vacuum-packaged hot-smoked fish and sous vide foods (Tables 6 and 7). Based on the safety evaluation, heat processes controlling the growth and toxin production from $10^{5.3}$-10^6 spores of nonproteolytic *C. botulinum* type E in fish (III) and type B in sous vide meat (V) were developed.

4.4.1 Inoculation of REPFED products with *Clostridium botulinum* (III-V)

The hot-smoked fish products were rainbow trout fillets and whole whitefish (Table 6), and the sous vide foods consisted of meat alone, or meat, vegetables, and rice (Table 7). The pH and the concentration of NaCl added in the foods varied in the range of 4.7 to 7.6 and 0.2 to 2.0%, respectively. The product size varied from 530 to 1500 g. The products were inoculated with $10^{5.3}$ to 10^6 spores of nonproteolytic *C. botulinum* types B, E, and F, in the mixtures described in 4.1.

4.4.2 Thermal processing of REPFED products (III-V)

The fresh unpackaged fish were processed in an electrically controlled smoking chamber (Vemag, Kerres, GmbH, Sulzbach/Murr, Germany) and the packaged sous vide products were processed in a water autoclave (Stock Pilot Rotor 900 G, Hermann Stock Maschinenfabrik GmbH, Neumünster, Germany). All products were exposed to heat treatments equal to processing at 85ºC for 0 to 515 min (Tables 6 and 7) as determined by converting the temperature data measured during the processes into the equivalent process times at 85ºC by using the formula $10^{(T-Tref)/z}$, where T is the actual temperature and T_{ref} is 85ºC. The z-values of 10.1-10.4ºC (as determined in study III) were used in study III, and a value of 7ºC (ECFF, 1996) was used in studies IV and V. In study III, relative humidity (%RH) inside the smoking chamber was chosen as an additional process parameter. The RH during each hot-smoking process was between 10 and 80%. After thermal processing the fish were cooled at room temperature for 2 h until packaging (III) and the sous vide products (IV, V) were chilled at 2ºC.

4.4.3 Packaging and storage of REPFED products (III-V)

The hot-smoked and cooled fish (III) and sous vide products (IV, V) were vacuum-packaged (Multivac A 300/16 1986, Multivac Verpackungsmaschinen, Wolfertschwenden, Germany) in nylon-polyethylene pouches prior to or after thermal processing, respectively. The fish were stored at 8ºC for 35 d, and the sous vide foods were stored at 4 or 8ºC for 9 to 37 d. The storage times corresponded to the commercial product shelf lives employed in Finland or these times extended by 1 to 2 weeks.

4.4.4 Sampling procedures (III-V)

After storage periods of 9 to 37 d at 4 or 8ºC, the REPFED products were analysed for the presence and number of viable nonproteolytic *C. botulinum* organisms by PCR (Franciosa *et al*., 1994; Hielm *et al*., 1996; Sciacchitano and Hirshfield, 1996) combined with MPN technique (Finnish Standards Association, 1994). The presence of botulinum neurotoxin in the products was determined according to the Nordic Committee on Food Analysis (Nordic Committee on Food Analysis, 1991a), with slight modifications in study IV (Hyytiä *et al*., 1997). The bioassays were approved by the Committee on Animal Experimentation of the

Faculty of Veterinary Medicine, University of Helsinki. In addition, the pHs of all products were measured (Microprocessor pH 537, Wissenschaflich-Technische Werkstätten, Weilheim, Germany) in studies III-V. The NaCl content of the fish was measured in study V according to the International Organization for Standardization (1989).

4.4.5 Sensory evaluations (III-V)

The sensory quality of the REPFED products was determined by trained panels 1 to 3 d after processing (III-V) as well as during and at the end of the storage (IV, V). All the products were served at a temperature typical of the product: hot-smoked fish at room temperature and sous vide products at 65ºC. In studies III and V a quantitative profiling of various sensory attributes (Lawless and Heymann, 1998), including flavour, odour, juiciness, firmness, and the degree of cooking in study III, and brownness, greyness, aroma, flavour, and juiciness in study V, of products exposed to various heat treatments at 85ºC for 0 to 515 min was used. In study IV, a quantitative ranking of the suitability of the sous vide foods, stored at 8ºC for various times, to human consumption in reference to a fresh product, was employed using appearance and aroma as the sensory attributes (Meilgaard *et al.*, 1987). The details of the analytical procedures have been described in the original publications III-V.

4.4.6 Predictive microbiological models (IV)

In study IV, predictions for the lethal effect of the heat treatments on spores of nonproteolytic *C. botulinum* in the sous vide foods were generated by the Food Micro Model (FMM, version 2.5, Leatherhead Food Research Association, Leatherhead, Surrey, UK). Moreover, the FMM and Pathogen Modelling Program (PMP, version 5.0, US Department of Agriculture Eastern Regional Research Center, Wyndmore, PA, USA) were used to predict the lag time for growth and time-to-turbidity, respectively, from the nonproteolytic *C. botulinum* spores. The FMM thermal death model for nonproteolytic *C. botulinum* type B, predicting the estimated minimum decrease in the spore number, uses process temperature (80 to 95ºC), water-phase NaCl concentration (0 to 5%), and pH (4.0 to 7.4) as controlling factors. The two growth models (FMM nonproteolytic *C. botulinum* types B, E, F; PMP type B) take into account the storage temperature (FMM 4 to 30ºC, PMP 5 to 28ºC), pH (FMM 5.1 to 7.5, PMP 5.0-7.0), water-phase NaCl content (FMM 0 to 4.5%, PMP 0 to 4%), and the initial number of spores (PMP 1 to 10^5 CFU/product unit).

4.5 Statistical analyses (I, III, V)

The sensitivity and specificity of the biochemical test systems for *C. botulinum* evaluated in study I were determined (Martin *et al.*, 1987). In study III, linear regression analysis was employed to generate the biphasic thermal destruction curves for nonproteolytic *C. botulinum* type E spores heated in model fish media at 75 to 93ºC. Analysis of variance, taking into account the heat treatment, duplicate, and the storage time (study V only) was applied in the sensory evaluation in studies III and V to yield the significance of differences between various

sensory attributes of vacuum-packaged hot-smoked fish products and sous vide meat foods treated by various heat processes with respect to their lethality to nonproteolytic *C. botulinum* spores.

Table 6. Vacuum-packaged hot-smoked fish products used in study III and the effect of various thermal processes on the growth and toxin production from 10^6 nonproteolytic *Clostridium botulinum* spores in the fish products stored at 8ºC for 35 d.

Product ingredients, storage time (d)	$P_{85°C}$ (min) using different z-values [a]		RH (%) [b]	Time (d) to detection of *C. botulinum*	pH	NaCl (%[w:v])	Time to toxicity (d)
	10.1-10.4°C	7°C					
Rainbow trout fillet	1.5 [c]	1.8 [c]	60	<7	6.4-6.9	<0.5	>35
Rainbow trout fillet	25	119	25	<7	6.4-6.9	<0.5	>35
Rainbow trout fillet	26	127	70	<35	6.4-6.9	<0.5	>35
Rainbow trout fillet[d]	**34**	**189**	**75**	**>35**	**6.4-6.9**	**<0.5**	**>35**
Whole whitefish	1.5 [c]	1.8 [c]	50	<7	6.7-7.6	<0.5	35
Whole whitefish	**42**	**220**	**80**	**>35**	**6.7-7.6**	**<0.5**	**>35**
Whole whitefish	44	235	10	<7	6.7-7.6	<0.5	35
Whole whitefish	**62**	**386**	**80**	**>35**	**6.7-7.6**	**<0.5**	**>35**

[a] The z-values 10.1-10.4°C were obtained in the present study, and 7ºC was according to ECFF (1996).
[b] The mean relative humidity (approximation to the nearest 5%) measured during the most effective 30-min period at the end of heat process.
[c] Heat process currently employed in the Finnish smoked fish industry.
[d] The products with no detectable *C. botulinum* or toxin during the entire storage period are written in **bold text.**

Table 7. Sous vide products and the effect of various thermal processes on the growth and toxin production from $10^{5.3}$ nonproteolytic *Clostridium botulinum* spores in the sous vide foods stored at 4 and 8°C for 9 to 37 d.

Product ingredients, storage time (d)	P$_{85°C}$ (min)[a]	*C. botulinum* detected 4°C	*C. botulinum* detected 8°C	Predicted log reduction in spore number by FMM	pH	NaCl (%[w:v])	Time to toxicity (d) 4°C	Time to toxicity (d) 8°C	Predicted time to turbidity at 8°C by PMP (d)	Study
Pork cubes, 41-21	15 [b]	Yes	Yes	NE[c]	6.0-6.3	0.7	>21	21	<2.4[d]	IV
Pork cubes, 30-37	103 [b]	Yes	Yes	1.4	5.8-6.1	0.7	>37	>37	7	IV
Beef cubes, 21-28	336 [b]	Yes	Yes	>12.0	5.8-6.1	0.2	>28	>28	>90	IV
Beef cubes, 14-21	126 [b]	No	Yes	1.6	5.6-6.1	0.7	>21	>21	9	IV
Pork fillet, 10-17	7.1 [b]	Yes	Yes	NE	5.8-6.0	2.0	>17	>17	<2.3[d]	IV
Beef roast, 30-37	13 [b]	Yes	Yes	NE	5.7-6.1	1.6	>37	>37	<2.2[d]	IV
Beef roast, 9-16	3.7 [b]	Yes	Yes	NE	5.6-5.8	1.9	>16	>16	<2.5[d]	IV
Ground beef, 21-28	<0.1 [b]	Yes	Yes	NE	5.5-6.0	0.2	>21	21	<3.5[d]	IV,V
Beef liver cubes, 21-28	304 [b]	Yes	Yes[e]	>12.0	6.1-6.3	0.3	>28	>28	>90	IV
Broiler fillets, marinade, 21-28	56 [b]	Yes[e]	No	0.5	5.9-6.1	1.4	>28	>28	3	IV
Rice, vegetables, pork, seafood, 21-28	69 [b]	No	Yes	2.2	5.8-6.0	1.9	>28	>28	13	IV
Rice, water, milk, 21-28	ND [b, f]	Yes	Yes	>12.0	6.1-6.7	1.1	>28	>28	>90	IV
Beef, pork, water, vegetables, 21-28	1.7 [b]	Yes	Yes	NE	4.8-5.1	1.3	>28	>28	<4.5[d]	IV
Beef, vegetables, water, 30-37 [g]	**236 [b]**	**No**	**No**	**6.1**	**4.7-5.3**	**1.3**	**>37**	**>37**	**>90**	**IV**
Water, potatoes, beef, vegetables, 21-28	250 [b]	Yes	Yes[e]	>12.0	5.3-5.8	1.0	>28	>28	>90	IV
Pork, vegetables, water, 21-28	**80 [b]**	**No**	**No**	**3.8**	**4.9-5.3**	**1.0**	**>28**	**>28**	**43**	**IV**
Ground beef, 21	67	No	Yes	ND	6.0	0.2	>21	>21	ND	V
Pork cubes, 14	2 [b]	Yes	Yes	ND	6.2	0.7	>14	14	ND	V
Pork cubes, 14	**515**	**No**	**No**	**ND**	**6.2**	**0.7**	**>14**	**>14**	**ND**	**V**

[a] Assuming a z-value of 7°C. The P$_{82.2°C}$ values originally determined in study IV were converted to P$_{85°C}$ values by using the formula $10^{(T-Tref)/z}$, where T is the actual temperature and T$_{ref}$ is 85°C.

[b] Heat process employed in the Finnish sous vide industry.

[c] NE, no effect; process temperature was below the model limits.

[d] The initial number of organisms was outside the model limits.

[e] Viable *C. botulinum* counts were detected with a low inoculum level of 10^2 spores/kg.

[f] ND, not determined.

[g] The products with no detectable *C. botulinum* or toxin during the entire storage period at any temperature are written in **bold text**.

5. RESULTS

5.1 Applicability of biochemical test systems in identification of *Clostridium botulinum* (I)

None of the three test systems was able to identify both group I and group II *C. botulinum*. Neither were they capable of distinguishing *C. botulinum* from their nontoxigenic counterparts. The API 20 A test alone identified most of the proteolytic *C. botulinum* strains (78%) and all the *C. sporogenes* strains correctly as *C. botulinum*/*C. sporogenes*. However, this test system misidentified all group II *C. botulinum* strains, most of them (68%) being identified as *Clostridium perfringens*. The Rapid ID 32 A and RapID ANA II misidentified all group I *C. botulinum* strains, whereas they both correctly identified the majority of group II *C. botulinum* strains (97 to 99%). However, they also falsely classified almost all nontoxigenic group II strains (97%) as *C. botulinum*.

5.2 Multiplex PCR detection of *Clostridium botulinum* (II)

The multiplex PCR yielded amplification fragments of 782 bp for *C. botulinum* type A strains alone, 205 bp for type B alone, 389 bp for type E alone, and 543 bp for *C. botulinum* type F strains alone. Other bacterial strains included in the study did not yield a PCR product. The sensitivity of the multiplex PCR, when using DNA from pure *C. botulinum* cultures as template, was 10 cells for type B and 10^2 cells for types A, E, and F. When present in the multiplex PCR, >0.2, >1.0, and >2.0 µg/µl of minced meat inhibited the amplification of type A fragment, types E and F fragments, and type B fragment, respectively. Fish and faeces did not inhibit the PCR at the level of 10 µg/µl reaction mixture. The detection limit of the entire assay including the two-step enrichment procedure was lowest, 10^{-2} spore/g, for *C. botulinum* type A in minced beef and for type B in minced beef and fish. The highest detection limit was 10^3 spores/g of faeces observed for type E. The optimal enrichment time varied from 1 to 5 d depending on the *C. botulinum* type and sample material, with only a 1-d incubation being required for the detection of *C. botulinum* type B in beef and type E in fish. Of the 72 naturally contaminated food samples, *C. botulinum* type A was detected in two fish heads (2.8%), types B and E in two vegetable sausages (2.8%), and type B in a can of deer meat (1.4%).

5.3 Heat resistance of nonproteolytic *Clostridium botulinum* type E spores in model fish media (III)

Biphasic thermal destruction curves were observed when heating 10^6 spores of nonproteolytic *C. botulinum* type E in model media consisting of rainbow trout or whitefish at the temperature range of 75 to 93ºC, followed by incubation in the presence of lysozyme at 30ºC for 90 d. The D-values obtained for the heat-resistant spore fractions (Table 8) were 10-to-50-fold longer than those obtained for the non-heat resistant spores. The spore heat resistance was greater in rainbow trout medium than in whitefish medium. The z-values obtained for the

heat-resistant spore fractions were approximately 10ºC (Table 8). The total heating times required to destroy the entire inoculum of 10^6 nonproteolytic type E spores were 149 min at 85ºC and 6.2 min at 93ºC for trout, and 55 min at 81ºC and 8.6ºC at 90ºC for whitefish.

Table 8. D and z-values of heat-resistant fraction of nonproteolytic *Clostridium botulinum* type E spores heated in model fish media at 75 to 93ºC.

Medium	D-values (min) at 75 to 93ºC					z-value
	75	81	85	90	93	(ºC)
Rainbow trout	255	ND[a]	98	ND	4.2	10.4
Whitefish	ND	55	ND	7.1	ND	10.1

[a] ND, not determined.

5.4 Safety evaluation and development of thermal processes employed in the REPFED industry (III-V)

5.4.1 Vacuum-packaged hot-smoked fish products (III)

The safety evaluation of the hot-smoking processes employed by five Finnish smoked fish companies revealed that the processes corresponded to heating at 85ºC for 6.1 min or less. In the inoculated pack study, a similar process of 1.5 min at 85ºC failed to eliminate 10^6 spores of nonproteolytic *C. botulinum* type E, and resulted in elevated viable counts in vacuum-packaged hot-smoked rainbow trout and whitefish, and toxin production in whitefish stored at 8ºC for 35 d. Moist heat treatments of 34 and 42 min at 85ºC, with RH>70%, were observed to control the growth and toxin production from 10^6 type E spores in smoked rainbow trout and whitefish, respectively, stored at 8ºC for 35 d (Tables 6 and 9). The high RH (>70%) in the smoking chamber was observed to significantly (p<0.01) enhance the thermal destruction of nonproteolytic type E *C. botulinum* spores; in whitefish processed at 85ºC for 44 min but in the presence of 10% RH, elevated viable *C. botulinum* counts and toxin production were observed within 35 d at 8ºC (Table 6).

5.4.2 Sous vide products (IV, V)

The safety evaluation of 16 sous vide products revealed that the processes employed by the Finnish sous vide industry corresponded to heating at 85ºC for <0.1-336 min. Only two of these processes with $P_{85ºC}$ values of 80 and 236 min were regarded as safe in the inoculated pack study. These processes controlled the growth and toxin formation from $10^{5.3}$ spores of nonproteolytic *C. botulinum* type B in pork and beef-based mixed foods stored at 8ºC for 28 and 37 d, respectively (Tables 7 and 9). All the other processes with $P_{85ºC}$ values of <0.1-336 min allowed for the survival of *C. botulinum* spores and subsequent elevation of viable *C. botulinum* counts. Furthermore, following processing at 85ºC for 15 min or less, toxin formation was observed at 8ºC in pork cubes and ground beef within 14 and 21 d, respectively

(Table 7). In study V, an attempt to improve the heat processing of the high-risk pork cube and ground beef products revealed that a heat process with a $P_{85°C}$ value of 515 min was sufficient to control botulinal growth and toxin formation in pork cubes stored at 8°C for 14 d. With beef, the increased process of 67 min at 85°C was sufficient at a storage temperature of 4°C, but allowed for elevated viable type B counts, although not toxin production in 21 d at 8°C.

Table 9. Heat processes observed to control the growth and toxin formation from $10^{5.3}$-10^6 spores of nonproteolytic *Clostridium botulinum* in REPFED products stored for 2-5 weeks at 8°C.

Product	Safe storage time (d)	$P_{85°C}$[a]	Study
Vacuum-packaged hot-smoked rainbow trout	35	34[b]	III
Vacuum-packaged hot-smoked whitefish	35	42[b]	III
Sous vide beef, vegetables, water	37	236	IV
Sous vide pork, vegetables, water	28	80	IV
Sous vide pork cubes	14	515	V

[a] Assuming a z-value of 10.1-10.4°C (study III) for fish and 7°C (ECFF, 1996) for sous vide foods.
[b] Provided that the RH inside the smoking chamber was higher than 70%.

5.4.3 Applicability of predictive microbiological models (IV)

The thermal death model by FMM was inconsistent with the PCR results obtained in the inoculated pack study (Table 7). More than 12-D reductions were predicted for products showing viable *C. botulinum* counts at sell-by date and/or 1 week after. The lag time for growth model by FMM and time to turbidity model by PMP (Table 7) were also inconsistent with the PCR results. Fail-safe predictions were generated in the majority of the cases, but with a beef liver product a 90-d safe storage time was predicted, while viable *C. botulinum* counts were detected in several samples stored at 4 and 8°C (Table 7).

5.5 Sensory evaluation of REPFED products (III-V)

All sensory attributes of the vacuum-packaged hot-smoked rainbow trout samples treated at 85°C for 42 min at >70% RH (study III) were significantly weaker than those of the samples processed for 0.3 min. However, all products were perceived to be optimally cooked. In whitefish products, only the juiciness was slightly decreased by processing at 85°C for 42 min in the presence of >70% RH in comparison to the mild processing. All products were considered to be cooked to the correct degree.

In reference to fresh products, the sensory quality of sous vide foods stored at 8°C for various times remained acceptable for human consumption during the entire storage period (study IV). As for sous vide meat products, heat treatments at 85°C for 67-515 min ensured equal or increased colour, flavour, aroma, juiciness, and tenderness when compared to heating at 85°C for 2 min or less (study V). Moreover, the effective heat treatment resulted in a decreased greyness in the ground beef.

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6. DISCUSSION

6.1 Evaluation of biochemical test systems (I)

The biochemical test systems, API 20 A, Rapid ID 32 A, and RapID ANA II were shown to be inappropriate for the identification of *C. botulinum* (study I). While the API 20 A system could only correctly identify the proteolytic *C. botulinum* strains, the Rapid ID 32 A and RapID ANA II systems misidentified all those strains but were accurate for the nonproteolytic *C. botulinum*. As group I and group II *C. botulinum* are phenotypically completely distinct (Smith and Sugiyama, 1988; Whitmer and Johnson, 1988) the result with API 20 A is not surprising since it only provides a single identification of '*C. botulinum*/*C. sporogenes*', and thus does not differentiate between the organisms of group I and II. The capability of API 20 A to identify *C. difficile* was greatly improved when the test strips were incubated for 48 h instead of 24 h (Gresser *et al*., 1984). In the present study, the extension of incubation time from 24 to 48 h improved the identification of some *C. botulinum* strains but impaired that of other strains, indicating that the enzymatic reactions by individual bacterial strains occur at different speeds. The Rapid ID 32 A and RapID ANA II test systems provide a possibility to identify both group I and II *C. botulinum*, but both of these tests failed to recognize the group I strains. This is in agreement with a previous report on the Rapid ID 32 A system that readily identified group II *C. botulinum* but failed with group I organisms (Brett, 1998). As reported with *C. difficile*, the presence of oxygen in the test environment may drastically affect the activity of some enzymes used in the identification with RapID ANA II (Peiffer and Cox, 1993). In the present study the hydrolysis of proline, phenylalanine, and arginine by the group I organisms was reduced in RapID ANA II as compared to the prediction provided by the test. This might be due to the aerobic incubation of the RapID ANA II test strips as instructed by the manufacturer.

None of the tests were able to distinguish *C. botulinum* from the nontoxigenic strains, which suggests that these strains are very similar to their toxigenic counterparts in terms of phenotypical characteristics. The genotypical relatedness between *C. botulinum* and the nontoxigenic strains has been shown (Lee and Riemann, 1970a, b; Nakamura *et al*., 1977).

6.2 Development of multiplex PCR assay (II)

The multiplex PCR assay developed in study II enabled sensitive and specific simultaneous detection of *C. botulinum* types A, B, E, and F in pure cultures as well as in food and faecal material. Four new primer pairs were designed in order to ensure optimal annealing at 60ºC. In the previously reported PCR protocols (Franciosa *et al*., 1994; Szabo *et al*., 1994; Sciacchitano and Hirshfield, 1996; Takeshi *et al*., 1996) the great variation between the melting temperatures of single primers would not allow multiplex reactions. The smallest size of the type B amplification fragment probably explains the sensitivity of the assay being 10-fold better for type B than for the other types. Moreover, the amplification of the type B fragment was not as likely to be inhibited by the sample materials as was that of types A, E, and F (II Table 3). Since the multiplex PCR assay was developed, two years' experience with

various types of sample materials has shown the amplification of the type A fragment to be more sensitive to inhibition than that of types B, E, and F. In study IV, as discussed in 6.4, it is probable that the greater amplification fragment size of *C. botulinum* types B and F as opposed to that of type E (Franciosa *et al*., 1994; Hielm *et al*., 1996) impaired the PCR detection of types B and F. Therefore, the multiplex PCR assay developed in the present work provides an appropriate tool for challenge tests employing a mixture of nonproteolytic *C. botulinum* types B, E, and F.

The detection limit of the entire assay including two-step enrichment was best (10^{-2} spores/g) for types A and B being detected in beef, and for types B and F detected in whitefish. However, the optimal enrichment times for type A in beef and type F in fish were 5 d, whereas only 1-d enrichment was required for the detection of type B in beef and type E in whitefish (II Table 4). As *C. botulinum* type B seems to be naturally present in meats and particularly type E in fish (Tables 1 and 2), it is possible that the natural niches also support the germination and growth of these organisms better than those of the other *C. botulinum* strains, which results in improved PCR detection. Toxin formation observed only in the vacuum-packaged hot-smoked whitefish products but not in trout products in study III also agrees with the finding on whitefish supporting *C. botulinum* type E growth. In faeces the detection limit was relatively high, indicating the suppression of botulinal growth by the presence of other microflora. Using the multiplex PCR assay, the prevalence of *C. botulinum* types A, B, and E in various foods sold in Finland were shown to be 3-9%, which is of the order of the figures reported earlier (Tables 1 and 2).

6.3 Heat resistance of nonproteolytic *Clostridium botulinum* type E spores in model fish media (III)

The heat resistance of nonproteolytic *C. botulinum* type E spores was greater in rainbow trout medium than in whitefish medium (Table 8), probably due to greater fat and lysozyme content in trout. The D-values measured for the heat resistant spore fraction in trout were also greater than those in fish and seafood reported in the literature; a $D_{75°C}$-value of 255 min was observed in this study, while earlier D-values for type E at 73.9-76.7 °C vary between 0.7 min in oyster homogenate (Chai and Liang, 1992) and 58.5 min in cod homogenate (Gaze and Brown, 1990) (Table 3). The difference may be explained by the effect of lysozyme, as lysozyme was not applied in the earlier studies. In phosphate buffer with lysozyme, a $D_{75°C}$-value of 273 min was obtained for nonproteolytic type B spores (Peck *et al*., 1993).

The reported $D_{85°C}$-values for types B and E in seafood without lysozyme supplement vary in the range of 0.2 (Cockey and Tatro, 1974) to 8.8 min (DePantoja, 1986), while those reported for type B in phosphate buffer in the presence of lysozyme are 48.5-100 min (Peck *et al*., 1993). The latter D-values are in agreement with the 98 min obtained for type E in trout in the present study. The present $D_{93°C}$-value of 4.2 min in trout medium is similar to those reported in phosphate buffer with lysozyme (Alderton *et al*., 1974; Peck *et al*., 1993), but also similar to those obtained in crabmeat in the absence of lysozyme (Peterson *et al*., 1997) (Table 3). The $D_{81°C}$-value of 55 min measured in whitefish medium corresponds to those reported in phosphate buffer with (Scott and Bernard, 1985) and without lysozyme (Smelt, 1980; Scott

and Bernard, 1982) at the temperature range of 80 to 80.2ºC (Table 3). The only earlier study conducted with whitefish reported D-values at 80ºC of 1.6 to 4.3 min without lysozyme (Crisley et al., 1968). The present $D_{90ºC}$-value in whitefish was 7.1 min, and is in the order of those reported for nonproteolytic type B spores heated in phosphate buffer (Smelt, 1980) and crabmeat (Peterson et al., 1997) without added lysozyme, but shorter than those measured for spores of types B and E in phosphate buffer in the presence of lysozyme (Peck et al., 1993) (Table 3). As lysozyme is probably not completely destroyed by heating (Garibaldi, 1986), it is possible that the great heat resistance observed in crabmeat (Peterson et al., 1997) is due to the activity of natural lysozyme or some other enzyme with a similar activity.

The z-values of approximately 10ºC obtained in the two fish media are somewhat higher than those generally reported for the nonproteolytic C. botulinum (ECFF, 1996). However, similar or greater z-values of 9.4 to 14.5ºC have been measured for nonproteolytic C. botulinum type E in phosphate buffer (Smelt, 1980), crawfish (DePantoja, 1986), turkey slurry (Juneja et al., 1995), and in carrot homogenate (Gaze and Brown, 1990), and for type B in phosphate buffer (Smelt, 1980; Scott and Bernard, 1982), turkey slurry (Juneja and Eblen, 1985), and in carrot homogenate (Gaze and Brown, 1990) (Table 3). The present z-values obtained in fish media were used in the inoculated pack study with fish products (III) to evaluate the processes with respect to the lethality to nonproteolytic C. botulinum type E spores. In comparison to the $P_{85ºC}$ values obtained with a z-value of 7ºC (ECFF, 1996), much shorter $P_{85ºC}$ values for the smoking processes were obtained with the present z-values (Table 6). This demonstrates the great importance of determining the heat resistance parameters for each heating medium in order to estimate better the thermal inactivation, and survival, of nonproteolytic C. botulinum in various media. Using a false z-value in estimating the lethality of an unknown process may therefore lead to significant misjudgement of process lethality with respect to C. botulinum spores.

6.4 Safety evaluation and development of thermal processes employed in the REPFED industry (III-V)

The safety evaluation of hot-smoking (III) and sous vide processes (IV) revealed that the majority of the processes employed in the Finnish REPFED food industry represented considerable under-processing with respect to lethality to nonproteolytic C. botulinum. Only two sous vide products possessed complete protection against nonproteolytic C. botulinum type B at 8ºC. The $P_{85ºC}$ values of the safe heat processes were 80 and 236 min. All hot-smoking processes (Table 6; III Table 1) and four sous vide products (Table 7; IV Table 4) were considered to possess a high risk related to botulinal growth and subsequent toxigenesis. The $P_{85ºC}$ values of the high-risk processes were 15 min or shorter. Based on $D_{85ºC}$-values determined for type E in fish media in the present study (III) and those for type B in meat (Scott and Bernard, 1985; Juneja and Eblen, 1995; Juneja et al., 1995; Juneja 1998), the high-risk processes were estimated to cause a less than 10^3 reduction in the number of nonproteolytic C. botulinum spores. This estimation was supported by the results obtained in the inoculated pack studies (III-V).

The pH value had a marked effect on the probability of growth of *C. botulinum* in the sous vide products. As opposed to the safe sous vide foods with $P_{85°C}$ values of 80 and 236 min, pork cubes, beef cubes, and beef liver cubes processed with $P_{85°C}$ values of 103-336 min, allowed botulinal growth at 4 and/or 8ºC. This is very probably explained by the difference between the pH measured in the products: the pH in the safe products varied in the range of 4.9-5.8, partly inhibiting the growth of nonproteolytic *C. botulinum* (Segner *et al*., 1966), while in the pork, beef and liver cubes pH was 5.8-6.1. As confirmed by others (Lund *et al*., 1985, 1990; McClure *et al*., 1994; Graham *et al*., 1996a, b; Graham *et al*., 1997), minor changes in the pH of a growth medium may influence the probability of growth and toxin formation by nonproteolytic *C. botulinum*. The pH and buffer capacity in different types of foods and raw materials varies greatly, and thus may even vary inside a single package of a ready-to-eat meal. In the REPFED food industry where a number of ready-to-eat meals are produced with a variety of ingredients, uncontrolled pH variations inside food packages may lead to the growth of pathogenic micro-organisms.

Due to the evident safety hazard presented by nonproteolytic *C. botulinum* in the REPFED products manufactured in Finland, an effort to improve product safety identified heat treatments providing protection against 10^6 nonproteolytic spores in vacuum-packaged hot-smoked rainbow trout and whitefish stored at 8ºC for 35 d (III) (Tables 6 and 9) and in sous vide processed pork cubes and ground beef stored at 4 and/or 8°C for 14-28 d (V) (Tables 7 and 9). In general, high-moisture (RH >70%) heat treatments equal to processing at 85ºC for 34 and 42 min were required for rainbow trout and whitefish products, respectively. A high RH of >70% inside the smoking chamber was an absolute prerequisite for the product safety; a 44-min heat process at 85ºC but with an RH of 10% resulted in toxigenesis in whitefish stored at 8ºC. An enhancing effect of moist heat on the thermal destruction of *C. botulinum* spores has been described (Alderman *et al*., 1972; Pace *et al*., 1972), and it is apparently due to an increased a_w on the fish surface. As study III indicated, the Finnish smoked fish industry has the technology required to control RH in the smoking chambers during processes. The implementation of hot-smoking processes with a high RH described in the present work is therefore strongly recommended in order to improve the safety of vacuum-packaged hot-smoked fish products. However, as the intrinsic control factors in hot-smoked fish products are limited, strict adherence to refrigerated storage must be stressed.

The moist heat treatments required to control nonproteolytic *C. botulinum* type E in fish were greater than the heat treatment of 18.1-23.3 min at 85ºC, reported to control the growth from 10^6 spores of nonproteolytic types B, E, and F in meat medium supplemented with lysozyme and stored for 48-64 d at 8ºC (Graham *et al*., 1996a; Fernández and Peck, 1999) (Table 4). In the present study, a 15-min process at 85ºC resulted in toxin formation in pork cubes stored for 21 d at 8ºC. Based on earlier studies on spore heat resistance with or without lysozyme (Smelt, 1980; Scott and Bernard, 1982, 1985; Peck *et al*., 1993; Juneja *et al*., 1995; Juneja, 1998), nonproteolytic type B spores seem to be more heat resistant than type E spores (Table 3). Therefore, as the observed heat resistance of type E spores in the fish (III) was much greater than that reported for type B spores in meat, it may be assumed that rainbow trout and whitefish provided protection for the type E spores, increasing their heat resistance.

Fat and protein are known to protect *C. botulinum* spores from heat (Lücke, 1985); this is particularly relevant for rainbow trout in which the fat content may be up to 20%.

As opposed to the relatively short heat treatments required for the fish products, a 515-min process at 85°C, regardless of the RH, was sufficient to control nonproteolytic *C. botulinum* type B in the sous vide pork cube product. In sous vide ground beef, heating at 85°C for 67 min combined with a storage temperature of 4°C was sufficient, but this combined with storage at 8°C allowed a slight elevation of viable *C. botulinum* counts, but not toxigenesis. In reference to the earlier *in vitro* studies with meat medium, it is evident that the meat products studied in IV and V increased the heat resistance of the nonproteolytic type B spores: while heating at 85°C for 18.1 min controlled nonproteolytic type B in meat medium with lysozyme at 8°C for 64 d (Graham *et al.*, 1996a; Fernández and Peck, 1999), a similar process at 85°C for 15 min resulted in toxin formation in pork cubes within 21 d at 8°C. Based on these findings, the importance of challenge testing with food products over *in vitro* experiments and predictive models was clearly demonstrated. This was supported by the observation that the predictions for the spore reduction, lag time to growth, and time to toxigenesis, generated by the predictive models FMF and PMP were inconsistent with the data obtained in the inoculated pack study (IV).

The heat treatments required to control *C. botulinum* in fish were generally shorter than those for sous vide products. Apart from the significant effect of RH enhancing the spore thermal destruction in fish, the obvious difference in the heat resistance between nonproteolytic type B and E spores (Table 3) has probably contributed to the process requirements. Furthermore, the sous vide products were packaged prior to processing, while the fish were processed unpackaged. Therefore, it is possible that the polyethylene film covering the sous vide foods retarded the heat penetration into the products. In order to simulate natural contamination of the products, the spore inoculum was mixed into the sous vide products with a relatively small particle size, while that in the fish was sprayed on the fish surface, more readily exposed to heat and moisture. A comparison between the two fish products also supported this theory: although the heat resistance of type E spores in trout medium was greater than in whitefish medium, all trout fillets remained nontoxic during the entire storage at 8°C, while whole whitefish allowed toxin formation at the same temperature. The rate of heat, moisture, and smoke transfer on the fillets was apparently greater than that in the whole fish carrying the spore inoculum inside the abdominal cavity. Additionally, evidence of whitefish supporting the growth of nonproteolytic *C. botulinum* type E is also reported in the study II.

The z-values of 10.1-10.4°C obtained in study III were used to determine the $P_{85°C}$ values of the fish hot-smoking processes required to control *C. botulinum* growth. Based on these values, relatively short heat treatments of 34-42 min at 85°C, in comparison to the 80-515 min at 85°C required with sous vide foods, were required to control the botulinum toxin formation in the fish products. By using the recommended z-value of 7°C (ECFF, 1996), the $P_{85°C}$ values of the safe heat treatments for trout and whitefish were 189 and 220 min, respectively (Table 6). Such processes would be of the order of those observed to ensure the safety of sous vide foods, and would probably deteriorate the sensory quality of fish. As the z-values of approximately 10°C were experimentally observed for nonproteolytic *C. botulinum*

type E spores in fish (III), these values and the corresponding $P_{85°C}$ values are probably closer to reality than the recommended values (ECFF, 1996) obtained for nonproteolytic type B spores *in vitro*. The short $P_{85°C}$-values of the safe smoking processes are explained by the effect of RH that is not taken into account when estimating pasteurization values.

The sensory quality of all sous vide foods remained acceptable during the entire storage period of 9-37 d at 8°C. Moreover, positively from the sensory aspect, the intensified heat processing of sous vide beef and pork products enhanced the sensory attributes of these food products. With fish products the effect of intensified processing was less favourable. All safely processed trout and whitefish products were perceived to be cooked to the correct degree. Naturally the prolonged heating of unpackaged fish at an increased temperature will result in a decrease in juiciness, while in the sous vide foods, packaged prior to processing, the juiciness was actually enhanced by the intensified heat treatments. Apart from a loss of juiciness, the safely processed whitefish were considered to be similar to the mildly processed whitefish products (III, Fig. 5). The sensory quality of the rainbow trout seemed to be somewhat more sensitive to the intensified heating combined with a high RH (III, Fig. 4). However, the use of the intensified high-RH processes in the fish industry, that currently employs too mild processes as determined in study III, is essential to improve the safety of vacuum-packaged hot-smoked fish products. Alternative control strategies such as restricted product shelf lives should otherwise be established to ensure the safety of vacuum-packaged hot-smoked fish products.

The PCR method combined with two-step enrichment (Franciosa *et al.*, 1994; Hielm *et al.*, 1996; Sciacchitano and Hirshfield, 1996) employed in the detection of viable *C. botulinum* counts in studies III-V, is very sensitive but does not differentiate between spores and vegetative cells of *C. botulinum*. To obtain more data on the growth behaviour of *C. botulinum* in the products, sampling at a shorter interval throughout the entire storage period would be desirable. However, conducting extensive safety evaluations, such as in study IV, with the PCR method used (Franciosa *et al.*, 1994; Hielm *et al.*, 1996) is very laborious and expensive, as a single serotype out of the three tested can be detected at a time. Moreover, as demonstrated in Tables 2 and 3 of study IV, the probability of detecting *C. botulinum* type E in the samples seemed to be greater than that for types B and F. This is probably due to inhibition of PCR, since the heat resistance of nonproteolytic *C. botulinum* type B and F spores seems to be greater than that of type E (Table 3). The use of the novel multiplex PCR method described in study II would therefore markedly facilitate safety evaluations.

7. CONCLUSIONS

1. None of the three commercially available biochemical test systems (API 20 A, Rapid ID 32 A, and RapID ANA II) were able to identify both group I and group II *C. botulinum*, neither were they capable of distinguishing group I and II *C. botulinum* from their nontoxigenic counterparts. Thus, these test systems are not suitable for the identification of *C. botulinum*.

2. The multiplex PCR assay developed provides a sensitive and specific tool for the simultaneous detection of *C. botulinum* types A, B, E, and F in pure cultures, as well as in food and faecal material. The method improves the diagnostics of the *C. botulinum* strains pathogenic to humans.

3. Biphasic thermal destruction curves were observed when recovering the heated spores in the presence of lysozyme, yielding greater D-values for the heat resistant spores permeable to lysozyme than for the non-heat-resistant spores. The z-values obtained for nonproteolytic *C. botulinum* type E in the fish media were approximately 10ºC. The heat resistance of nonproteolytic *C. botulinum* type E spores was affected by the heating medium, being greater in rainbow trout than in whitefish medium. This indicates that rainbow trout provides more protection against heat for *C. botulinum* spores than whitefish.

4. All fish hot-smoking processes and most of the sous vide processes evaluated, employed by the Finnish food industry, represented considerable under-processing with respect to elimination of nonproteolytic *C. botulinum* spores. Heat treatments equal to processing at 85ºC for 15 min or less were shown to possess a high risk, and resulted in growth and toxin formation from $10^{5.3}$-10^6 nonproteolytic spores in sous vide foods and in vacuum-packaged hot-smoked whitefish and in the inoculated pack studies.

5. Thermal processes controlling the growth and toxin production from $10^{5.3}$-10^6 spores of nonproteolytic *Clostridium botulinum* in vacuum-packaged hot-smoked fish products and sous vide products stored at 4 and 8ºC were identified. Moist heat treatments at 85ºC for 34 and 42 min, combined with RH>70%, controlled the growth and toxin formation from nonproteolytic type E spores in rainbow trout and whitefish, respectively, for 35 d at 8ºC. Heating at 85ºC for 515 and 67 min controlled the toxigenesis from nonproteolytic type B spores in sous vide pork cubes and ground beef, respectively, at 8ºC. However, elevated viable *C. botulinum* counts were detected in ground beef. The use of the described processes in combination with proper refrigeration is strongly recommended in order to improve the safety of REPFED products.

6. The sensory quality of the sous vide foods remained acceptable during the entire storage period at 8ºC. Intensified heating with processes shown to ensure the product safety with respect to nonproteolytic *C. botulinum* enhanced the sensory attributes of sous vide meat products, did not markedly affect those of hot-smoked whitefish, and slightly decreased those of hot-smoked rainbow trout products.

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