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PREVALENCE, MOLECULAR EPIDEMIOLOGY AND GROWTH OF *CLOSTRIDIUM BOTULINUM* TYPE E IN FISH AND FISHERY PRODUCTS

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This is not the end; this is only the beginning. It is time to move on and confront new challenges.

ABSTRACT

The prevalence of *Clostridium botulinum* type E in five commercially important raw fish species and seven various types of fishery products was determined by using quantitative PCR-based detection method. The applicability of randomly amplified polymorphic DNA (RAPD) analysis and repetitive element sequence-based PCR (rep-PCR) in subtyping *C. botulinum* strains was evaluated. Additionally, 92 type E isolates from different types of fish and seafood samples were characterized by pulsed-field gel electrophoresis (PFGE) and RAPD to determine the genetic biodiversity of type E strains. Challenge tests and predictive models were used to evaluate the safety of certain types of vacuum-packaged fishery products that were found positive for type E spores in the prevalence study. Additionally, the ability of sodium nitrite ($NaNO_2$) and potassium nitrate (KNO_3) to prevent the outgrowth and toxigenesis by nonproteolytic *C. botulinum* was assessed.

Both farmed and wild fish species in Finland were found to be contaminated by *C. botulinum* type E, with prevalence levels of 17% and 20%, respectively. Eight percent of fish roe samples, 5% of vacuum-packaged and 3% of air-packaged fishery product samples were positive for type E.

RAPD analysis was found to be a discriminating and reproducible method for the genotyping of type E strains. The highest discrimination was achieved by combining RAPD analysis with PFGE. The distinct advantages of RAPD as compared to PFGE were the 100% typeability and rapid performance. Rep-PCR lacked the discrimination to be a useful tool in *C. botulinum* typing.

High genetic biodiversity was observed among type E isolates regardless of the isolation source or geographical origin. Extensive genetic variation was observed between strains isolated from different fish species as well as among isolates from one fish species or even from one individual fish. Processing did not appear to favor survival of any particular genotype. The wide genetic biodiversity observed among *C. botulinum* type E strains was concluded to facilitate the use of DNA-based typing methods as a tool in contamination studies in food industry and in investigations of botulism outbreaks.

Using inoculums corresponding to the natural type E contamination level of fish, vacuum-packaged unprocessed and cold-smoked rainbow trout stored at 8^{o}C became toxic in two and three weeks, respectively. Even at 4^{o}C, the cold-smoked product turned toxic after

four weeks. Vacuum-packaged raw pickled (gravad) rainbow trout stored at 6°C was evaluated to be safe with respect to type E, provided that the declared shelf life was within reasonable limits. The results strongly indicated that unless the constant maintenance of a chill chain temperature below 3°C cannot be guaranteed, the shelf life of vacuum-packaged cold-smoked rainbow trout in its current formulation should be limited to ten days. However, the use of nitrite or nitrate in vacuum-packaged cold-smoked rainbow trout was found to reduce the hazard arising from *C. botulinum* type E at slightly abusive storage temperatures.

The applicability of currently available predictive microbiological models for safety evaluation of fishery products with respect to *C. botulinum* type E was found to be highly questionable. The models tested were hampered by the limitations of the controlling environmental factors set by the programs which had an adverse effect on the reliability of predictions.

LIST OF ORIGINAL PUBLICATIONS

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

I. Hyytiä, E., Hielm, S. and Korkeala, H. (1998) Prevalence of *Clostridium botulinum* type E in Finnish fish and fishery products. Epidemiol. Infect. 120, 245-250.

II. Hyytiä, E., Björkroth, J., Hielm, S. and Korkeala, H. (1999) Characterisation of *Clostridium botulinum* groups I and II by randomly amplified polymorphic DNA analysis and repetitive element sequence-based PCR. Int. J. Food Microbiol. In press.

III. Hyytiä, E., Hielm, S., Björkroth, J. and Korkeala, H. (1999) Biodiversity of *Clostridium botulinum* type E strains isolated from fish and fishery products. Appl. Environ. Microbiol. 65, 2057-2064.

IV. Hyytiä, E., Hielm, S., Mokkila, M., Kinnunen, A. and Korkeala, H. (1999) Predicted and observed growth and toxigenesis by *Clostridium botulinum* type E in vacuum-packaged fishery product challenge tests. Int. J. Food Microbiol. 47, 161-169.

V. Hyytiä, E., Eerola, S., Hielm, S. and Korkeala, H. (1997) Sodium nitrite and potassium nitrate in control of nonproteolytic *Clostridium botulinum* outgrowth and toxigenesis in vacuum-packed cold-smoked rainbow trout. Int. J. Food Microbiol. 37, 63-72.

1. INTRODUCTION

Development of new-generation foods, which are mildly processed, contain few or no preservatives, are packaged in vacuum or modified atmospheres to ensure long shelf life and rely primarily on refrigeration for preservation, has raised concerns of potential increases in botulism risk caused by psychrotrophic nonproteolytic group II *Clostridium botulinum* (Peck, 1997). In the fish industry, this hazard was recognized as early as in the 1960s, soon after the introduction of vacuum-packaging technology to fishery product manufacturing. Three successive serious *C. botulinum* type E outbreaks caused by vacuum-packaged hot-smoked fish from the Great Lakes area in the Unites States (Anonymous, 1960; Anonymous, 1963; Anonymous, 1964; Conner et al., 1989) lead to surveys on type E prevalence in aquatic environments both in North-America and Europe. These studies demonstrated that type E was most prevalent at cold and temperate regions of the northern hemisphere, with particularly high contamination levels in the Baltic Sea area (Johannsen, 1963; Cann et al., 1965; Huss et al., 1980). A high prevalence of type E in fish farms was also observed (Huss et al., 1974a; Cann et al., 1975; Tjaber and Håstein, 1975). In the United States attempts were made in 1964 to improve the safety of fishery products when guidelines for the production of smoked fish were established and vacuum-packaging was severely restricted (City of Milwaukee, 1964). Despite wide recognition of the emerging risk, few other actions were taken to improve the safety of fishery products or to further elucidate the epidemiology of type E. In Finland, only one small-scale survey including two fish farms was performed (Ala-Huikku et al., 1977). Interest in the issue gradually decreased, since additional significant outbreaks had not occurred. However, a cluster of four outbreaks (Anonymous, 1991; Öberg, 1994; Anonymous, 1998; Korkeala et al., 1998) associated with vacuum-packaged hot-smoked fish in northern Europe in the 1990s confirmed the emerging botulism risk which appeared to be increasing with wider introduction of various types of minimally processed foods.

In northern Europe, raw or mildly processed fishery products are commonly consumed and widely exported which increases the possibility that botulism outbreaks, like most other foodborne outbreaks, will affect more than one small geographic area or country (Majkowski, 1997). The negative publicity caused by a botulism outbreak can severely damage the economics of the fish industry in a small country such as Finland. Therefore, appropriate measures to control and decrease the risk caused by type E needs to be undertaken. Contamination studies in fish farms and fish manufacturing plants should to be performed to

facilitate the introduction and maintenance of the hazard analysis critical control point (HACCP) system. Before this can be accomplished, the basic epidemiology of type E must be investigated to obtain updated data on the prevalence of type E in different fish species and fishery products. Furthermore, the genetic biodiversity of type E strains must be studied in order to evaluate the applicability of DNA-based typing methods in epidemiological studies concerning type E. The diagnostics of type E has traditionally concentrated on the detection of botulinal neurotoxin (BoNT) from food and clinical samples by a mouse lethality assay (Hatheway, 1995). During this past decade, PCR-based techniques have facilitated the detection of *C. botulinum* without laborious toxin detection (Szabo et al., 1992; Campbell et al., 1993; Fach et al., 1993; Hielm et al., 1996). However, very few efforts have been made to develop modern research tools, which could be used to type botulinal strains to the subspecies level (Lin and Johnson, 1995; Hielm et al., 1998a).

Development of new types of products and even minor changes in formulation of existing products warrants a fresh look into their microbiological safety. Traditionally, the risk of the growth of pathogenic microorganisms and possible toxin production in foods has been determined through the use of inoculated pack studies (Roberts, 1997). Now, however, there are too many products, alternative ingredients, and process variations to conduct a complete laboratory evaluation of each possible contingency and potential foodborne pathogen for each product. Therefore, predictive food microbiology, the modeling of microbial populations, particularly those of foodborne pathogens, has become an active field of research. However, before predictive models can be considered reliable, they must be thoroughly validated by conducting challenge tests using various types of products (Whiting and Buchanan, 1994). With respect to *C. botulinum* type E in fishery products, validation data are limited. Additionally, the toxin production mechanism of *C. botulinum* in foods remains unclear due to the laborious nature of the conventional enumeration procedure, which employs the combination of either most probable number (MPN) method or conventional plating and the mouse lethality assay (Doyle, 1991; Austin et al., 1998). Moreover, there is a need to evaluate the use of additional hurdles to preserve fishery products and improve their safety.

2. REVIEW OF LITERATURE

2.1 Foodborne botulism

2.1.1 Pathogenesis and clinical symptoms

Clostridium botulinum is the causative agent of botulism. Because botulinal spores are widely distributed in the environment, they may be introduced into processed foods through raw materials or by post-processing contamination of foods. Foodborne botulism is an intoxication that results from the consumption of food in which *C. botulinum* has grown and produced potent neurotoxins. Botulinal toxins are unquestionably among the most potent poisons of biological origin (Lamanna, 1959). As little as 25-50 ng of neurotoxin can be lethal (Peck, 1997). Consumption of 0.1 g of food in which this bacterium has grown and produced toxin can result in severe illness. Based on the antigenic characteristics of the neurotoxin, seven serotypes (A-G) are recognized. Types A, B, E and F have been associated with human foodborne botulism cases. After ingestion, the botulinal toxin enters the circulation system via lymphatic system. It binds to receptors on nerve endings and blocks the release of the neurotransmitter acetylcholine at the neuromuscular junction, which causes the progressive descending flaccid paralytic symptoms typical for botulism (Hatheway, 1990; Rhodehamel et al., 1992). The usual incubation period is 18 to 72 h. The most common symptoms include gastrointestinal signs, dysphagia, dry mouth, diplopia, dysphonia, generalized weakness, blurred vision, dilated fixed pupils, ptosis, dizziness and respiratory impairment (Rhodehamel et al., 1992; Hatheway, 1995). The duration of acute symptoms ranges from one to ten days or more, depending on the resistance of the host, the type and amount of toxin ingested, and the type of food. The treatment consists of administration of therapeutic antitoxin and intensive supportive care, particularly respiratory assistance. Recovery involves the regeneration of new nerve endings. Therefore, full recovery may take several weeks to months. The estimated case/fatality ratio in foodborne botulism outbreaks worldwide is 20% (Hatheway, 1995).

2.1.2 Epidemiology

In recent years, an average of 450 outbreaks of foodborne botulism with 930 cases have been reported annually worldwide (Hatheway, 1995). Thirty-four per cent of the outbreaks were due to type A, 52% type B, and 12% type E. Type F is an extremely rare cause of foodborne botulism. 72% of the outbreaks and 48% of the cases were reported from Poland. Other countries with relatively frequent occurrences of foodborne botulism are China, Iran, the United States, Germany, France and Italy (Hauschild, 1992). A close association between the frequency and type of botulism outbreaks and the occurrence of *C. botulinum* in the environment has been shown (Hauschild, 1989). *C. botulinum* type E is a common cause of botulism in colder regions of the northern hemisphere (Hauschild, 1992). These outbreaks are mostly associated with fish and marine mammals. Types A and B are generally the cause of outbreaks in more temperate and warmer zones, with one type often predominating over the other. Proteolytic types A and B are linked to the majority of the outbreaks in the United States, China, South America and southern European countries, and the most frequently implicated foods are vegetables. In Central Europe the vehicle is most often a meat product with the causative organism being nonproteolytic type B strains. More than 90% of all outbreaks are caused by home-prepared or home-preserved foods (Hatheway, 1995).

2.1.3 Diagnostics

Foodborne botulism is first diagnosed on the basis of the patient's symptoms and food history. Diagnosis is confirmed by detecting toxin in patient serum sample or stool or in food consumed before the onset of illness (Hatheway, 1995). Recovery of *C. botulinum* from stools or gastric samples with symptoms and signs indicative of botulism is usually sufficient for confirmation. Recovery of the organism from food that does not contain demonstrable toxin is inconclusive. A recent study of foodborne botulism patients in the United States found toxin in the serum of 37% of the patients and the stool samples of 23% (Woodruff et al., 1992). *C. botulinum* was recovered from the stools of 51% and from the gastric aspirates of 45% of the patients whose samples were cultured. In 32% of the patients laboratory confirmation of illness failed, reflecting both inadequate sensitivity of diagnostic laboratory testing and delays in obtaining specimens. Electrophysiologic studies can provide a presumptive diagnosis of botulism in patients with clinical signs of botulism (Cherington, 1998) and can be especially helpful when laboratory tests are negative.

The mouse bioassay with seroneutralization is the standard procedure for determining the presence and type of *C. botulinum* toxin in foods, faeces, clinical specimens and enriched cultures (Hatheway, 1988). Although enzyme-linked immunosorbent assays (ELISA) for detecting botulinum neurotoxins have been developed (Notermans et al., 1982; Potter et al., 1993; Doellgast et al., 1994), none have been evaluated adequately or made commercially available for laboratories involved in investigating botulism outbreaks. Study conducted by Del Torre et al. (1998) described some of the problems associated with the use of ELISA tests by reporting cross reaction with metabolic products of other clostria present in samples. Recovery of the organism from stool and food samples is performed according to conventional culture methodology (Hatheway, 1988; Rodriguez and Dezfulian, 1997). Rapid test kits based on phenotypic characteristics of anaerobic bacteria have been developed, but their sensitivity and specificity have been shown to be inadequate for *C. botulinum* identification (Brett, 1998; Lindström et al., 1999). Recently, PCR-based analysis methods detecting the botulinal neurotoxin (BoNT) gene have been introduced in an attempt to replace the time consuming conventional methods (Szabo et al., 1992; Campbell et al., 1993; Fach et al., 1993; Hielm et al., 1996; Aranda et al., 1997).

2.2 DNA-based typing methods

Typing assays are necessary because several strains of a single species of microorganisms share overlapping niches or thrive under identical environmental conditions (Van Belkum, 1994). A bacterial species is an assemblage of isolates which originated from a common ancestor population in which a steady generation of genetic divergence resulted in clones (Maslow et al., 1993a). Clones are defined as genetically related isolates that are indistinguishable from each other by a variety of molecular typing methods (Farber, 1996). The genetic divergence of strains arise from random, nonlethal mutations that accumulate over time (Maslow et al., 1993a).

Genotyping methods involve the analysis of chromosomal or extrachromosomal DNA. Based on the technical aspects, six different categories of genotyping methods can be established (Tenover et al., 1997): plasmid fingerprinting; restriction endonuclease analysis (REA) of plasmid DNA; REA of chromosomal DNA by using frequent cutting enzymes and conventional electrophoresis; restriction fragment length polymorphism (RFLP) analysis by using DNA probes; macrorestriction pattern (MRP) analysis by using infrequently cutting

enzymes and pulsed-field gel electrophoresis (PFGE); and nucleic acid amplification-based typing methods by using PCR.

Several criteria must be considered in the evaluation of any typing system. The most important features are typeability, reproducibility, discriminatory power, ease of performance and ease of interpretation (Maslow et al., 1993a). The reliability of the typing system depends on the discriminatory power and the reproducibility of the method used. Problems encountered in plasmid analyses result from the fact that plasmids are extrachromosomal elements, which occasionally are very unstable (Maslow et al., 1993a). Additionally, not all organisms contain plasmids. REA of chromosomal material, on the other hand, is hampered by difficulties in interpretation due to numerous restriction fragments produced. By using probe-based techniques, the number of fragments seen in REA is significantly reduced making the interpretation easier, but the discriminatory power of this method is only moderate (Maslow et al., 1993a; Farber, 1996). The characteristics of PFGE and some PCR-based typing methods are considered in more detail in the following sections.

2.2.1 Pulsed-field gel electrophoresis (PFGE)

PFGE was first described in 1984 as a tool for examining the chromosomal DNA of eukaryotic organisms (Schwarz and Cantor, 1984). Subsequently, PFGE has proven to be a highly effective molecular typing technique for many different bacterial species (Maslow et al., 1993a; Tenover et al., 1997). In this method, the bacterial genome, which typically is 2000 to 5000 kb pairs in size, is digested with a restriction enzyme that has relatively few recognition sites and thus generates approximately 10-30 restriction fragments ranging from 10 to 800 kb (Maslow et al., 1993a).

Due to the large size of DNA fragments generated by infrequently cutting restriction enzymes, a special methodology to prepare unsheared DNA is applied (Finney, 1988): the intact microorganisms are embedded in agarose plugs, where the cells are lysed with suitable lytic agents. Contaminating cellular material and chemicals used to accomplish the lysis are removed with extensive washing. The isolated DNA is then digested *in situ.* The selection of restriction enzyme is usually based on GC %-content of the bacterial species being analyzed (Logonne, 1993). DNA fragments larger than 25 kb are not separated or are separated poorly by conventional gel electrophoresis. Therefore, PFGE applies a modification which involves periodically changing the orientation of the electric field across the gel (Finney, 1988). This is commonly done either by simply alternating the electric field between the forward and reverse

direction (field inversion gel electrophoresis, FIGE), or by generating uniform electric fields at 120^{o} to each other using hexagonal electrodes (contour-clamped homogenous electric field, CHEF) (Logonne, 1993).

In general, PFGE is one of the most reproducible and highly discriminatory typing techniques available and is currently the typing method of choice for many species (Tenover et al., 1997). The interpretation of PFGE gels is relatively straight forward, and consensus guidelines for correlating variations in macrorestriction patterns (MRPs) with epidemiological relatedness have been published (Tenover et al., 1995). Recently, a national network for subtyping foodborne pathogenic bacteria by PFGE was developed in the United States to facilitate a rapid recognition and/or confirmation of common source outbreaks (Swaminathan, 1998).

The main disadvantages of PFGE are the technical demands and the long time of performance required by the procedure (Tenover et al., 1997). However, recently a rapid DNA preparation procedure has been developed (Matushek et al., 1996). Other problems encountered with PFGE include inadequate cell lysis causing incomplete restriction digestion of the DNA (Farber, 1996), and the production of endogenous DNases by organisms such as *Campylobacter jejuni* (Gibson et al., 1994) and *Clostridium difficile* (Kato et al., 1994), resulting in degradation of the DNA before PFGE.

2.2.2 PCR-based typing methods

During the past decade, PCR-based DNA fingerprinting of microorganisms has been developed through the use of a wide variety of techniques and primer designs (Van Belkum, 1994). The basis of PCR-based DNA fingerprinting is that the primers can bind to specific regions of DNA, and when this binding occurs in the proper orientation and within an optimum distance, species or strain-specific amplification products, detected as amplification fragment length polymorphism (AFLP), are generated (Caetano-Anollés, 1993). The resulting PCR products will represent a variety of different-sized DNA fragments that are visualized by agarose gel electrophoresis. In comparison with other genotyping methods, PCR techniques are, in general, simpler, faster and require much less DNA for analysis (Kerr, 1994). However, standard guidelines for interpretation of PCR-fingerprints are not yet available (Tenover et al., 1997).

Randomly amplified polymorphic DNA assay (RAPD), also referred to as arbitrarily primed PCR (AP-PCR), employs a single short (typically 10 bp) primer that is not targeted to

amplify any specific bacterial DNA sequence (Williams et al., 1990). The primer will hybridize at multiple random chromosomal locations and initiate DNA synthesis. Amplification is conducted at low annealing temperatures, which allows for mismatches and thus permits arbitrary primer sequences to bind nonspecifically as well as specifically to the DNA template. Identification of a suitable primer, that provides consistent results, is difficult and must be done empirically (Tyler et al., 1997). The discriminatory power of the method is greatly dependent on the primers used (Kerr, 1994).

Due to its arbitrary nature, RAPD-PCR is susceptible to technical variation which may cause problems in reproducibility (Tyler et al., 1997). Arbitrary amplification protocols are affected by a number of factors, which include DNA extraction methods, ratio of DNA template concentration to primer concentration, batch-to-batch variation in primer synthesis, Mg^{2+}-concentration, PCR conditions, model of thermocycler used, and supplier and concentration of *Taq* DNA polymerase. Most of these problems can be overcome by optimizing the reaction conditions for each organism analyzed (Penner et al., 1993; Berg et al., 1994).

As an alternative to the arbitrary approach applied in RAPD, known conserved regions can be amplified with single or multiple primers in a way which gives rise to polymorphic DNA fingerprints. Bacterial genomes harbor repetitive sequences that can be used as primer targets in a technique known as repetitive element sequence-based PCR (rep-PCR) (Versalovic et al., 1991). These sequences are characterized by a length of 20-400 bp, presence throughout the entire genome but rarely within open reading frames and widespread occurrence among bacterial species (Van Belkum, 1994). Examples of these repetitive DNA motifs are repetitive extragenic palindromes (REP) and enterobacterial repetitive consensus elements (ERIC), both discovered in the genomes of *Escherichia coli* and *Salmonella typhimurium* (Stern et al., 1984; Hulton et al., 1991); the BOX element, which was discovered in the genome of *Streptococcus pneumoniae* (Martin et al., 1992) and RepMP3, which was first detected in the genome of *Mycoplasma pneumoniae* (Wenzel and Herrmann, 1988). All of these motifs are genetically stable and differ only in their copy number and chromosomal locations between species, thus making them desirable target for strain differentiation (Hulton et al., 1991). Due to defined primer sequences, high stringency amplification conditions can be applied in rep-PCR, and therefore, reproducibility of the method is not as much of a concern as in RAPD (Tyler et al., 1997). The discriminatory power of the method is generally considered to be moderate (Tenover et al., 1997).

2.2.3 Typing of *Clostridium botulinum* by DNA-based methods

The first report about genomic characterization of *C. botulinum* was published in 1995 and included MRP analysis of four type A strains by PFGE (Lin and Johnson, 1995). Recently, Hielm et al. (1998a) described the use of PFGE in genomic analysis of group II *C. botulinum* and found it to be highly discriminating and reproducible. However, not all strains were typeable either due to DNA degradation by active endonucleases or resistance of the cell wall to lysis.

The application of rRNA gene restriction pattern analysis (ribotyping) for the genomic characterization of *Clostridium botulinum* group I and II strains was also recently reported (Hielm et al., 1999). However, the discriminatory power was found to be lower than that of PFGE and there were some difficulties in the interpretation of patterns generated by certain restriction enzymes. Therefore, ribotyping was concluded to be suitable only for taxonomic purposes in *C. botulinum* species identification.

There are no reports concerning the use of PCR-based typing methods in the characterization of *C. botulinum* genomes. However, RAPD analysis has been used to type *C. difficile* isolates (McMillin and Muldrow, 1992; Killgore and Kato, 1994). Due to the limited number of studies focusing on genotyping *C. botulinum* strains, there is very little information available on the genetic biodiversity of type E strains.

2.3 *Clostridium botulinum* type E

2.3.1 Phenotypic characteristics

Clostridium botulinum type E is a gram-positive anaerobic spore-forming rod. Due to its phenotypic characteristics, such as an inability to digest casein, it belongs to the nonproteolytic *C. botulinum* group II (Lynt et al., 1982). The minimum temperature for growth and toxin production has been reported to be 3.3^{o}C (Schmidt et al., 1961). However, in a recent study, growth and toxin production were described at 3.0-3.2^{o}C (Graham et al., 1997). The temperature range of 25-37^{o}C is optimal for growth (Cato et al., 1986). Type E tolerates waterphase NaCl concentrations up to 5%, which corresponds to a_W value of 0.97 (Sperber, 1982). The minimum pH for growth is 4.8, with the optimum being 6.8 to 7.0 (Hobbs, 1976; Sperber, 1982). However, the effect of pH is dependent on the composition of the growth media (Sperber, 1982). Similarly, the maximal oxidation-reduction (Eh) values permitting

growth are dependent on the media. The highest value reported is 250 mV (Huss et al., 1980), but the optimal growth occurs at Eh below -198 mV (Ando and Iida, 1970).

Spores of type E demonstrate moderate heat resistance with $D_{80°C}$ values ranging from 0.6 to 3.3 min depending on the heating substrate and other variables such as pH, a_W, and salt content (Hobbs, 1976). Inclusion of lysozyme in the recovery medium to enumerate the survivors substantially increases the apparent heat-resistance of a small fraction of type E spores, resulting in biphasic survival curves (Peck et al., 1992). The spore coats of this subpopulation are naturally permeable to lysozyme, which permits lysozyme to diffuse from the recovery medium into heat-damaged spores to induce germination. The proportion of heated spores that are permeable to lysozyme varies from 0.1 to 20% depending on the strain (Peck et al., 1993). For example, 1.4% of the spores of type E strain Beluga were lysozyme-permeable with a $D_{85°C}$ value of 46 min, as opposed to lysozyme impermeable spores with $D_{85°C}$ value of < 1 min.

The concentration of botulinal toxin often encountered in toxic foods is at the level of 10^4 MLD_{50}/g (Hauschild, 1990). Huss et al. (1979) reported $5x10^3$ MLD_{50}/g in fresh herring inoculated with type E. Lethal doses of ingested toxin for humans are estimated as the toxin content of the food consumed by fatal or near-fatal cases of botulism without effective treatment. These range from $1x10^5$ to $5x10^5$ MLD_{50} for type E strains (Hauschild, 1990). The type E neurotoxin is heatlabile. However, the thermal inactivation of botulinal toxins is nonlinear and dependent on the heating media. For safe thermal inactivation of botulinal toxins up to 10^5 MLD_{50}/g in foods, Woodburn et al. (1979) recommended time / temperature combinations of 20 min at 79°C or 5 min at 85°C.

2.3.2 Prevalence in environment

Type E is the most prevalent *C. botulinum* serotype in aquatic environments of the northern sub-arctic and temperate zones. High incidences have been found from marine sediments in the coastal waters of Canada, Alaska, Greenland and Russia (Craig and Pilcher, 1967; Eklund and Poysky, 1967; Kravchenko and Shishulina, 1967; Nickerson et al., 1967; Miller et al., 1972; Huss, 1980). The Baltic Sea area appears to carry the highest contamination level in the world, with 81% (Hielm et al., 1998c) to 100% (Johannsen, 1962; 1963; Cann et al., 1965) of sediment samples being positive for type E. Freshwater sediments in various parts of the world, such as the United States (Bott et al., 1968), Japan (Yamakawa and Nakamura, 1992), Sweden (Johannsen, 1963) and Finland (Hielm et al., 1998c), have also been found to be

contaminated by type E. Studies performed in Germany (Bach et al., 1971), Denmark (Huss et al., 1974a), and Finland (Hielm et al., 1998b) have shown contamination of trout farm bottom sediments at a level of 29 to 68%.

It has been suggested that type E in the marine environment is of terrigenous origin and the spores were carried to the sea in water discharged from large land masses (Johannsen, 1962; Laycock and Loring, 1972). However, Bott et al. (1968) and Huss (1980) concluded that type E was a true aquatic organism, as few type E spores were found in terrestrial soils. Data collected by Hielm et al. (1998c) favored this theory in that type E counts positively correlated with low bottom oxygen content, depth and absence of bioturbation activity. Type E grows well in carrion of fish, marine mammals and invertebrates and these organisms also carry spores in their intestines. Water currents are another significant factor influencing in the distribution of spores through the marine environment (Huss, 1980).

2.3.3 Prevalence in raw fish

It has been demonstrated that inappropriately handled trash fish used for feeding farmed trout may cause severe losses due to type E intoxication of the trout (Huss and Eskildsen, 1974). However, the germination and growth of *C. botulinum* in live fish intestines has not been demonstrated. Thus, the presence of type E spores in fish must be regarded as a passive contamination and a reflection of the general contamination of the environment, feed and water of the harvest area. Therefore, extremely variable prevalences have been detected in different parts of the world. Houghtby and Kaysner (1969) reported low type E prevalences of 1 and 7% in salmon caught from rivers and coastal waters in Alaska and the Pacific Northwest of the Unites States, respectively. They also noticed that the contamination level was higher in fish caught from rivers than in fish caught from the ocean. When moving south along the Pacific cost, type E was virtually absent from fish and seafood, as revealed by the study of Baker et al. (1990a) in that only 0.6% of the samples were positive for type E. Five per cent of flounders and pollacks caught from the Atlantic cost of the United States were contaminated by type E (Nickerson et al., 1967). Bott et al. (1966) described an extremely variable prevalence in the freshwater fish caught from different parts of the Great Lakes with the prevalences varying from 0.7 to 57%.

In Europe, studies have mainly focussed on the harvest areas of the North Sea and the Baltic Sea. Fish caught from the North Sea appeared to be free of type E contamination (Cann et al., 1965; Cann et al., 1967). However moving towards Scandinavia, type E prevalence in

fish increased. Cann et al. (1965; 1967) reported that 54% of herrings caught from the Norwegian Sea were positive for type E. Johannsen (1963) reported a contamination level of 81% for herrings caught from the Swedish coastal waters in the Baltic Sea. Huss and Pedersen (1979) studied herrings from the Danish coastal waters and found only 4% of the samples positive. However, a considerably higher contamination level of 39% was detected in cods, plaices and eels caught from the same harvest area. The authors concluded that the bottom-feeding habit characteristic of these fish species was the reason for the high contamination level, as opposed to herring which are plankton-feeding fish. They suggested the findings indicate that the sea bed is the primary source of contamination.

Studies on the prevalence of type E in European freshwater wild fish are limited. The only study performed to date failed to detect any fish contaminated by type E (Huss and Pedersen, 1979). However, several studies have demonstrated high prevalences in European freshwater farmed trout with levels of 7% in Great Britain (Cann et al., 1975), 11% in Norway (Tjaberg and Håstein, 1975), 65% in Denmark (Huss et al., 1974a), and 16% in Finland (Hielm et al., 1998b).

Limited attempts have been made to quantitate type E spore counts in fish. By using MPN techniques, results varied from less than 5 cfu/kg to 5300 cfu/kg (Cann et al., 1966; Nickerson et al., 1967; Huss et al., 1974a, Baker et al., 1990b). The latter spore load was detected in Danish farmed trout and is the highest contamination level found in food to date.

Huss et al. (1974a) and Huss and Pedersen (1979) also examined the localization of contaminating spores in fish and found that some differences exist between fish species. Trout were mainly contaminated in intestines, as opposed to herrings which appeared to be contaminated on outer surfaces. Huss and Pedersen attributed this difference in localization of spores to a difference in biology. As pelagic plankton feeders, herrings are more likely to harbor spores on gills and outer surfaces, while farmed trout are often fed with heavily contaminated feed, such as trash fish, and are therefore more likely to harbor the major contamination in the viscera.

2.3.4 Prevalence in fishery products

Despite the substantial evidence of frequent contamination of fresh fish by *C. botulinum* type E in some regions, only a small amount of updated data is available concerning the prevalence of the organism in fishery products. Most surveys were performed in the 1960s and since then fish processing practices have been modernized and new regulations concerning food hygiene

have been implemented. Pace et al. (1967) studied the hot-smoking process of Great Lakes chub and showed that although the smoking process (82.2°C, 30 min) reduced contamination level, appreciable numbers of fish were still contaminated by type E spores after processing. An examination of 240 samples of various types of smoked fishery products manufactured by 28 small processors in the Pacific Northwest of the United States showed that 4.6% were contaminated by type E (Hayes et al., 1970). However, a more recent study by Heinitz and Johnson (1998) failed to detect positive samples in any of 201 vacuum-packaged cold- and hot-smoked fish and shellfish retail products in different regions of the United States. Similarly, none of hundred samples of cold- and hot-smoked fish obtained from retail outlets in the Toronto region of Canada contained *C. botulinum* (Dodds et al., 1992).

In Europe, type E prevalence has only been studied in the United Kingdom and in some Baltic Sea area countries. *C. botulinum* was not detected in over 200 samples of vacuum-packaged fishery products purchased at random from shops in England (Hobbs et al., 1965). In another study, 0.8% of 646 samples of vacuum-packaged cold-smoked herring was contaminated by type E (Cann et al., 1966). In a more recent study, all 82 samples of vacuum-packaged hot-smoked trout and mackerel purchased from retail outlets in the UK were negative for *C. botulinum* (Gibbs et al., 1994). In Sweden, Johannsen (1965) studied 144 samples of hot-smoked herring and found 4.2% positive for type E. Smoked Swedish eel processed at 55°C for 2 hours and at 60°C for further 30 minutes was still contaminated with type E spores (Abrahamsson, 1967). In Denmark, 5% of hot-smoked trout was found to be positive for type E immediately after processing (Huss et al., 1974b). There is no information available concerning the prevalence of type E in Finnish fishery products. Overall, the spore levels were reduced considerably in hot-smoked products as compared to fresh fish. The spore counts detected ranged from 0.1 cfu/kg to 10 cfu/kg (Pace et al., 1967; Abrahamsson, 1967).

2.3.5 Fish-related botulism outbreaks caused by type E

Botulism resulting from the consumption of fish has been recorded in the Russian literature since 1818 (Hobbs, 1976). During this century, the majority of type E outbreaks have been the result of poor home processing or the consumption of raw products (Hauschild, 1992). In Norway, during the 30-year period from 1961 to 1990, nine type E outbreaks were associated with the consumption of raw fermented trout, “rakefisk” (Hauge, 1970; Hauschild, 1992). Similarly, in Sweden, different types of raw fish products have been implicated in a number of type E outbreaks (Öberg, 1994). In a review of foodborne botulism in Alaska from 1947 to

1985, Wainwright et al. (1988) reported that 73% of the 44 laboratory confirmed outbreaks involved type E toxin. The implicated vehicles were native Alaskan foods, which included home processed fish, fish roe and products from marine mammals. These foods were prepared by traditional methods that involved minimal processing and/or slow cold-fermentations, and were consumed without further cooking. Recent modifications in traditional fermentation techniques have increased the risk of botulism in certain subgroups of Alaskan natives (Shaffer et al., 1990).

Before the early 1960s it was generally accepted that commercial processing of foods had addressed the botulism hazard associated with fishery products reasonably well. However, during the period from 1960 to 1963 commercially prepared vacuum-packaged hot-smoked fish from the Great Lake area was implicated in three outbreaks of type E botulism in the United States (Anonymous, 1960; Anonymous, 1963; Anonymous, 1964; Conner et al., 1989). These outbreaks served to demonstrate *C. botulinum* hazards associated with products not processed to the point of commercial sterility and subsequently subjected to temperature abuse. As a consequence, strict regulations delineating processing and distribution practices of vacuum-packaged hot-smoked fish in the Great Lake area were introduced (City of Milwaukee, 1964; Pace and Krumbiegel, 1973). These included heating fish to a minimum temperature of 82.2^{o}C for 30 minutes, a ban on vacuum-packaging, required refrigerated storage below 4.5^{o}C and a shelf life limitation to seven days. The high botulism risk associated with vacuum-packaged hot-smoked fishery products was further demonstrated by subsequent European outbreaks: vacuum-packaged hot-smoked trout and whitefish were implicated in one outbreak in Germany (Baumgart, 1970), in three outbreaks in Sweden (Anonymous, 1991; Öberg, 1994), and in two recent outbreaks in Germany (Anonymous, 1998; Korkeala et al., 1998). Commercially canned fish has been the source of type E botulism in a few outbreaks in the United States and Poland (NFPA, 1984; Hauschild, 1992). In most cases, post-process contamination through can leakage was the suspected cause of the outbreak.

"Kapchunka" is a commercially produced ready-to-eat salt-cured, air-dried, whole uneviscerated whitefish, which is an ethnic food of the Jewish Community (Rhodehamel et al., 1992). There have been three separate type E outbreaks during the period 1981-1987 due to consumption of this product in the United States and Israel (Slater et al., 1989; Rhodehamel et al., 1992). Kapchunka production involves a minimum refrigerated brining period of 25 days followed by a 3- to 7-day air-drying phase at ambient temperatures. In Egypt in 1991, similar type of a product caused one of the largest type E outbreaks known (Weber et al.,

1993) with at least 92 cases and 20 deaths being reported. The implicated food was a traditionally prepared fish product called "faseikh", which is prepared from fresh, uneviscerated mullet fish by fermenting for several hours to days, and then dry-salting in barrels.

2.4 Methods to control *Clostridium botulinum* type E in foods

The prevalence of *Clostridium botulinum* type E in processed foods is generally low. To maintain an indication of consumer risk obtained by routine sampling would require testing of an inordinately large number of samples (Hobbs, 1976). Control of botulism must therefore be achieved by adequate processing and storage control. Food processors have two main objectives in controlling *C. botulinum*: to destroy all botulinal spores in the product and to prevent the growth and subsequent toxin production by spores that have survived the processing. Control methods can be divided into physical and chemical treatments.

2.4.1 Physical treatments

The growth of *C. botulinum* type E can be inhibited by reducing water activity (a_W) below the level of 0.97 by drying or by adding food ingredients such as salt or sugar. The types of solutes used to decrease a_W may influence the minimum a_W for growth and toxin production. For example, Baird-Parker and Freame (1967) reported that germination and growth occurred at a lower a_W when the a_W was adjusted with glycerol rather than salt.

Pasteurization is the mild heat treatment of foods which kills the vegetative cells of most microbial species, and may reduce the number of *C. botulinum* type E spores. However, pasteurization alone is not sufficient to destroy type E spores although heat-injured spores are more susceptible to the inhibitory effects of other preservative treatments (Eklund, 1982). Spores are generally more sensitive to heat at extreme levels of pH than at values near neutrality. Dry spores are substantially more resistant to heat than moist spores, and it has been shown that reducing the water activity with salt increases the heat resistance of spores in mild heat treatments (Bucknavage et al., 1990). Similarly, Pace et al. (1972) showed that relative humidity at the level of 70% or above enhanced the quantitative destruction of type E spores in hot-smoking process in which the internal temperature of the fish reached 82^{o}C. Lysozyme, which is present in a large variety of raw foods (Lund and Peck, 1994), increases the heat-resistance of a subpopulation of spores of many type E strains in that the spore coat of

these spores is permeable to lysozyme which diffuses into the spore and replaces the heat-damaged germination system (Peck et al., 1992; Peck et al., 1993).

Thermal sterilization is a process in which low acid foods (pH > 4.6) that are packaged in hermetically sealed containers are processed at temperatures around 121^{o}C to destroy pathogens, including the spores of *C. botulinum*. The thermal process used to ensure the safety and stability of low-acid canned foods has been designed to provide at least a 12 log reduction in heat-resistant spores of *C. botulinum*, thus giving a rise to the classic 12-D (botulinum cook) concept, where one D equals 90 % destruction of the microbial population present (Rhodehamel et al., 1992).

C. botulinum spores can also be destroyed by radappertization (sterilization by radiation) by using high doses of irradiation. Irradiation does not inactivate botulinum toxin. Pasteurization by irradiation or radurization process has little or no effect on the spores. Eklund (1982) showed, in fact, that toxin production can be slightly more rapid in irradiated fish than untreated fish. Irradiation of fishery products has not been approved for commercial use anywhere (Farkas, 1998). However, in a number of countries, including France, the Netherlands, the United Kingdom and the United States clearances have been issued for radiation decontamination of poultry and red meats. The eventual acceptance of a radiation process will depend largely on whether the criteria laid down by health authorities governing the wholesomeness of irradiated foods can be satisfied. The usefulness of radiation sterilization will also depend on the successful control of undesirable organoleptic changes induced in foods by the high levels of irradiation required (Farkas, 1998).

Refrigerated or frozen storage is the most effective method of controlling botulism in mildly preserved low-acid foods. Freezing prevents germination, growth and toxin production. It has a slight lethal action on vegetative cells, but it does not affect spores or toxin (Hobbs, 1976). Refrigerated storage of products at < 3^{o}C is an adequate control of growth and toxigenesis by type E (Graham et al., 1997) but once again, the preformed toxin is stable at refrigerated temperatures. Spores can germinate relatively rapidly at temperatures as low as 2^{o}C, although the optimum temperature for full germination of type E spores is 9^{o}C (Grecz and Arvay, 1982).

Several studies have indicated that vacuum-packaging is more conducive to botulinum toxin production than CO_2-enriched modified atmospheres (MA) or storage in air (Cann et al., 1980; Post et al., 1985; Garcia et al., 1987). However, Baker et al. (1990a; 1990b) found that growth and toxin production by type E was only slightly faster in fish homogenate packaged in vacuum than that packaged in 100 % CO_2 MA. Larson et al. (1997) showed that the rate

and level of toxigenesis was equal in vegetables packaged under modified atmosphere in bags with differing oxygen and carbon dioxide transmission rates.

2.4.2 Chemical treatments

Growth and toxin production by type E do not occur above a waterphase NaCl concentration of 5% (Sperber, 1982) which corresponds an approximate NaCl level of 3.8% (w/w) in typical smoked fishery products. The preserving action of salt in most foods is closely related to other factors in the curing process. For example, use of nitrite, lowering the level of pH and an increase in the processing temperature can decrease the level of salt required to inhibit the growth of type E strains (Lynt et al., 1982)

Acidification is widely used to control *C. botulinum* in foods. A pH of 4.6 is generally considered to be the dividing line between low-acid and acid foods. Growth of type E does not occur at pH value below 4.8 (Sperber, 1982). Food products are acidified by the addition of acids or by production of acid by starter cultures.

Sodium nitrite is a multifunctional food additive that has been used for decades in combination with NaCl for curing fresh meats because it effectively inhibits the growth and toxigenesis of *C. botulinum* (Pierson and Smoot, 1987). Moreover, it is responsible for the development of typical cured meat color and flavor and also functions as an antioxidant. However, the use of nitrite is restricted or prohibited in many countries (Pelroy et al., 1982; European Parliament and the Commission of the European Communities, 1995) due to its capability under certain conditions to react with amines to form carcinogenic nitrosamine compounds (Walters, 1992). Nitrite exerts its antibotulinal effect in two different ways: it can form undissociated nitrous acid (HNO_2), which is able to pass the ion barrier of bacterial cell wall and disrupt the function of iron and sulfur containing enzymes (Pierson and Smoot, 1987). In heat treated products, nitrite forms the Perigo Type factor (PTF) which is inhibitory to *C. botulinum* (Perigo et al., 1967; Christiansen et al., 1973). The antibotulinal effect of nitrite is enhanced by an increase in NaCl concentration and a decrease in pH. The antibotulinal effect of nitrite in fishery products has been studied by Pelroy et al. (1982), who reported that when NaCl was the only preservative, the inhibitory concentration of waterphase NaCl was 3.8% for hot-processed salmon that had been inoculated with 100 type E spores/g and stored at 25^{o}C for seven days. However, when 100 mg/kg nitrite was included in the process, the necessary inhibitory concentration of NaCl lowered to 2.5%. Similar observation in corresponding experimental design was made by Cuppett et al. (1987) in hot-smoked

whitefish. The effect of nitrite on type E growth and toxigenesis in cold-smoked fishery products has not been studied. Nitrite levels in meat decrease during storage with the depletion rate being dependent on temperature and product formulation (Nordin, 1969; Christiansen, 1980). Ascorbate decreases the rate of nitrosamine formation (Bowen et al., 1974), and reduces the concentration of nitrite required to inhibit *C. botulinum* outgrowth (Tompkin et al., 1978).

Nitrate is used as an additive in the manufacture of cheeses to prevent "blowing" of the cheese caused by outgrowth of clostridia (Skovgaard, 1992). It is also used as an additive in the production of certain semi-preserved pickled fishery products to control the microbial activity during storage in barrels (Knøchel and Huss, 1984). Nitrate *per se* has no inhibitory effect on *C. botulinum* (Greenberg, 1972; Roberts, 1975), but when added to foods, it may act as a reservoir for nitrite, provided that nitrate-reducing bacteria are present (Skovgaard, 1992). Moreover, both nitrate and nitrite inhibit the growth of obligatory anaerobes by delaying the depletion of trimethylaminoxide (TMAO) in fish which results in a positive oxidation-reduction potential (Eh). There is no information available on the applicability of nitrate for preserving hot- or cold-smoked fishery products.

Smoke contains components such as formaldehyde, acetic acid, antioxidants and cresols, which may prevent spore germination and growth of certain microorganisms (Fretheim et al., 1980; Rhodehamel et al., 1992). Although smoking of meat reportedly had little effect on *C. botulinum* (Hauschild, 1989), Eklund et al. (1982) indicated significant effects of both natural and liquid smoke on *C. botulinum* type E in hot-processed whitefish.

Polyphosphates are used in brine-cured meat products to increase water-holding capacity, improve cooking yields and enhance functional qualities (Sofos and Busta, 1980). A study by Tompkin et al. (1979) showed evidence of antibotulinal properties of certain polyphosphates. The sequestering of iron and other cations is presumed to be the mechanism of the inhibitory action.

Among other compounds, potassium sorbate and sorbic acid (Sofos et al., 1979), nisin (Scott and Taylor, 1981) and sodium lactate (Meng and Genigeorgis, 1994) have been reported to posses some levels of antibotulinal activity. A number of these compounds may never be used as antibotulinal additives in foods because of toxicological considerations, high cost, impracticality in application, solubility characteristics and effects on product quality characteristics.

Due to the potential adverse health effects, the use of preservative chemicals has been decreasing over time and there has been an increased interest in the possible use of

biopreservation in the food industry (Conner et al., 1989). A technique known as Wisconsin process (Tanaka et al., 1980) involves the incorporation of lactic acid bacteria (*Pediococcus acidilactici*) and a fermentable carbohydrate into the food product. When the product is properly refrigerated, the added lactic acid bacteria remain largely inactive. However, if the product is temperature-abused, the lactic acid bacteria will grow and produce acid in amounts sufficient to preclude toxigenesis by *C. botulinum*. The application of this technique requires challenge studies on a product-to-product basis since the efficacy of biopreservation depends on the initial pH, inoculum level and type, level of carbohydrate, buffering capacity of the product, and the presence of inhibitory compounds.

2.4.3 Combination of preservative factors (hurdles concept) and predictive microbiology

Leistner and Rodel (1976) described the hurdles concept as a method to control the growth of microorganisms in foods. Several inhibitory parameters (hurdles) are used at subinhibitory levels instead of a single inhibitory level parameter (hurdle) to control growth and possible toxin production of foodborne pathogens. Roberts et al. (1981) used a combination of hurdles that included salt, nitrite, pH and heat treatment for inhibiting *C. botulinum* in shelf-stable, canned-cured meats. Tanaka et al. (1986) increased the safety of pasteurized processed cheese spread by controlling moisture content and pH and by adding phosphates and salt. Theoretically, *C. botulinum* must pass all of these barriers in order to grow and produce toxin. The effects of different combinations of heat treatment, pH and NaCl on the time for growth of type E in broth at different incubation temperatures have been reported recently (Fernández and Peck, 1997; Graham et al., 1997; Stringer et al., 1997). The effects of other preservative factors, such as lactate (Meng and Genigeorgis, 1993; Meng and Genigeorgis, 1994), nisin and other bacteriocins (Scott and Taylor, 1981), and modified atmospheres (Baker et al., 1990b; Reddy et al., 1997a) have also been evaluated in various types of food.

The number of factors affecting microbial growth is extensive. Therefore, it is impossible to simultaneously test all combinations of treatments. Moreover, it is very important to conduct challenge studies in an appropriate food system, since interaction effects may be different from laboratory media (Conner et al., 1989). An important step in quantifying the response of type E and other *C. botulinum* toxin types to combinations of preservative factors are predictive models. Predictive models are equations which can evaluate the information available in a large database to predict growth under defined conditions. Use of predictive models reduces the number of inoculated pack studies, which are time

consuming and expensive (Whiting and Buchanan, 1994). Additionally, predictive models can be used to develop monitoring devices, such as chemical or physical indicators, electronic integrators and loggers, which monitor the environmental factors that influence microbial growth during processing, storage and distribution (McMeekin et al., 1992; Skinner and Larkin, 1998). Initial models developed for *C. botulinum* were probability models which estimated the probability of toxin formation directly, by using a polynomial expression incorporating the environmental variables (Roberts et al., 1981; Lindroth and Genigeorgis, 1986; Tanaka et al., 1986). Whiting and Call (1993) and Whiting and Oriente (1997) developed time-to-growth models for *Clostridium botulinum* by using nonlinear regression to estimate the probability of growth at a given time. The most recent development in predictive microbiology with respect to *C. botulinum* is the waiting time modeling approach which allows the quantification of the customized margin of safety in analysis (Schaffner et al., 1998). Two growth models for *C. botulinum* are currently commercially available: Food MicroModel (Leatherhead Food Research Association, Leatherhead, Surrey, UK) and Pathogen Modeling Program (USDA Eastern Regional Research Center, Wyndmoor, PA, USA). However, predictive models cannot be used with confidence until they are validated by comparing the predictions to data obtained from inoculated pack studies (Whiting and Buchanan, 1994). The commercially available predictive models have not been validated in fishery products with respect to type E.

2.4.4 Recommendations to ensure the safety of refrigerated processed foods of extended durability (REPFEDs) with respect to nonproteolytic *Clostridium botulinum*

Refrigerated processed foods of extended durability (REPFEDs) rely on a mild heat treatment followed by storage at chill temperature for microbiological safety and quality. Recent research has identified combinations of mild heat treatments and subsequent refrigerated storage that, when combined with a specified shelf life, provide a limited safety margin with respect to nonproteolytic *C. botulinum* (Fernández and Peck, 1997; Graham et al., 1997; Stringer et al., 1997). Based on these research results, the Advisory Committee on the Microbial Safety of Food (ACMSF) (1992) and European Chilled Food Federation (ECFF) (1996) recommended procedures to ensure the safety of REPFEDs. According to ACMSF and ECFF, no requirements in terms of heat treatment or preservation system are specified, if the product is either stored at or below 3^{o}C or the product is stored at 3-5^{o}C for less than ten days or at 5-10^{o}C for less than 4-5 days. The microbial safety of products with a shelf life longer than ten days at chill temperatures (<10^{o}C), should be controlled by one or more of the

following factors: (1) minimum heat treatment of 90°C for 10 minutes or equivalent, (2) pH of 5.0 or less throughout the food, (3) waterphase NaCl level of 3.5% throughout the food, (4) a_W of 0.97 or less throughout the food, (5) a combination of heat treatment and preservative factors which have been shown to consistently prevent growth and toxin production by *C. botulinum*. ACMSF recommends that heat treatments or combination processes should reduce the number of viable spores by a factor of 10^6 (a 6-D-process).

2.5 Risk factors predisposing fishery products to growth and toxigenesis by *Clostridium botulinum* type E

In order for a foodborne type E botulism outbreak to occur, the following four prerequisites must be met (Eklund, 1982): (1) the raw material of the food is contaminated by type E spores from the environment; (2) the processing of the food is inadequate to inactivate type E spores, or the product is contaminated after processing; (3) the prevailing conditions in the food are favorable for the growth and toxigenesis of *C. botulinum;* (4) the food is consumed without cooking or after insufficient heating.

Data concerning type E prevalence in fresh fish indicate that the raw material of fishery products must be assumed to potentially contain type E spores in certain regions of the world. The role of post-process contamination is unknown since there are no reports evaluating type E contamination at fish processing plants. However, it is feasible to believe that if fish with low contamination levels are continuously being processed in a factory, there is a possibility of a build-up of manufacturing environment contamination by type E spores (Eyles and Warth, 1981; Huss, 1997).

Most fishery products are minimally or partially processed in order to preserve the sensorial quality of products (Richardson, 1994). These processes have virtually no effect on type E spores. For example, the raw pickling process includes only a short cold-fermentation of brined fillets with dry salt, sugar and spices (Jeppesen and Huss, 1992) and in the cold-smoking process, mildly brined fillets are subjected to temperatures ranging from 18-25°C (Huss et al., 1995). Additionally, hot-smoking process includes a stage where the temperature is raised between 70-90°C for a short period of time to mildly cook the flesh (Dillon et al., 1994) but the internal temperature of the product only reaches 65-85°C. Type E spores have been shown to survive a smoking process of whitefish where the internal temperature of the product exceeded 82°C (Pace et al., 1972).

Several factors favor the growth and toxin production by type E in the above named fishery products after processing. A main concern is possible temperature abuse which has

been shown to be common throughout the distribution and retail market, and at the consumer level. A survey performed in the United States found that 4-16% of retail store coolers and display cases were maintained at 13°C or above (Harris, 1989). Similarly, in a Greek study Sergelidis et al. (1997) concluded that domestic and retail store refrigerators are critical points of the cold chain in that 55 and 32% of domestic and retail refrigerators, respectively, functioned at temperatures 9°C or higher. Due to consumer demand for convenience and market demand for a long shelf life, fishery products are often packaged in high-barrier packaging material under vacuum or various modified atmospheres (Richardson, 1994). As a consequence, the growth of aerobic spoilage bacteria is restricted, and instead, the growth of anaerobic bacteria is favored (Genigeorgis, 1985). Moreover, long shelf lives ensure that even the heat-injured spores have time to recover. Increased consumer demands for healthier foods that are low in NaCl and calories and contain no preservatives have led the manufacturers to reformulate their products which traditionally were salty and heavily smoked (Rhodehamel et al., 1992). Due to public health concerns, the use of preservatives other than NaCl in fishery products is strictly regulated in many countries (Pelroy et al., 1982; European Parliament and the Commission of the European Communities, 1995).

Most fishery products are ready-to-eat delicacies that are consumed without heating which could inactivate the toxin (Huss et al., 1995). Since type E is a nonproteolytic organism, it produces no off-odors or off-flavors that would render the product unacceptable to the consumer and prevent consumption of a toxic product (Eklund, 1982).

The ability of type E to grow and produce toxin has been studied in vacuum-packaged raw fish of several different species (Huss et al., 1979; Post et al., 1985; Baker and Genigeorgis, 1990; Garren et al., 1995), in vacuum-packaged cold-smoked herring (Cann et al., 1980) and in vacuum-packaged hot-smoked salmon (Pelroy et al., 1982), whitefish (Cuppet et al., 1987), herring (Cann et al., 1980; Huss et al., 1980) and mackerel (Cann et al., 1980). However, in several of these studies the type E inoculums were grossly above the natural contamination level encountered in fish and often the inoculation was performed after the processing. Additionally, none of these studies quantified the growth of type E during the storage period.

3. OBJECTIVES OF THE PRESENT STUDY

The present substudies were conducted in order to

1. determine the prevalence of *C. botulinum* type E in Finnish raw fish and fishery products,

2. evaluate the applicability of randomly amplified polymorphic DNA analysis (RAPD) and repetitive element sequence-based PCR (rep-PCR) in subtyping *C. botulinum* type E as compared to PFGE,

3. examine the genetic biodiversity of *C. botulinum* type E strains using a combination of PFGE and RAPD to evaluate the effect of fish species, geographical location and processing on the genetic variation of the organism,

4. evaluate the capability of *C. botulinum* type E to grow and produce toxin in commercially important vacuum-packaged fishery products, and determine the association of growth with toxigenesis,

5. evaluate the ability of two currently available modeling software programs to predict the growth and toxin production by *C. botulinum* type E in vacuum-packaged fishery products,

6. determine the ability of sodium nitrite ($NaNO_2$) and potassium nitrate (KNO_3) to control the growth and toxin production of nonproteolytic *C. botulinum* in vacuum-packaged cold-smoked rainbow trout and investigate the effect of these preservatives on the microbiological and sensorial quality of the product.

4. MATERIALS AND METHODS

4.1 Sampling in prevalence study (I)

During the period from December 1994 to September 1996, 438 raw fish samples from five different fish species and 208 roe samples from four fish species were examined. The raw fish samples consisted of 178 intestinal, 157 surface and 103 whole fish samples. Whole intestines were included in the intestinal sample, and the surface sample consisted of skin, gills, fins and peritoneum of the fish. With small-sized fish species, such as Baltic herring and vendace, whole fish composite samples of 100-200 g were used. Sixty two per cent of the raw fish samples were non-farmed and were purchased from local market places or retail outlets as whole or gutted fish. The burbot, whitefish and Baltic herring were mainly of marine origin, while the vendace mainly originated in freshwater harvest areas in Finland. Rainbow trout intestines were obtained from six freshwater and nine marine trout farms from various locations in Finland. Rainbow trout surface samples originated in marine trout farms and were purchased from local market places or obtained directly from the farms. Overall, 20% of non-farmed and 33% of farmed raw fish samples were of freshwater origin. In terms of geographical distribution, the origins of the raw fish samples were representative of major Finnish harvest and fish farming areas. Of the fish roe samples 54 were fresh with no preservatives, while 154 were frozen and contained approximately 3% NaCl (w/w). All frozen and some fresh roe samples were purchased from local retail outlets. The fresh burbot roe samples were obtained from the intact roe pouch before evisceration.

During the period from November 1995 to January 1997, 214 vacuum-packaged raw pickled, cold- and hot-smoked rainbow trout and salmon and hot-smoked whitefish samples, and 123 air-packed hot-smoked Baltic herring, vendace and river lamprey samples were examined. The samples were purchased from local retail outlets. The vacuum-packaged fishery product samples were produced by 25 Finnish manufacturers. The NaCl concentration of the products as stated by the manufacturers varied from 1.1 to 4.1% (w/w). No other preservatives were included, with the exception of sodium benzoate in the hot-smoked products of some manufacturers. Neither the manufacturer's names nor the NaCl concentration of the air-packaged products were provided to the investigators.

4.2 *Clostridium botulinum* strains genotyped (II, III)

Fifteen *Clostridium botulinum* group I and 21 group II strains were used in the evaluation of the applicability of RAPD and rep-PCR in subtyping *C. botulinum* strains. The details of the origins of the strains are given in Table 1 of paper II.

In order to study the genetic biodiversity of *C. botulinum* type E strains, the following isolates were characterized: 56 isolates from fresh fish caught or farmed at various geographical locations in Finland; 11 isolates derived from Finnish fishery product samples produced by six different manufacturers; 15 strains isolated from samples of German farmed fresh fish; ten strains of North American and North Atlantic origin (31-2570 E, RS-1 E, Beluga E, C-51 E, C-60 E, C-94 E, 250 E, 36208 E, KA-2 E, and 4062 E), which were isolated from different types of seafoods over a period of 60 years, as shown by Table I of paper II.

4.3 Detection, quantification and isolation of *Clostridium botulinum* (I-V)

Samples were examined for the presence of *Clostridium botulinum* by using a quantitative serotype-specific PCR analysis, which was based on a 3-tube most probable number (MPN) procedure (Hielm et al., 1996). Briefly, logarithmic dilutions of samples were inoculated into tubes of tryptone-peptone-glucose-yeast extract (TPGY) broth (Difco, Detroit, MI, USA; Food and Agriculture Organization, 1991) and incubated anaerobically at 26^{o}C for three days. Washed and boiled cells from the overnight cultures were used as template DNA for PCR. DynaZyme^{TM} DNA polymerase (cloned from *Thermus brochianus*; Finnzymes, Espoo, Finland) and a 96-well PTC-100 thermal cycler (MJ Research, Watertown, MA, USA) were used for PCR. The molecular weight of the amplified PCR products were determined by agarose gel electrophoresis with comparison to standard DNA fragments (DNA molecular weight marker VI; Boehringer Mannheim GmbH, Mannheim, Germany). Results were reported as the mean counts of parallel samples.

C. botulinum type E strains were isolated from PCR-positive samples following the method of the Nordic Committee on Food Analysis (1991b) with the modification of using PCR detection instead of a mouse bioassay.

4.4 Toxin analysis (I, IV, V)

The procedure for the assay of botulinum toxin followed the Nordic Committee on Food Analysis protocol (1991a), with minor modifications. Comminuted fish samples (20-25 g) were homogenized with gelatin phosphate buffer in a ratio 1:2 (w/v) for two minutes in a Stomacher 400 Lab Blender (Seward Medical, London, UK). The homogenate was centrifuged at 10,000-15,000 x g for 15 minutes at 6°C (Sigma 3K 30; Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). Trypsin activated extracts (1.0 ml) were injected intraperitoneally into two white mice (20-25 g). The mice were observed for botulinal symptoms for seven days. If death with typical symptoms was recorded for one or both of the mice, the injection was repeated with a heat-treated extract. The bioassays were approved by the Committee on Animal Experimentation of the Faculty of Veterinary Medicine.

4.5 Cultivation of *Clostridium botulinum* strains and isolation of chromosomal DNA (II, III)

The cultures were grown for three days on anaerobic egg yolk agar (American Public Health Association, 1992) from which TPGY broth was inoculated. Group I cultures were incubated at 37°C in a culture jar with a model BR 38 gas-generating kit (Oxoid, Basingstoke, UK). Group II strains were incubated at 26°C in an anaerobic cabinet with an internal atmosphere of 85% N_2, 10% CO_2 and 5% H_2 (MK III; Don Whitley Scientific Ltd., Shipley, UK).

Conventional DNA isolation for RAPD and rep-PCR was performed according to the method by Marmur (1961) with several modifications. Cells were harvested by low-speed centrifugation (1500 x g) at 4°C (Sigma 3K 30; Sigma Laborzentrifugen GmbH) from a 5-ml volume of overnight culture (14 h). The cells were resuspended in 600 µl of TE (10 mM Tris [pH 7.5], 1 mM EDTA [pH 8.0]) containing 8.3 mg of lysozyme per ml (Sigma St. Louis, MO, USA) and 167 U of mutanolysin per ml (Sigma) and incubated at 37°C with gentle shaking either for 15 min (group I) or 2 h (group II). To obtain complete lysis, 9.5 mM EDTA (pH 8.0), 0.24 M NaCl, 49 µg of proteinase K per ml (Finnzymes) and 0.8% (v/v) sodium dodecyl sulfate were added and the mixture was incubated with gentle shaking at 60°C for 1 h. Subsequent phenol/chloroform/isoamyl alcohol (25:24:1, v/v) and chloroform/isoamyl alcohol (24:1, v/v) extractions were performed and DNA was precipitated by ice-cold ethanol (95%,

v/v), air dried and redissolved in TE buffer (10:1, v/v) overnight. RNase-treatment was performed by the addition of 164 µg of RNase per ml (Sigma) and incubation of the mixture with gentle shaking at 37°C for 40 min. 0.21 M NaCl was added and subsequently chloroform/isoamyl alcohol extraction and ethanol precipitation were performed. The DNA was resuspended in 100 µl of TE buffer (10:1, v/v) and quantified with a spectrophotometer (Hitachi U-2000; Hitachi Ltd., Tokyo, Japan). For RAPD-PCR and rep-PCR analyses the DNA was diluted in TE buffer to a final concentration of 5 ng/µl and 50 ng/µl, respectively.

Agarose-embedded DNA intended for PFGE analysis was isolated according to Maslow et al. (1993b), with the modifications presented by Hielm et al. (1998a). Briefly, overnight TPGY cultures in late log phase (16 h) were chilled on ice and resuspended in PIV (10 mM Tris [pH 7.5], 1 M NaCl) containing 3.5-4.0% (v/v) formaldehyde solution and placed on ice for 1 h. Cell suspensions were mixed with an equal amount of 2% (w/v) low melting temperature agarose (InCert agarose; FMC Bioproducts, Rockland, ME, USA) and cast in GelSyringe dispensers (New England Biolabs, Beverly, MA, USA). The resulting plugs were lysed for 2 h in lysis solution (6 mM Tris [pH 7.5], 1 M NaCl, 100 mM EDTA [pH 8.0], 0.5% brij-58, 0.2% deoxycholate, 0.5% sodium lauroyl sarcosine, 20 µg/ml RNase, 1 mg/ml lysozyme, 10 U/ml mutanolysin) with gentle shaking at 37°C and DNA isolation was completed with a 1 h ESP (0.5 M EDTA [pH 8.0], 10% sodium lauroyl sarcosine, 100 µg/ml proteinase K) wash at 50°C, followed by phenylmethylsulfonyl fluoride inactivation of proteinase K.

The DNA of all strains was isolated a minimum of two times from separate colonies with both methods.

4.6 PCR-based typing methods (II, III)

RAPD analysis was performed by using Ready-To-Go RAPD Analysis Beads™ (Pharmacia Biotech AB, Uppsala, Sweden), as described by the manufacturer, carefully observing factors affecting reproducibility (Tyler et al., 1997). Amplifications were performed in a PTC-100 thermal cycler (MJ Research) for 45 cycles of 1 min at 95°C, 1 min at 36°C and 2 min at 72°C, with a 5 min initial denaturation at 95°C and a 5 min final extension at 72°C. The sample volume of 25 µl contained 10 ng of DNA and 25 pmol of a single oligonucleotide primer. Amplification products were electrophoresed in 2.0% (w/v) agarose gels (MetaPhor Agarose; FCM BioProducts) in 1 x TAE buffer (Amresco, Solon, OH, USA) at 80 V for 5 h. The gels

were stained for 20 min in 1.5 l of distilled water containing 0.5 mg of ethidium bromide and destained for 40 min in distilled water before photography by standard procedures (Sambrook et al., 1989). DNA molecular weight marker VI (Boehringer Mannheim GmbH) was used as a fragment size marker. To identify the most discriminatory primers, 27 arbitrary primers belonging to two commercially available primer sets (RAPD Analysis Primer SET 1-6, Pharmacia Biotech; Operon RAPD 10-mer Kit J 1-20, Operon Inc. Alameda, CA, USA) were tested with eight representative strains. The four primers exhibiting the highest discriminatory power (Pharmacia 5, OPJ 6, OPJ 13 and OPJ 16) were selected for use in method evaluation (paper II). Primers OPJ 6 and OPJ 13 were used to study the biodiversity of type E strains (paper III).

Rep-PCR analysis was performed according to the method of Versalovic et al. (1991), with minor modifications and carefully observing factors affecting reproducibility (Tyler et al., 1997). Ready-To-Go PCR Beads™ (Pharmacia Biotech) were used for PCR reactions. Amplifications were performed in a PTC-200 thermal cycler (MJ Research) for 35 cycles of 30 s at 90^{o}C, 1 min at 40^{o}C and 8 min at 65^{o}C, with a 7 min initial denaturation at 95^{o}C and a 16 min final extension at 65^{o}C. The sample volume of 25 µl contained 100 ng of DNA and 50 pmol of each primer. Amplification products were electrophoresed in 2.0% (w/v) agarose gels (NuSieve 3:1 Agarose; FCM BioProducts) in 1 x TBE buffer (Amresco) at 80 V for 5 h. The gels were stained for 1 h in 1.0 l of distilled water containing 0.5 mg of ethidium bromide, destained for 1 h in distilled water and photographed with standard procedures (Sambrook et al., 1989). DNA molecular weight marker VI was used as a fragment size marker. Two opposing degenerate primers REP1R-Dt (5'-IIINCGNCGNCATCNGGC-3') (N=A, T, C or G; I=iosine) and REP2R-Dt (5'-NCGNCTTATCNGGCCTAC-3') and two single oligo-nucleotide primers BOXA1R (5'-CTACGGCAAGGCGACGCTGACG-3') and RW3A (5'-TCGCTCAAAACAACGACACC-3') were initially evaluated by using different annealing temperatures in amplifications with respect their applicability in *C. botulinum* genotyping with eight representative strains. Only the degenerate primers (REP1R-Dt and REP2R-Dt) were found to be discriminatory. All primers were synthesized by Pharmacia Biotech (Vantaa, Finland).

The reproducibility of both methods was verified by repeating all amplifications from two isolates of the same strain a minimum of two times with all strains. The banding patters were interpreted visually. Patterns with two or more fragment size differences were classified as belonging to different clones. Faint bands were included in the fingerprint only if they were detected reproducibly from two isolates of the same strain.

4.7 Macrorestriction analysis by PFGE and cluster analysis (III)

Restriction endonuclease digestion of the agarose embedded *Clostridium botulinum* DNA was performed as described by the manufacturer by using three rare-cutting restriction enzymes (*Sma*I, *Xho*I and *Xma*I [New England Biolabs]). All samples were electrophoresed with a Gene Navigator system (Pharmacia Biotech) with a hexagonal electrode through a 1% (w/v) agarose gel (SeaKem Gold; FMC Bioproducts) in a 0.5 x TBE buffer (Amresco). Switch times were ramped from 1 to 24 s over 22 h at 14^{o}C and 6 V/cm. Low Range PFG marker (New England Biolabs) was used for fragment size determination. The gels were stained for 30 min in 1 liter of running buffer containing 0.5 mg of ethidium bromide and destained in running buffer until appropriate contrast was obtained for standard photography (Sambrook et al., 1989) or/and digital imaging with Alpha Imager 2000 documentation system (Alpha Innotech, San Leandro, CA, USA).

*Sma*I/*Xma*I and *Xho*I MRPs in the molecular size range of 50-350 kb were analyzed by using GelCompar software (version 4.0; Applied Maths, Kortrijk, Belgium). The similarity between all MRPs was expressed as Dice coefficient correlations, according to the equation $S_D = [2n_{AB} / n_A + n_B] \times 100$, where n_{AB} is the number of matched fragments, and $n_A + n_B$ the total number of fragments in profiles A and B (Applied Maths, 1996). The position tolerance for band matching was set at 1.4% of the total length of the pattern (300 kb), with no increase. Arrangement of *Sma*I/*Xma*I and *Xho*I MRPs into dendrograms was accomplished by using the unweighted pair group method with arithmetic averages (UPGMA). The genotypes resulting from MRP analyses were clustered at a similarity level of 96% with *Sma*I/*Xma*I digests and 90% with *Xho*I digests, referring to possible epidemiological relatedness according to the guidelines set out by Tenover et al. (1995). These similarity levels corresponded roughly to a three band difference.

4.8 Challenge tests (IV, V)

Inoculated pack studies were performed by using vacuum-packaged rainbow trout and the following treatments: (a) no processing, (b) raw pickling (gravad), (c) NaCl-treatment and cold-smoking, (d) NaCl+$NaNO_2$ -treatment with two different nitrite concentrations (d1 and d2) and cold-smoking, and (e) NaCl+KNO_3 -treatment with two different nitrate concentrations (e1 and e2) and cold-smoking. Additionally, (f) depletion studies of nitrite and

nitrate together with microbiological and sensorial analyses were carried out with uninoculated samples. In studies d, e and f, nitrite- and nitrate-treated samples were compared to NaCl-treated controls. Later in the text, the studies will be referred to by using the corresponding letter or letter-number combination.

4.8.1 Fish (IV, V)

For the inoculation studies and the depletion study, rainbow trout *(Oncorhyncus mykiss)* were obtained from a local fish manufacturing plant where they had been beheaded and filleted. The fillets had an average weight of 600-900 g.

4.8.2 Brining (IV, V)

Brining was carried out by the injection method. The pressure used in the brine injection machinery (Fomaco 44/176; Fomaco Food Machinery Company A/S, Køge, Denmark) was 1.6 bars and the concentration of the brine was 21%. The fillets for raw pickled product were dry-salted overnight at 3^{o}C after curing by the injection method. The waterphase NaCl concentrations of the final raw pickled product and the final cold-smoked product were 6.7% ± 0.26 [mean ± standard error (SE)] and 3.2% ± 0.08, respectively. In studies d1, e1, and f, the $NaNO_2$ and KNO_3 (Riedel-deHaën AG, Seelze, Germany) concentrations of the curing solutions were 3 g/l and 13 g/l, respectively, yielding nitrite and nitrate concentrations of 166 mg/kg ± 9 and 686 mg/kg ± 67 (mean ± SE) in the final cold-smoked product, respectively. In studies d2 and e2, $NaNO_2$ and KNO_3 concentrations of the curing solutions were 2 g/l and 10 g/l respectively, yielding nitrite and nitrate concentrations of 109 mg/kg ± 7 and 347 mg/kg ± 29 in the final cold-smoked product, respectively.

4.8.3 Sample inoculation (IV, V)

Details of the strains and inoculums used in studies a, b and c are presented in Table 1 of paper IV. In studies d1 and e1, a mixture of five strains of nonproteolytic *C. botulinum* was used with low and high inoculums of 810 cfu/kg and 20210 cfu/kg The spore mixture contained three strains of type E (Beluga E, 211 E, C-60 E), one strain of type B (2 B) and one strain of type F (FT 10 F). In studies d2 and e2, three strains of type E were used (Beluga E, 211 E, C-94 E) with low and high inoculums of 140 cfu/kg and 4140 cfu/kg. The strains were

obtained from the National Serum Institute (Copenhagen, Denmark) and from the collection of Dr. Seppo Lindroth which has been maintained at the National Veterinary and Food Research Institute (Helsinki, Finland). Spore suspensions of individual strains were prepared according to the method of Food and Agriculture Organization (1991). Before inoculation, the suspensions were enumerated according to Doyle (1991). The spore mixture used for inoculation contained an equivalent number of non-heat-shocked spores of each strain diluted in sterile distilled water; 1 ml of dilution contained the required spore load for one sample. Before inoculation the brined fillets were sliced into portions weighing 300 g (d1, e1) or 200 g (all the other inoculation studies). The samples were surface-inoculated immediately before processing.

4.8.4 Processing and storage conditions (IV, V)

The raw pickling process used in study b was performed by slicing and then vacuum-packaging fish fillets that had been ripened overnight in dry-salt. Dill and other spices typical of raw pickled fishery products were not used in order to minimize uncontrolled factors in the samples. Cold-smoking of the inoculated fillets in studies c, d and e was performed in an electronically controlled, electrically heated smokehouse equipped with an external smoke generator (Vemag; Kerres GmbH, Sulzbach am Murr, Germany) at 18-21^{o}C for 20 h. In all inoculation studies the fillets were vacuum-packaged (Multivac A 300/16 1986; Multivac Verpackungsmaschinen, Wolfertschwenden, Germany) in polyamide/polyethylene films (Wipak Oy, Nastola, Finland) with an oxygen permeability of 31 cm^3/m^2/24 h (23^{o}C, 50% RH) and a water vapor permeability of 1.6 g/m^2/24 h (38^{o}C, 90% RH) immediately after inoculation and/or processing.

Uninoculated fillets used for nitrite and nitrate depletion studies and for microbiological and sensorial analyses (f) were cold-smoked in an electronically controlled, electrically heated smokehouse equipped with an external smoke generator (Alpas; Alpas GmbH, Bremen, Germany) at 18-21^{o}C for 20 h. Fillets were then divided in half and vacuum-packaged (Multivac R 7000 1976; Multivac Verpackungsmaschinen) in polyethylene/polyamide films (Suomen Union Verpackungs Oy, Sipoo, Finland) with an oxygen permeability of 29-45 cm^3/m^2/24 h/atm (23^{o}C, 50% RH) and a water vapor permeability of 10-15 g/m^2/24 h (38^{o}C, 90% RH).

Samples were then stored at 8^{o}C for five weeks, at 6^{o}C for six weeks and at 4^{o}C or 8^{o}C for six weeks in studies a, b and c, respectively. In studies d and e, the samples were stored at

4^oC or 8^oC for six weeks and in study f, at 4^oC or 8^oC for eight weeks. In Finland, a shelf life of 3-4 weeks is usually established for products corresponding to those used in the studies described in papers IV and V.

4.8.5 Sampling procedures (IV, V)

In study a, *Clostridium botulinum* type E counts and botulinum toxin were analyzed immediately after inoculation and thereafter once a week for five weeks from five parallel samples for both inoculums for a total of 60 samples. Immediately after inoculation, water activity (a_W) was determined from a composite of five parallel samples for both inoculums and pH was determined from five parallel samples for both inoculums. In study b, both type E counts and botulinum toxin were analyzed immediately after inoculation and then once a week for six weeks from four parallel samples for a total of 28 samples. Four samples were stored for an extended period of 26 weeks and were analyzed for type E count and toxigenesis. NaCl concentration and pH were determined at the time of inoculation from ten and four parallel uninoculated samples, respectively. In study c, type E counts were determined immediately after processing and then weekly for six weeks from two parallel samples for each inoculum level and storage temperature for a total of 56 samples. Botulinum toxin was analyzed after 3, 4, 5, and 6 weeks storage from three parallel samples for each inoculum and storage temperature for a total of 48 samples. NaCl concentration and pH were determined immediately after processing from five parallel uninoculated samples.

In study d, botulinum toxin was analyzed after 4 and 6 weeks storage from three parallel samples for each inoculation level, curing treatment and storage temperature for a total of 72 samples. In study e, *C. botulinum* counts were determined immediately after processing and thereafter once a week from two parallel samples for each inoculation level, curing treatment and storage temperature for a total of 168 samples. Botulinum toxin was analyzed after 3, 4, 5, and 6 weeks storage from three parallel samples for these treatments for a total of 144 samples.

Nitrite and nitrate determinations of study f were carried out immediately after processing and thereafter twice a week on five parallel samples for each curing treatment and storage temperature for a total of 330 samples. The reduction of nitrate to nitrite was determined by analyzing both nitrite and nitrate concentrations from the samples treated with KNO_3+NaCl. Microbiological analyses and sensory evaluation were carried out once a week

on two parallel samples for each curing treatment and storage temperature for a total of 96 samples.

4.8.6 Microbiological analyses and sensory evaluation (V)

A 10 g sample of cold-smoked rainbow trout was homogenized with 90 ml of 0.1% (w/v) peptone water and serial 10-fold dilutions were used for microbiological analyses. The aerobic plate count (APC) and lactic acid bacteria were determined by the methods of the Nordic Committee on Food Analysis (1986; 1991c) by using Plate Count Agar (Difco) and MRS agar (Oxoid), respectively.

The sensory evaluation panel consisted of 7-10 trained judges. The sensitivity of taste and smell of the judges had been checked. The uninoculated samples were evaluated for aroma and taste by using the method described by Amerine et al. (1965). The scale was from zero to five; a score of two points or less meant that the judge had considered the sample unfit for human consumption. The sample was deemed spoiled if two or more judges considered it unfit.

4.8.7 Physical and chemical determinations (IV, V)

pH was determined from homogenates of minced fish and distilled water in a ratio 1:1 (w/v) by using a digital Microprocessor pH 537 measuring device (Wissenschaftlich-Technische Werkstätten, Weilheim, Germany). A_W was analyzed from minced samples by a Durotherm measuring apparatus (Lufft GmbH, Fellbach, Germany).

Waterphase NaCl concentrations were determined according to the method of the Nordic Committee on Food Analysis (1974). Nitrite and nitrate were analyzed by using the method of Eggers and Cattle (1986), the only modification being that activated carbon was not used. The samples were analyzed with a high performance liquid chromatograph (Hewlett-Packard 1090 Series M LC; Hewlett-Packard GmbH, Waldbronn, Germany). Chromatographic separations were carried out by using a 4.6 mm x 50 mm IC-Pak^{TM} Anion column (Waters Millipore Corp.; Milford, MA, USA). The LC mobile phase was 10 mM K_2HPO_4 (pH 8.0) containing 15% acetonitrile at a flow rate of 0.9 ml/min. Nitrite and nitrate were detected with a diode array detector at 215 nm wavelength. The analysis time was eight minutes and the limit of determination for both nitrite and nitrate was 2.5 mg/kg. The precision values of the method calculated as CV% (coefficient of variance) after extraction

and analysis of the same fortified samples six times were 12.0% (concentration = 10 mg/kg) and 2.5% (100 mg/kg) respectively for nitrite and 14.3% and 2.8% respectively for nitrate.

4.8.8 Predictive models (IV)

Two predictive microbiological modeling programs, Food MicroModel version 2.5 (Leatherhead Food Research Association; Leatherhead, Surrey, UK) and Pathogen Modeling Program version 5.0 (USDA Eastern Regional Research Center; Wyndmoor, PA, USA) were used for the generation of growth and time-to-toxicity predictions.

The growth model for nonproteolytic *C. botulinum* types B, E and F by the Food MicroModel program uses temperature, pH and a_W or waterphase NaCl concentration as controlling factors. The model does not provide predictions for the lag time of toxin production. The predictions for growth in studies a, b and c were determined by using the controlling factors present in each study with the exception of waterphase NaCl concentration and initial number of organisms which were set by the limitations of the program. The input values of the controlling factors used for the predictions were in study a: temperature = 8°C, pH = 6.5 (inoculum A) or 6.4 (inoculum B), a_W = 0.985 (inoculum A) or 0.992 (inoculum B), initial number of organisms = 1 log cfu/g (minimum value); in study b: temperature = 6°C, pH = 6.1, NaCl (w/v) = 4.5% (maximum value), initial number of organisms = 2.3 log cfu/g; and in study c: temperature = 4°C or 8°C, pH = 5.9, NaCl (w/v) 3.2%, initial number of organisms = 1 log cfu/g (minimum value).

The lag time predictive model for nonproteolytic *C. botulinum* toxin production in vacuum-packaged raw fish by the Pathogen Modeling Program uses temperature, aerobic plate count (APC) and initial number of organisms as controlling factors and was used in study a. The input values of the controlling factors were: temperature = 8°C, APC = -2 log cfu/g (minimum) or 3 log cfu/g (maximum), initial number of organisms = 2.3 log cfu/sample (inoculum A) or 2.4 log cfu/sample (inoculum B). The result was reported as the time to detectable toxin production. The time-to-turbidity predictive model for nonproteolytic *C. botulinum* type B uses temperature, pH, waterphase NaCl level and initial number of organisms in the food as controlling factors and was used in studies b and c. Limits were set by the program for waterphase NaCl concentration and storage temperature. The input values of the controlling factors in study b: temperature = 6°C, pH = 6.1, NaCl (w/v) = 4.0% (maximum value), initial number of organisms = 4.6 log cfu/sample; and in study c: temperature = 5°C (minimum value) or 8°C, pH = 5.9, NaCl (w/v) 3.2%, initial number of

organisms = 1.4 log cfu/sample (inoculum A) or 2.9 log cfu/sample (inoculum B). The result was reported as the time (tau) when the probability of growth reached half of the maximum probability of growth over the entire storage period (study c) or the lower 95% confidence limit of the tau value (study b).

4.8.9 Statistical analyses (V)

Student's t-test and analysis of variance (ANOVA) were computed by the Microsoft Excel 4.0 statistical system (Microsoft Corp., Redmont, WA, USA). The bacterial counts were log-transformed before the statistical analysis.

5. RESULTS

5.1 Prevalence of *Clostridium botulinum* type E in Finnish raw fish and fishery products (I)

Of 438 raw fish samples, 82 (19%) were positive for the BoNT/E gene by PCR analysis. Of the five fish species studied, the highest prevalence of *C. botulinum* type E was detected in Baltic herring with 40% of the samples being contaminated (I, Table 1). The estimated number of type E spores per kg ranged from 30 to 2730 (mean ± standard deviation: 180 ± 390) in PCR-positive raw fish samples. Positive samples were evenly distributed throughout the main Finnish catching areas. The prevalence of type E in the non-farmed marine and freshwater raw fish samples was 23% and 9%, respectively. The contamination level of the farmed raw fish samples at both marine and freshwater farms was 13%. Fish samples from five of nine marine farms and four of six freshwater farms studied were contaminated by type E. The total prevalence of type E (either intestinal or surface sample or both were positive in an individual fish) in wild fish was 20% and in farmed fish 17%.

Sixteen (8%) of the 208 fish roe samples studied were positive for the BoNT/E gene. Of the different roes included in the study, burbot roe had the highest contamination level of 14% (I, Table 1). The estimated number of type E spores per kg was 30-120 (60 ± 40).

Of the 214 vacuum-packaged and 123 air-packaged fishery product samples studied, 10 (5%) and 4 (3%) were positive for the BoNT/E gene, respectively. The highest prevalence was detected in vacuum-packaged hot-smoked whitefish samples which showed a contamination level of 10% (I, Table 1). The estimated count of type E spores per kg ranged from 30 to 290 (60 ± 80) in PCR-positive vacuum-packaged samples and from 30 to 60 (40 ± 10) in PCR-positive air-packaged samples.

The presence of bacteria was confirmed by the isolation of *C. botulinum* type E strains from PCR-positive samples. Mouse bioassays did not detect any botulinum neurotoxin in PCR-positive vacuum-packaged fishery product samples.

5.2 Application of RAPD and rep-PCR typing techniques for characterization of group I and II *Clostridium botulinum* (II)

5.2.1 RAPD

Of four primers used in amplifications, OPJ 6 was found to be the most discriminative. It generated 9-17 bands of variable intensity with a size range of 150-1800 bp depending on the *C. botulinum* serotype. Nonproteolytic group II serotypes were discriminated at the strain level (II, Fig. 1) with 4, 11 and 2 distinct banding patterns being observed among five type B, 13 type E and three type F strains, respectively. Proteolytic type A and F strains were discriminated at the serotype level (II, Fig. 2). For three proteolytic type B strains, OPJ 6 generated two distinct banding patterns. Use of additional primers did not increase the discriminatory power of the method (II, Table 2). Primer OPJ 13 generated 7-10 intensively stained bands and fewer small fragments than the other primers (II, Fig. 3). Additionally, a serotype specific fragment of approximately 1300 bp was observed for type E strains. Patterns for individual strains were consistent throughout repeated amplifications of one isolate and in amplifications of multiple isolates.

5.2.2 Rep-PCR

Depending on the *C. botulinum* serotype, REP primers yielded 6-8 intensively stained fragments with a size range of 400-2200 bp and several additional faint bands. Proteolytic strains were discriminated at the serotype level and a group I specific fragment of approximately 1300 bp was observed (II, Fig. 4). Nonproteolytic serotypes B and E were discriminated at the strain level (II, Fig 5). The REP -primer pair generated two and three distinct fingerprints for five type B and 13 type E strains, respectively. All nonproteolytic type F strains had an identical banding pattern. A group II specific fragment of approximately 650 bp was detected. Patterns for individual strains were consistent throughout repeated amplifications of one isolate and in amplifications of multiple isolates. Of the two other primers tested, RW3A did not yield any banding patterns, even when the annealing temperature was considerably decreased while BOXA1R distinguished groups I and II, but was unable to discriminate the strains at the serotype level.

5.3 Biodiversity of *Clostridium botulinum* type E strains isolated from fish and fishery products (III)

5.3.1 Macrorestriction digests and cluster analysis

There was a distinct difference in the capability of restriction enzymes *Sma*I/*Xma*I and *Xho*I to digest *C. botulinum* type E DNA. Of the 30 isolates that were undigestible by *Sma*I, 13 were digested by *Xma*I, an isoschizomer of *Sma*I with the same restriction site but different cleavage site. Seventeen isolates (18%) were undigestible by both *Sma*I and *Xma*I with 13 of these isolates being of German origin. Only one isolate (K-36; isolated from a fishery product) was undigestible by *Xho*I, and it was also not digested by *Sma*I/*Xma*I.

The *Sma*I/*Xma*I digests (III, Fig. 1) of the 75 typeable strains generated 33 different MRPs (I-XXXIII), forming 23 clusters at a similarity level of 96% (III, Fig. 2). The discriminatory power of *Xho*I was distinctly better with 51 different MRPs (I-LI) forming 37 clusters at a similarity level of 90% being detected among the 91 typeable strains analyzed (III, Fig. 4). Combining the results of *Sma*I/*Xma*I and *Xho*I digests only slightly increased the discriminatory power by yielding 56 different subtypes. The reproducibility of the banding patters between different DNA lots was excellent with each enzyme used. Extensive genetic biodiversity was observed between the strains isolated from different fish species as well as among isolates from one fish species (III, Table 1). In some cases, strains isolated from intestinal and surface samples of the same fish (III, Fig. 2 and 4, K-21 and K-22) produced different MRPs. In both dendrograms (III, Fig. 2 and 4), the ten typeable Finnish fishery product isolates (K-19, K-33, K-34, K-37, K-38, K-46, K-76, K-117, K-125, and K-126) mainly clustered together along with other epidemiologically unrelated isolates. When the results of both macrorestrictions were combined, three main PFGE types were observed (III, Table 1): clone VIII included 12 Finnish rainbow trout isolates that were digested by *Xma*I but not by *Sma*I; clone XL was composed of 11 German isolates, and clone XXXIII was composed of five Finnish isolates from Baltic herring. Indistinguishable PFGE types were also found in several epidemiologically unrelated sample pairs, such as in two different fish species, and in raw fish and prepared product.

The Finnish type E isolates also exhibited high local geographical biodiversity in macrorestriction analysis (III, Table 2). Isolates originating in fish from lakes, fish farms and manufacturing plants of interior Finland appeared to exhibit more extensive genetic variation than isolates from seawater fish and coastal Finland. Strains with differing genetic profiles

could be isolated from fish originating in the same farm and from products of the same manufacturing plant. On the other hand, isolates with clonal MRPs were detected from distant geographical locations in Finland (III, Table 2, PFGE type VIII). Additionally, some Finnish isolates (K-6, K-20, K-54, and K-126) also belonged to the same clusters (III, Fig. 4, clusters 1 and 6) as the German isolates, which were genetically very homogenous. Similarly, the North American isolate 250 E clustered with some Finnish isolates in both macrorestrictions (III, Fig. 2, cluster 9; Fig. 4, cluster 28).

5.3.2 RAPD analysis

All 92 strains were typeable by RAPD with both primers. Interpretation of RAPD banding patterns was difficult due to a large number of small fragments and frequent occurrence of faint bands (III, Fig. 5 and 6). Therefore, RAPD fingerprints were not used for computerized cluster analysis. Primers OPJ 6 and OPJ 13 generated 27 and 19 different banding patterns, respectively. Despite of the occurrence of faint bands, the reproducibility of the banding patterns was good between different DNA lots. When the results of the primers were combined, 38 different RAPD types (I-XXXVIII) were observed (III, Table 1). Fifty-six isolates (61%) belonged to the five most prevalent RAPD types (I-V), which were distributed throughout different types of samples. In five cases the discriminatory power of RAPD was superior to that of PFGE. For example, strains K-33 and K-34 were isolated from the same package of frozen salted whitefish roe, and appeared to be clonal according to *Sma*I and *Xho*I MRPs (III, Fig. 2 and 4). However, a two band difference was reproducibly observed in fingerprints generated by primer OPJ 13. When the results of PFGE with two restriction enzymes and RAPD analysis with two primers were combined, 62 different genetic profiles were detected among the 92 type E isolates analyzed.

5.4 Predicted and observed growth and toxigenesis by *Clostridium botulinum* type E in vacuum-packaged fishery product challenge tests (IV)

5.4.1 Vacuum-packaged unprocessed rainbow trout

The observed type E growth at 8^{o}C over the course of five weeks was substantially slower than that predicted by the Food MicroModel (IV, Fig. 1a). The predicted time until detectable toxin production by the Pathogen Modeling Program varied from five to eight days depending

on the level of APC used as the controlling factor (minimum 1 x 10^{-2} cfu/g, maximum 1 x 10^{3} cfu/g). There was approximately a 2 log increase in *C. botulinum* type E count when the first samples became toxic at two weeks of storage at 8°C.

5.4.2 Vacuum-packaged raw-pickled (gravad) rainbow trout

According to the prediction by the Food MicroModel there would be no growth in raw pickled rainbow trout during six weeks of storage at 6°C (IV, Fig. 1b). The time to toxin production was predicted to be eight days by the Pathogen Modeling Program. The observed *C. botulinum* type E count declined during the first four weeks of storage and showed approximately a 2 log increase thereafter, but did not return to the initial inoculation level. No toxin production was detected throughout the storage period. The four samples which were stored for 26 weeks at 6°C were all positive for toxin, with no increase observed in the *C. botulinum* count (50 cfu/g ± 100; mean ± SD).

5.4.3 Vacuum-packaged cold-smoked rainbow trout

No increase in *C. botulinum* type E counts was detected at either 4°C or 8°C during six weeks of storage even though the Food MicroModel predicted exponential growth after two weeks at 8°C (IV, Fig. 2a and 2b). Time to toxin production was predicted to be > 90 days by the Pathogen Modeling Program at both storage temperatures. However, toxic samples were observed after three weeks storage at 8°C and four weeks at 4°C.

5.5 Application of sodium nitrite ($NaNO_2$) and potassium nitrate (KNO_3) to control outgrowth and toxigenesis by nonproteolytic *Clostridium botulinum* in vacuum-packaged cold-smoked rainbow trout (V)

5.5.1 Effect of nitrite and nitrate on the *Clostridium botulinum* growth and toxigenesis

In studies d2 and e2, the *C. botulinum* counts fell below the detection threshold (20 cfu/kg) in the samples treated with $NaNO_2$+NaCl and with KNO_3+NaCl during the six weeks storage time (V, Table 3). The highest counts were detected in the NaCl-treated controls.

In studies d1 and e1 (V, Tables 1 and 2), toxicity occurred after four weeks in both NaCl- and $NaNO_2$+NaCl -treated samples, but was higher in the NaCl-treated samples at the higher storage temperature and with the higher inoculum. After six weeks the number of toxic

samples had increased considerably; it was lowest in the KNO_3+NaCl-treated samples. On the other hand, in studies d2 and e2 (V, Table 3) the samples treated with $NaNO_2$+NaCl and with KNO_3+NaCl remained non-toxic throughout the six-week storage period. The NaCl-treated controls were toxic after three weeks at 8°C.

5.5.2 Effect of nitrite and nitrate on the microbiological and sensorial quality of the product

Within two weeks the APCs had reached a level of 7.0 log cfu/g in NaCl-treated controls stored at 4°C and in samples treated with NaCl and with $NaNO_2$+NaCl stored at 8°C (V, Fig. 2). Differences in APCs between the three curing methods were significant after two weeks storage at 4°C. In the samples treated with $NaNO_2$+NaCl and with KNO_3+NaCl, the APCs were 2-3 and 1 log units lower respectively than in the NaCl-treated controls. At 8°C the differences between the curing methods were smaller. The NaCl-treated samples were considered unfit for human consumption after three weeks at both storage temperatures. The rejection characteristics described by the judges were "putridity" and "an ammoniac taste and smell". The sensorial spoilage of the samples treated with $NaNO_2$+NaCl and with KNO_3+NaCl took place much later (V, Fig. 2) and was characterized by rancid off-flavors. The lactic acid bacteria counts followed closely the APCs in the samples treated with $NaNO_2$+NaCl and with KNO_3+NaCl. The relative amount of lactic acid bacteria as compared to the APC was considerably lower in the NaCl-treated controls than in those treated with $NaNO_2$+NaCl or with KNO_3+NaCl.

5.5.3 Nitrite and nitrate depletion in the product

During eight weeks of storage, the nitrite concentration decreased from the input level of 166 mg/kg ± 9 (mean ± SE) to the final concentration of 34 mg/kg ± 2 and 11 mg/kg ± 2, at 4°C and 8°C respectively (V, Fig. 1). After five days, the depletion rate was more rapid ($P<0.05$) at the higher storage temperature. The nitrate concentrations decreased from 686 mg/kg ± 67 to the final level of 465 mg/kg ± 140 and 427 mg/kg ± 33, at 4°C and 8°C respectively. The nitrate depletion rate was not affected by temperature. The reduction of nitrate to nitrite began earlier at the higher temperature, but there was no significant difference between the storage temperatures in the nitrite concentrations attained after 26 days of storage (V, Fig. 1).

6. DISCUSSION

6.1 Prevalence of *Clostridium botulinum* type E in Finnish raw fish and fishery products (I)

A moderately high *Clostridium botulinum* type E prevalence of 20% in Finnish wild fish was detected in the present survey. Earlier studies conducted in the Baltic Sea area (Johannsen, 1962; Zaleski et al., 1978; Huss and Pedersen, 1979) had shown variable prevalences depending on the fish species examined, but in general the contamination level has been found to be high. In this study, *C. botulinum* type E was isolated for the first time in wild freshwater fish, vendace, in Europe. The feeding habits of the fish appeared to have an influence on the level of contamination. The lowest prevalence was recorded for vendace which feed only on plankton. Of the other wild fish species studied, burbot is a predatory fish and whitefish a bottom feeder. During the early stage of its life, Baltic herring is a plankton feeder but later also bottom feeds on crustaceans and fish fry (Koli, 1990). Huss and Pedersen (1979) reported a considerably higher prevalence of type E in bottom-feeding fish species as compared to plankton-feeders, and thereby concluded that the sea bed was the primary source of contamination. However, in their study Huss and Pedersen classified Baltic herring as a plankton feeder and did not observe substantial type E prevalence in this species.

The prevalence of type E did not differ significantly between wild and farmed fish. Nor was there any difference in contamination levels between freshwater and marine trout farms. A study of Danish freshwater trout farms showed a very high type E prevalence of 65% in whole rainbow trout (Huss et al., 1974a). A considerably lower contamination level of 11% was detected in Norwegian freshwater trout farms (Tjaberg and Håstein, 1975). A recent Finnish study by Hielm et al. (1998b) reported contamination levels of 19% and 6% in fish intestinal samples originating in freshwater earth ponds and shelf-cleaning ponds, respectively. Fourteen per cent of the intestinal samples from fish held in marine net cages were positive for type E. The variation in contamination levels between different countries may be due to differences in fish farm construction and feeding systems. At Finnish rainbow trout farms one potential source of contamination is Baltic herring which is widely used as a cheap feed additive and has a high type E contamination level of 40%.

The presence of *C. botulinum* type E was also demonstrated for the first time in Finnish fish roe and fishery products, although the numbers of spores found were generally lower than in fresh fish samples. The results of this study clearly indicate the need for urgent

improvements in the safety of fishery products since the present processing practices are inadequate to eliminate the natural contamination of fish with type E spores. The contamination levels detected in raw fish appeared to have little effect on the contamination levels of the processed fishery products. For example, type E was not demonstrated in hot-smoked Baltic herring samples, although fresh herrings were heavily contaminated. The small size of herrings probably contributes to the even distribution of temperatures high enough to destroy spores during the hot-smoking process. On the other hand, vacuum-packaged hot-smoked whitefish was identified as a potential risk product with a type E contamination level of 10%. Eviscerated whitefish are typically smoked whole. Spores are more likely to survive better in the gills or on the peritoneum of a large whole fish than on the surface of a fillet. In addition, the spore load of a fillet would be lower after proper cleaning. The hot-smoking process also eliminates the competing microflora and may actually heat-shock the botulinal spores, which is not the case in cold-smoked or raw pickled products. Surveys conducted in the 1960s and 1970s in the Baltic Sea area reported prevalences of 13 and 20% in Swedish hot-smoked Baltic herring (Johannsen, 1965) and eel (Abrahamsson, 1967), respectively, and a prevalence of 5% in Danish hot-smoked trout (Huss et al., 1974b). The hot-smoking processes for different fish species are likely to differ between countries and even between different manufacturers within one country with respect to the combinations of time, temperature, relative humidity and construction of smoking oven (modern vs. traditional). Moreover, in some herring smoking processes the brining takes place after heat treatment which gives rise to a significant risk of post-process contamination (Korkeala and Pakkala, 1988).

6.2 DNA-based characterization of isolated strains (II, III)

Hygienic processing and preparation of food has for many years been regarded as a basic requirement and first line defense against pathogenic microorganisms (Huss, 1997). There is now general agreement among scientists and regulatory agencies that use of a hazard analysis critical control point (HACCP) system as a preventive strategy is much more likely to provide assurances of food quality and safety than the sole microbiological testing of the final product. Set up and maintenance of a HACCP system requires knowledge about the contamination routes of products. Molecular typing methods have been shown to provide valuable information about different bacterial populations associated with food processing (Björkroth et al., 1998; Autio et al., 1999). However, genotyping of bacteria is only possible if the genetic

variation within bacterial species is large enough to facilitate the subtyping of strains. This study evaluated the applicability of different molecular typing methods in type E subtyping and additionally focussed on determining the amount genetic biodiversity among type E strains.

6.2.1 Application of different DNA-based typing techniques for characterization of group I and II *Clostridium botulinum*

Of the individual typing protocols used in this study, PFGE with *Xho*I macrorestriction demonstrated the highest discriminating power. Use of *Sma*I/*Xma*I did not increase discrimination. The reproducibility among different DNA lots was excellent. However, the large number of isolates undigestible by *Sma*I was problematic. Hielm et al. (1998a) suspected CG-methylation as a cause for undigestion and addressed the problem by replacing *Sma*I with its isoschizomer *Xma*I. However, only 13 of 30 isolates undigestible by *Sma*I were digested by *Xma*I. These isolates were clonal both by *Xma*I (III, Fig. 2, cluster 23) and *Xho*I (III, Fig. 4, cluster 3) macrorestriction. The 13 German isolates undigestible by *Sma*I/*Xma*I were also closely related by *Xho*I macrorestriction (III, Fig. 4, cluster 1), and were clonal by RAPD analysis (III, Fig. 4, type III). Interestingly, three of the four Finnish isolates untypeable by *Sma*I/*Xma*I (K-6, K-20, and K-54) belonged to the same *Xho*I cluster as the German strains. Additionally, this cluster was related at a similarity level of 82% to *Xho*I cluster 3, which contained the *Xma*I-digested isolates. The close genetic relatedness of these epidemiologically unrelated isolates suggests that there is a specific genetic basis for undigestion by *Sma*I and to some extent *Xma*I. Samore et al. (1996) described a similar genetic relatedness between *C. difficile* isolates that were untypeable by *Sma*I, but typeable by restriction enzyme analysis and RAPD. They suggested that DNA degradation by endonucleases was the cause for undigestion. DNase activity has indeed been recognized in some clostridial species (Blaschek and Klacik, 1984; Kristjánsson et al., 1994). However, in this study it appeared that only one strain was untypeable due to active DNases, since it was not digested by either *Sma*I/*Xma*I or *Xho*I. The rest of the isolates untypeable by *Sma*I were digested by *Xho*I, which proved that the DNA was not severely degraded. Instead, it appears that the strains represented by these particular genotypes posses a specific DNA modification system, possibly methylation, that rendered the DNA undigestible by *Sma*I. Since the worldwide prevalence of this genotype is unknown, it is not advisable to use *Sma*I as the only restriction enzyme in the characterization of type E isolates.

All strains characterized by RAPD analysis in studies II and III were typeable with the arbitrary primers used. In general, the group II strains were discriminated at the strain level and group I strains at the serotype level. Primer OPJ 6 was most discriminating, but the use of primer OPJ 13 increased the number of RAPD subtypes observed in study III among type E isolates. Additional advantages gained from the use of primer OPJ 13 were the easy interpretation of banding patterns and the type E specific fragment which facilitated the serotype identification (III, Fig. 6). Good agreement of RAPD typing results with PFGE was seen in study II. For example, primer OPJ 6 distinguished 11 subtypes among the 13 epidemiologically unrelated type E strains. Two of these same strains were untypeable by PFGE and the rest were discriminated to eight different clones (Hielm et al., 1998a). The strains determined to be clonal by RAPD were also indistinguishable by PFGE, but in some cases RAPD could discriminate the clones created by PFGE. In study III, the discriminatory power of RAPD was inferior to that of PFGE. However, by combining the results of the two typing methods, an increase in the discriminatory power was observed. The typing data generated by RAPD and PFGE were in agreement for 67% of the isolates. The incongruity in the results for some sets of isolates reflects the fact that the molecular bases of PFGE and RAPD are very different. As a consequence, the discriminating power of the methods can vary considerably for particular sets of isolates (Samore et al., 1997). The poor discrimination of proteolytic *C. botulinum* strains in study II may be due to either small genetic diversity in group I strains or incorrect primer selection. The fact that the same primers which worked on nonproteolytic strains did not discriminate the proteolytic strains confirms the previous view that group I and II serotypes are genetically distinct from each other although they belong to the same species according to the current taxonomy (Collins et al., 1994; Collins and East, 1998).

The low discriminatory power of rep-PCR suggests that the genome of *C. botulinum* does not harbor repetitive sequences in large numbers. Since there appears to be fewer REP-like sequences in gram-positive bacteria (Versalovic et al., 1991), it was suggested that primers derived from repetitive sequences in gram-positive bacteria might increase the discriminating power of rep-PCR (Versalovic et al., 1993). This was not the case with *C. botulinum*, since both BOXA1R and RW3A, which are derived from *Streptococcus pneumoniae* and *Mycoplasma pneumoniae* respectively, failed to generate discriminatory banding patterns. Additionally, the lower annealing temperature required to generate banding patterns in this study and other studies reporting the use of rep-PCR for gram-positive bacteria (Jersek et al., 1996; Malathum et al., 1998) suggests that for gram-positives rep-PCR is

actually closer to RAPD analysis than the original rep-PCR described by Versalovic et al. (1991).

The conventional DNA isolation method described in the present study yielded high quality DNA from all *C. botulinum* strains studied, including those strains that were earlier reported to be untypeable by PFGE due to DNA degradation or resistance of the cell wall to lysis (Hielm et al., 1998a). Despite several reports concerning the poor reproducibility of PCR-based fingerprinting methods (Bassam et al., 1992; Caetano-Anollés, 1993; MacPherson et al., 1993), both RAPD analysis and rep-PCR were found to be reproducible, even in amplifications performed several months apart. By using the pre-optimized and pre-dispensed Ready-To-Go Beads and the same thermocycler reproducible banding patterns were generated despite the low stringency amplification conditions in RAPD-PCR and only moderately stringent conditions in rep-PCR. Ready-To-Go beads were very convenient to use and decreased potential pipetting errors and cross-contamination by minimizing pipetting steps. However, the current high cost of Ready-To-Go RAPD Analysis Beads™ may limit their use, particularly in large-scale experiments.

The general principles that are emerging for the interpretation of other molecular typing data, such as for PFGE, cannot be readily applied to PCR-based typing methods in which variation in banding patterns is not tightly coupled with specific genetic events (Tenover et al., 1997). Some researchers even claim that the resulting profile is a combination of artifactual variation mixed with true polymorphism (Tyler et al., 1997). It is especially difficult to establish the criteria for interpreting a change in the size of a single band or in the intensity of several bands. Also in this study, the variation in band intensity in RAPD profiles at matching positions among some strains and the generation of a large number of small fragments by all the other primers except OPJ 13 made the interpretation somewhat difficult. Analysis of all strains twice from separate colonies and use of agarose with a high resolution capacity for resolving the RAPD amplification products facilitated the interpretation of banding patterns. The REP profiles, on the other hand, were moderately simple to interpret due to the low number and large size of the bands.

6.2.2 Biodiversity of *Clostridium botulinum* type E strains isolated from fish and fishery products

The 92 *C. botulinum* type E strains characterized in the present study each belonged to one of three main groups: Finnish isolates, German isolates and North American / North Atlantic isolates. In general, high genetic biodiversity was demonstrated among the isolates regardless

of the isolation source or geographical origin, with the exception of the genetically homogenous German isolates. North American / North Atlantic isolates mainly grouped in the middle of both dendrograms. These ten strains, most of which were isolated several decades ago, belonged to seven different clusters in *Xho*I dendrogram, with some clusters containing Finnish isolates or demonstrating close relatedness with clusters containing Finnish and German isolates. Characterization of Finnish strains suggested that processing of fishery products did not seem to favor the survival of any particular genotype, in that all 11 isolates had different genetic profiles, even though some of the isolates originated in the same manufacturing plant or same product package. Moreover, isolates originating in narrow epidemiological fresh fish sources, such as rainbow trout from one farm or burbots caught from small harvest areas, had high genetic divergence. On the other hand, isolates that were clonal by all typing methods could be isolated from harvest areas or farms that were geographically distant from each other. These results arouse intriguing questions about the evolution of type E. As an environmental organism type E is in general not exposed to strong selection factors that would influence its genetic evolution and favor the survival of certain genotypes. However, a high mutation capacity might facilitate the adjustment of strains into several different ecological niches that exist in the aquatic environment and therefore result in high genetic biodiversity (Moxon et al., 1994).

As opposed to the wide genetic divergence observed among Finnish and North American / North Atlantic strains, the German isolates were found to be genetically homogenous. These isolates originated in the same fish farm and in four different fish species. There are no surveys available about the prevalence of type E in German freshwater sediments and in wild fish. However, in a small scale study performed in the early 1970s, Bach et al. (1971) were able to demonstrate *C. botulinum* type E in mud and fish samples originating from a German fish farm. Fish farming has been shown to maintain a reserve of botulinal spores, despite the low natural contamination levels in the surrounding environment (Cann et al., 1975). The few strains that are introduced into farms with fish obtained from outside the farm or with fish feed, become dominant, resulting in low genetic variation. Additionally, at this particular farm the practice of recycling water from one fish pond to another probably enhanced the spreading of this dominant genotype. Another possible reason for the genetic homogeneity of German isolates could be an unidentified selective factor or a combination of factors which favored the survival of only certain genotypes.

6.3 Safety evaluation of vacuum-packaged fishery products using challenge tests (IV, V)

In addition to HACCP system orientated contamination studies, which utilize molecular typing techniques, another approach to improve the microbiological safety of food products are challenge tests. The prevalence study identified several types of fishery products that were harboring type E spores and have never been evaluated with respect to their ability to support growth and toxigenesis by type E at abuse temperatures. This study also investigated the applicability of predictive models in safety evaluation of fishery products and assessed the ability of two preservatives, nitrite and nitrate, to improve safety of vacuum-packaged cold-smoked rainbow trout which was shown to support type E toxigenesis.

6.3.1 Growth and toxin production by *Clostridium botulinum* type E in vacuum-packaged fishery products

Direct measurement of *C. botulinum* growth in foods is difficult (Doyle, 1991), therefore information is limited concerning the correlation between increase in cell count and toxin production. The quantitative PCR-detection method used in this study allowed for the enumeration of *C. botulinum* type E and the plotting of growth curves. Toxigenesis occurred after a 2-3 log increase in cell count in unprocessed rainbow trout. The observed lag time of 14 days for toxin production at 8^{o}C shows good agreement with the results of Baker and Genigeorgis (1990) who reported type E toxigenesis after 15 days at 8^{o}C in vacuum-packaged fresh salmon homogenate with an inoculum of 1 cfu/g. The moderately slow growth of type E may have been due to the inhibiting influence of competing microflora. The use of non-heat-shocked spores and surface inoculation instead of deep inoculation may also have retarded the growth, but the experimental design of the present study was to simulate natural contamination of fish as closely as possible.

That no growth was observed in raw pickled rainbow trout was expected due to the high NaCl concentration (6.7%, w/v) of the product and that the maximum NaCl level in brine for the growth of nonproteolytic *C. botulinum* is 5% (Sperber, 1982). When placed in an adverse environment microbial populations decline over time (Whiting and Buchanan, 1994). Interestingly, toxicity occurred when storage time was grossly extended. Although growth and toxigenesis were highly unlikely, in theory, due to the inhibitory NaCl level, growth may have been possible in small pockets of microenvironments created by the uneven distribution of NaCl. The high SD of the mean NaCl concentration of raw pickled fish reflected the

difficulties that are encountered in the salting of fish fillets which results in significant package to package variation in the NaCl level. Simultaneous destruction of spores at one location in a sample and germination followed by growth at other location may explain why no increase in *C. botulinum* type E count was detected despite the presence of toxin production in vacuum-packaged raw picked and cold-smoked rainbow trout. In cold-smoked rainbow trout, the combination of a NaCl level of 3.2% (w/v), smoke and slight dehydration during smoking process together with a decrease in storage temperature from 8°C to 4°C extended the lag time for toxin production to four weeks as compared to two weeks observed in unprocessed rainbow trout. However, the results of this study strongly indicate that the combination of waterphase NaCl concentration above 3.5% and chill storage at 3-10°C recommended by the Advisory Committee on the Microbial Safety of Foods (1992) and the European Chilled Food Federation (1996) to ensure the safety of REPFEDs stored over ten days provides an inadequate margin of safety, since toxin was produced at a NaCl level of 3.2% (w/v) in three weeks at 8°C and in four weeks at 4°C.

6.3.2 Use of predictive models in safety evaluation

The generation of predictions for *C. botulinum* type E growth and lag times for toxin production in the three inoculation studies highlighted some of the limitations associated with the current predictive modeling programs. In the Food MicroModel the smallest possible initial number of organisms in food, 10 cfu/g, was unrealistic when considering the natural contamination level of *C. botulinum* type E in fish and fishery products (I, Table 1). Baker and Genigeorgis (1990) stated that increased spore loads have the greatest effect on shortening the time to toxin detection at refrigeration temperatures of < 8°C. Another limitation of the Food MicroModel was that it did not give lag time predictions for toxin production. If a product is considered safe until the growth of *C. botulinum* begins then the predicted and observed results in studies assessing the safety of vacuum-packaged unprocessed and cold-smoked rainbow trout were inconsistent. Based on the prediction, safe storage time for vacuum-packaged unprocessed rainbow trout at 8°C was less than two days. However, the first toxic samples did not occur until after two weeks of storage and by that time the predicted growth was already in the late exponential phase. Many predictive models are designed to be "fail-safe" which means that the growth rate predicted by the model will be faster or a predicted time-to-toxicity will be shorter than that which actually occurs in the food (Whiting and Buchanan, 1994). On the other hand, in cold-smoked product, no growth was predicted to

occur in vacuum-packaged cold-smoked rainbow trout during six weeks storage at 4°C. However, the first toxic samples were observed after four weeks. Predictions from all models are most accurate when all experimental factors are close to the midpoint of their overall range. As any factor changes, moving towards its limits, there will be greater variation in the predictions (Whiting and Buchanan, 1994). The confidence limits associated with predictions are markedly increased with increasing response times (McMeekin et al., 1997). In the study evaluating the safety of cold-smoked rainbow trout, both the low storage temperature of 4°C and the moderately high NaCl level of 3.2% contributed to long response time, and thus to an inaccurate prediction.

The main problem associated with the Pathogen Modeling Program was that no appropriate model for *C. botulinum* type E growth and toxin production in fishery products was included. The fish model was applicable only for time-to-toxicity predictions in unprocessed vacuum-packaged fish. It is worth speculating whether the time for detectable toxin production gained from the fish model would have been more accurate if the program had allowed for higher APCs than the maximum level of $1x10^3$ cfu/g. This level is approximately 2 log units too low for most fresh fish (Ikawa and Genigeorgis, 1987; Reddy et al., 1996; Reddy et al., 1997b). Garcia and Genigeorgis (1987) demonstrated increased lag times for *C. botulinum* when the level of initial microbial population (APC) of the fish was increased. The model for the nonproteolytic type B growth and toxin production was applicable for fishery products, but the predictions of this model cannot be regarded as reliable due to the limitations for temperature and NaCl concentration set by the program.

Dalgaard and Jørgensen (1998) evaluated the ability of Food MicroModel and Pathogen Modeling Program to predict the growth of *Listeria monocytogenes* in cold-smoked salmon and found the predictions to be inaccurate. Deviations from predictions, such as those detected in the present study and the study mentioned above, do not necessarily imply that the model is defective but more likely that knowledge of some food ecosystems is incomplete and factors other than those used in the model development have an effect on microbial behavior (McMeekin et al., 1997; Gould, 1999). Most models are developed in broths under constant conditions and do not account for changing environmental variables, such as fluctuating temperatures during distribution and storage, or internal package to package variation in intrinsic factors, such as level and distribution of NaCl. Additionally, models do not incorporate the effects of physiologic status of microbes, the activities of many commonly used antimicrobials, such as sorbates and bacteriocins, or the impacts of microbial competition or time of spoilage (Whiting and Buchanan, 1994; Evans et al., 1997; Schaffner

and Labuza, 1997). Therefore, the true applicability of currently available models to real life situations is questionable.

6.3.3 Use of nitrite and nitrate in vacuum-packaged cold-smoked rainbow trout

The extent of *C. botulinum* inhibition by nitrite can be explained as the result of a "race" between nitrite depletion and the death of germinated botulinal spores (Christiansen, 1980). When the level of nitrite is no longer inhibitory, germination and growth can occur. It is known that nitrate can potentate the inhibitory effect of nitrite (Christiansen et al., 1973). Additionally, nitrate keeps the oxidation-redox-potential of the fish containing TMAO at a high positive level and thereby inhibits the growth of obligatory anaerobes (Skovgaard, 1992). The inclusion of 166 mg/kg of nitrite reduced toxigenesis during the first four weeks of storage at 8^{o}C as compared to the NaCl-treated controls. After six weeks the residual nitrite level was low and not capable of inhibiting toxin formation. A nitrate concentration of 686 mg/kg appeared to be more inhibitory than a nitrite concentration of 166 mg/kg but did not completely inhibit toxigenesis during six weeks at 8^{o}C. Complete inhibition of toxigenesis was observed during the second study in which lower initial nitrite (109 mg/kg) and nitrate (347 mg/kg) concentrations were used. The higher toxigenesis in the first study with high nitrite and nitrate concentrations might be explained by the inoculum, which was approximately fivefold that used in the second study with low preservative concentrations and was composed of a mixture of three nonproteolytic *C. botulinum* serotypes. In the second study only type E strains were used. Differences are known to exist in toxin production capacity between different toxinotypes and even between different strains (Cann et al., 1980; Garcia and Genigeorgis, 1987; Garcia et al., 1987; Betts and Gaze, 1995). It is also likely that there are differences between the various strains in their tolerance of nitrite. Moreover, toxin production in a particular fishery product is determined by a large number of parameters. Variation in intrinsic fish-related factors, the initial bacterial population, concentration and distribution of NaCl, nitrite and nitrate within the fillet and the deposition of smoke together account for differences in the rate of toxin production in fish processed under otherwise standardized conditions.

Nitrite and nitrate appeared to have a positive effect on the microbial and sensorial quality of the product in that more rapid spoilage was observed in NaCl-treated controls as compared to samples cured with $NaNO_2$+NaCl or KNO_3+NaCl. At a storage temperature of 5^{o}C, Pelroy et al. (1994) also observed lower APCs and delayed sensorial spoilage in vacuum-

packaged cold-smoked salmon with an input nitrite level of 200 mg/kg as compared to NaCl-treated controls. In the present study, the larger relative proportion of APCs as compared to lactic acid bacteria may have contributed to the earlier spoilage of the NaCl-treated samples, which were obviously spoiled by organisms other than lactic acid bacteria. This was supported by the sensorial rejection characteristics of putridity and ammonia described by the judges in the sensorial analysis. In samples containing nitrite and nitrate, lactic acid bacteria constituted the major bacterial population. Low concentrations of nitrite and nitrate together with NaCl are known to be selective for lactic acid bacteria multiplication in vacuum-packaged meat and fishery products (Korkeala et al., 1992; Skovgaard, 1992; Lyhs et al., 1998). The extension of shelf life by nitrite and nitrate curing is obviously due to a phenomenon called delayed sensorial spoilage, in which the product is deemed unfit for human consumption much later than the time when the total bacterial count exceeds the maximum level (Korkeala et al., 1989; Jeppesen and Huss, 1992). Nitrite and nitrate appeared to inhibit the growth of spoilage organisms present in NaCl-treated controls and the rancid off-flavors at the time of sensorial rejection suggested that chemical spoilage was possibly involved.

Rapid depletion of nitrite was detected in cold-smoked fillets. Pelroy et al. (1982) did not report a similar observation in hot-smoked salmon steaks treated with $NaNO_2$+NaCl. They observed no change in the residual nitrite level during a 22-day storage period at 3.3^{o}C. In general, the fate of nitrite and its depletion rate differ from one system to another, since the situation involves several different depletion pathways and a number of variables, including product formulation, pH, and time-temperature relationships during processing and subsequent storage (Sofos et al., 1979). The results of the present study also indicate that in vacuum-packaged cold-smoked fish there apparently exists a bacterial population which is capable of reducing nitrate to nitrite. However, the increase in the nitrite concentration was found to be relatively slow. Additionally, there was a great variation in nitrate concentrations between the parallel samples of each sampling time and also between the successive sampling times which may indicate irregular binding of nitrate into the fillet and hamper its use as a preservative in fishery products.

7. CONCLUSIONS

1. Both farmed and wild fish species in Finland were found to be contaminated by *C. botulinum* type E, with prevalence levels of 17% and 20%, respectively. Eight percent of fish roe samples and 4% of various types of fishery products were positive for type E. Vacuum-packaged hot-smoked whitefish was identified as a particularly high risk product with a contamination level of 10%. The results indicate that current fish processing practices are inadequate to eliminate type E spores from fishery products.

2. RAPD analysis with two arbitrary primers (OPJ 6 and OPJ 13) was found to be a discriminating and reproducible method for genotyping type E strains. It had a higher discriminatory power for certain sets of isolates than PFGE with two macrorestriction enzymes (*Sma*I/*Xma*I and *Xho*I), although in general, MRPs generated by PFGE were more discriminating and easier to interpret. The distinct advantages of RAPD as compared to PFGE were 100% typeability and rapid performance. Rep-PCR was not discriminatory enough to be a useful tool for type E subtyping.

3. High genetic biodiversity was observed among type E isolates regardless of the isolation source or geographical origins. Combined results of PFGE and RAPD analyses yielded 62 subtypes among 92 isolates. Extensive genetic variation was observed among strains isolated from different fish species as well as among isolates from one fish species or even from one individual fish. Processing did not seem to favor survival of any particular genotype. The wide genetic biodiversity observed among *C. botulinum* type E strains facilitates the use of DNA-based typing methods as a tool in contamination studies in the food industry and in investigations of botulism outbreaks.

4. At the mildly abusive storage temperature of 8^{o}C, *C. botulinum* type E was observed to produce toxin in vacuum-packaged unprocessed and cold-smoked rainbow trout in two and three weeks, respectively, with an inoculum corresponding to the natural contamination levels of fish. Even at 4^{o}C, vacuum-packaged cold-smoked rainbow trout became toxic after four weeks. In each case, toxin production occurred with very

low or no detectable growth by type E. Vacuum-packaged raw pickled (gravad) rainbow trout stored at 6°C was determined to be safe with respect to type E, provided that the declared shelf life is within reasonable limits. The results strongly indicated that unless the constant maintenance of a chill chain temperature below 3°C can be guaranteed, the shelf life of vacuum-packaged cold-smoked rainbow trout in its current formulation should be limited to ten days.

5. The applicability of currently available predictive microbiological models in safety evaluation of fishery products with respect to *C. botulinum* type E was found to be highly questionable. The models tested were hampered by limitations of the controlling environmental factors set by the programs which had an adverse effect on the reliability of predictions.

6. The use of nitrite or nitrate in vacuum-packaged cold-smoked rainbow trout was found to reduce the hazard arising from *C. botulinum* type E at slightly abusive storage temperatures. Additionally, an extension in the microbiological and sensorial shelf life of the product was observed with both preservatives.

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