NEWSLETTER 06/2006

(Mars 2006)

Sommaire

1. **EDITORIAL** (Dr. L. Moor, Président)

Chers ami(e)s de WAVFH Wallonie-Bruxelles,

Comme nous vous l'avions déjà annoncé, nous avons l'ambition de faire de notre site web un lieu où il est possible d'échanger des idées, de faire paraître des informations que vous détenez et que vous voudriez faire partager, un lieu où il serait même possible d'appeler à l'aide, tant les matières deviennent complexes.
Le site internet de WAVFH Wallonie-Bruxelles est en train de subir les modifications nécessaires à la réalisation de cet objectif.
Et donc, nous vous convions, le jeudi 4 mai prochain, à inaugurer et, plus encore, à apprendre à se servir de ce nouvel outil, dans le cadre d'une démonstration « en live », où nous recueillerons à chaud vos impressions et peut-être aussi vos suggestions et vos attentes.

Cette démonstration sera précédée de notre assemblée générale, dont l'agenda sera le suivant :

1. Rapport des activités en 2005.
2. Présentation des comptes financiers en vue de leur approbation et désignation de 2 vérificateurs.
3. Renouvellement partiel du Conseil d'Administration : deux postes d'administrateurs sont déclarés vacants. Les candidatures seront adressées au secrétaire, Nicolas Korsak (E-mail : nkorsak@ulg.ac.be), pour le 29 avril au plus tard.

Si bien que le timing sera le suivant :
1. Assemblée générale.
2. Présentation et démonstration des nouvelles potentialités du site web
3. Le drink de l'amitié, sans lequel nos réunions ne seraient pas ce qu'elles sont !

Nous vous fixons rendez-vous au Sart-Tilman, le jeudi 4 mai 2006, à 19 h.30, au Département des sciences des denrées alimentaires de la Faculté de Médecine Vétérinaire (sous-sol du Bâtiment 43 bis).

N'oubliez pas non plus que pour faire fonctionner notre association, il nous faut vos encouragements, votre participation, votre adhésion, … et votre cotisation (40 € pour l'année 2006), que vous ne manquerez pas de virer au compte 310-0878666-29 de WAVFH Wallonie-Bruxelles.

En espérant vous revoir nombreux, je vous adresse mes sentiments les plus cordiaux.

Très cordialement,

Dr Lic. L. Moor, Président de WAVFH Wallonie-Bruxelles.

Le C.A. de WAVFH Wallonie-Bruxelles :

- ***Léon Moor, président;***
- ***Henri Vindevogel, vice-président (démissionnaire);***
- ***Guy Nolet, vice-président***
- ***Philippe Dodion, trésorier ;***
- ***Nicolas Korsak, secrétaire,***
- ***Georges Daube, François Verheven et Joël, Gustin membres.***

Website http://www.wavfh.be ou http://www.wavfh.ulg.ac.be/ (accès direct)

2. ACTUALITES

2.a. Microbiologie et hygiène des denrées alimentaires

***Clostridium botulinum* de type II**

(article sous presse :
M. Lindström, K. Kiviniemi and H. Korleala. Hazard and control of group II (non-proteolytic) Clostridium botulinum in modern food processing, Int. J. Food microbial., in press)

Résumé : *Clostridium botulinum* de type II pose un danger de sécurité dans l'industrie agro-alimentaire moderne, qui utilise de plus en plus un traitement de pasteurisation à base température, un conditionnement en anaérobiose, des produits à longue durée de conservation et le stockage réfrigéré. Ce risque élevé est confirmé par les nombres relativement élevés de cas provoqués par *Clostridium botulinum* de type II à partir de produits fabriqués durant les dernières décennies. Du fait de la prévalence élevée de *Clostridium botulinum* de type II dans l'environnement, les matières premières peuvent contenir des spores. Bien que les spores du groupe II sont moins thermorésistantes que les spores du groupe I (protéolytiques), elles peuvent supporter des traitements par la chaleur tels que mis en œuvre dans les entreprises agro-alimentaires utilisant le stockage réfrigéré. Certains composants

alimentaires peuvent effectivement fournir des spores avec une protection à la chaleur. La thermorésistance des spores devrait donc être investiguée pour chaque aliment en vue de déterminer l'efficacité des traitements thermiques industriels. Les souches du groupe II sont psychrotrophes et ont donc la capacité de croître à des températures de frigo. Le conditionnement en l'absence d'air et les produits à conservation prolongée fournissent des conditions favorables pour la croissance et la formation de toxines. Etant donné que l'utilisation de sel et d'additifs est limité dans les aliments, la sécurité microbiologique de tels aliments reposent principalement sur le stockage réfrigéré. Ceci pose de grands défis à l'industrie de préparation des aliments emballés et réfrigérés. Pour assurer la sécurité de tels aliments, plus d'un facteur doit être mis en œuvre pour contrecarrer la croissance des germes botuliniques et la formation de toxines.

cf. annexe

Article de revue : *Bacillus cereus* et ses toxines

(article sous presse :
J. Schoeni, A. C. Lee Wong. *Bacillus cereus* food poisoning and its toxins, Int. J. Food microbial., in press)

Résumé : Le genre Bacillus inclut des bactéries qui présentent une diversité très large en termes de physiologie, de niche écologique, de séquence d'ADN et de régulation génique. Es chercheurs commencent à comprendre la pathogénicité des espèces les plus intéressantes. Il semblerait que le génome de ces espèces soit fortement apparenté. *Bacillus anthracis* provoque l'anthrax alors que *Bacillus thuringiensis* est utilisé à grande échelle pour ses propriétés insecticides. Cette dernière espèce a déjà été impliquée dans des toxi-infections alimentaires. *Bacillus cereus* provoque principalement deux types de toxi-infections alimentaires collectives : un syndrome émétique et un syndrome diarrhéique. En plus, il a été associé à des infections locales et systémiques. Bien que dans cette revue, nous fournissons des informations sur le genre et sur une variété importante d'espèces, le point principal est constitué par les souches de *B. cereus* et leurs toxines impliqués dans les toxi-infections alimentaires collectives. *B. cereus* produit un grand nombre de facteurs de virulence potentiels, mais, pour la majorité d'entre eux, leur rôle exact n'a pas encore été clairement déterminé à ce jour. A ce jour, seulement le cereulide et l'hémolysine tripartite BL ont été clairement identifiés comme étant respectivement la toxine émétique et diarrhéique. L'entérotoxine non hémolytique, une molécule homologue à l'hémolysine BL, a aussi été associé avec le syndrome diarrhéique. Les récentes découvertes en rapport avec ces facteurs et avec d'autres entérotoxines présumées sont également présentés dans l'article.

cf. annexe

Article de revue : la température des réfrigérateurs en Irlande

(article :
J. Kennedy, V. Jackson, I.S. Blair, D.A. McDowell, C. Cowan, D.J. Bolton. *Food Safety Knowledge of Consumers and the Microbiological and Temperature Status of their Refrigerators*, J. Food Prot., 68, 2005, 1421-1430)

Résumé : les objectifs de cette étude ont été d'examiner chez les consommateurs les niveaux de connaissance en matière de sécurité alimentaire, d'établir les niveaux et l'incidence des contaminations bactériennes, de surveiller les températures opérationnelles des réfrigérateurs domestiques et, enfin, d'identifier les domaines dans lesquels des programmes d'éducation doivent être mis en place en Irlande. Un questionnaire de connaissance de la sécurité alimentaire appliqué à un échantillon représentatif de ménages (n=1020) a permis de mettre en évidence les manquements au niveau de la connaissance de la sécurité alimentaire. L'analyse d'écouvillons (n=900) réalisés dans les réfrigérateurs domestiques a montré les résultats suivants : 7,1 log ufc/cm² pour les germes totaux et 4.0 log ufc/cm² pour les coliformes totaux. L'analyse de tels écouvillons a permis de mettre en évidence les micro-organismes suivants : *Staphylococcus aureus* (41%), *Escherichia coli* (6%), *Salmonella enterica* (7%), *Listeria monocytogenes* (6%), et *Yersinia enterocolitica* (2%). *Campylobacter jejuni* et *E. coli* O157:H7 n'ont pas été détecté dans les frigos domestiques. Les profils de température ont été enregistrés dans un sous-échantillon par rapport à l'entièreté des réfrigérateurs échantillonnés (n=100). Ces températures ont été enregistrées pendant 72h et 59 % des frigos suivis avaient des températures moyennes supérieures à 5°C. Les enquêtes de connaissance et de température ont varié considérablement mais les consommateurs qui étaient le mieux classés sur le plan de la connaissance basique dans le domaine de la sécurité alimentaire avaient, au niveau de leurs frigos, des taux réduits de contamination bactérienne et rapportaient une incidence moindre d'affections liées aux aliments. Cette étude confirme les effets bénéfiques d'une connaissance basique en hygiène alimentaire et identifie des domaines spécifiques pour le développement de messages de communications ciblés à l'intention des consommateurs.

Article de revue : les produits frais et les TIAC aux USA

(article :
S. Sivapalasingam, C.R. Friedman, L. Cohen, R.V. Tauxe. *Fresh produce: a Growing Cause of Outbreaks of Foodborne Illness in the United States, 1973 through 1997*, J. Food Prot., 67, 2004, 2342-2353)

Résumé : les produits frais sont une composante principale de notre alimentation équilibrée. Durant les trois dernières décennies, le nombre de foyers provoqués par les micro-organismes pathogènes à partir de produits frais et tels que rapportés au Center for Disease Control (CDC) a augmenté considérablement. Nous avons analysé les données de 1973 à 1997 à partir du fichier du système de surveillance des foyers de Toxi-infections Alimentaires Collectives (TIAC). Nous avons défini un foyer comme étant la survenue d'au moins deux cas chez l'investigation épidémiologique avait révélé l'implication d'un fruit, d'un légume, d'une salade ou d'un jus non pasteurisés. Dans ce contexte, un total de 190 foyers a été rapporté, avec un total de 16.058 maladies, 598 hospitalisations et 87 mortalités. La part des TIAC provoqués par les produits frais non pasteurisés est passée de 0,7% dans les années 70 à 6% dans els années 90. Parmi les produits associés aux foyers, les aliments les plus impliqués étaient les salades, les laitues, les jus, les melons, les germes et les baies. Parmi 103 foyers (54%) causés par un pathogène identifié, 62 (60%) ont été causés par des bactéries pathogènes. Parmi ces 62 foyers, 30 ont été provoqués par *Salmonella*. Durant cette période d'étude, *Cyclospora* et *Escherichia coli* O157:H7 ont été reconnus comme étant des nouveaux pathogènes dans ces produits. Aux USA, les foyers de TIAC provoqués par les produits frais ont augmenté en valeur absolue mais également en proportion des foyers de TIAC rapportés. Les fruits et les légumes sont des composants majeurs d'une alimentation saine, mais consommer des produits frais non pasteurisés n'est pas sans risque. Des efforts supplémentaires sont nécessaires pour comprendre les interactions complexes entre les micro-organismes et les produits et les mécanismes par lesquels la contamination a lieu de l'exploitation à l'assiette du consommateur.

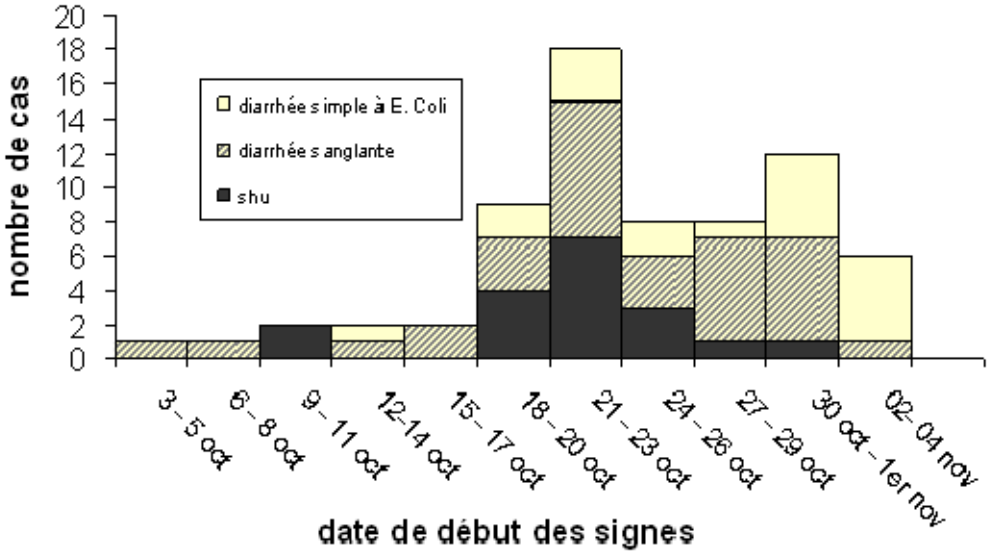
Article : impact des mesures de surveillance en France sur la diminution du nombre d de salmonelloses humaines
(article :
E. Poirier, L. Watier, E. Espié1, P. Bouvet, F.-X. Weill, H. de Valk, J.-C. Desenclos
Évaluation de l'impact des mesures prises dans les élevages aviaires sur l'incidence des salmonelloses en France, BEH, 2-3, 2006, 18-21)

Résumé : L'infection à salmonelle est l'infection bactérienne d'origine alimentaire la plus importante en terme d'impact sur la morbidité et la mortalité chez l'homme. En France, pour les années 90 on estime à 40 000 par an le nombre de salmonelloses confirmées biologiquement ; 5 500 à 10 200 hospitalisations et 90 à 550 décès par an pourraient être imputables à cette infection [1]. La transmission s'effectue principalement à partir d'aliments d'origine animale contaminés crus ou peu cuits. La surveillance des salmonelles chez l'homme en France s'effectue à partir du Centre national de référence (CNR) des Salmonella (Institut Pasteur) qui reçoit les souches isolées par un tiers des laboratoires d'analyse de biologie médicale (LABM) publics ou privés, ainsi que les compte rendus des sérotypages réalisés localement [3]. Cette surveillance a permis de mettre en évidence deux sérotypes majoritairement responsables d'infection chez l'homme, Salmonella sérotype Enteritidis (SE) et Salmonella sérotype Typhimurium (ST). Pour la période 1998-2003, ils représentent 70 % des souches de salmonelles isolées par an [3]. Plusieurs études ont pu déterminer la source de transmission principale de SE, une consommation d'oeufs ou de produits à base d'oeufs [2].

Pour en savoir plus :
http://www.invs.sante.fr/beh/2006/02_03/beh_02_03_2006.pdf

Cas groupés d'infections à E. coli O157:H7, Sud-Ouest de la France, octobre novembre 2005

Point final au 13 janvier 2006



Pour en savoir plus :
http://www.invs.sante.fr/presse/2006/le_point_sur/shu_130106/index.html

2.b. Législation

1/ REGLEMENT 2075/2005 DE LA COMMISSION DU 5 décembre 2005 fixant les règles spécifiques applicables aux contrôles officiels concernant la présence de Trichinella dans les viandes.

Pour en savoir plus :
http://europa.eu.int/eur-lex/lex/LexUriServ/site/fr/oj/2005/l_338/l_33820051222fr00600082.pdf

2.c. Risques chimiques

Avis de l'AFSSA (France) sur le cadmium

Incident en France à la suite de l'utilisation en alimentation animale de sulfate de zinc contaminé par du cadmium. L'AFSSA en a profité pour faire le point sur la problématique du cadmium et plus spécialement de la relation existant entre la consommation de reins et de foies et la survenue de cancers chez l'homme

Extrait du rapport : « *En 2001, la Commission européenne a fixé des teneurs maximales2 de Cd dans les denrées alimentaires, notamment dans le foie de bovin, mouton, porc et volaille à 0,5 mg/kg et dans les rognons de ces mêmes espèces à 1 mg/kg ainsi que dans la viande à 0,05 mg/kg.* »

Pour en savoir plus :
http://www.afssa.fr/Ftp/Afssa/34310-34321.pdf

2.d. Divers

Réaction du Dr. Chantal Rettigner (conférencière lors notre dernière après-midi d'études « le transport, un sujet chaud)

Agence fédérale
pour la Sécurité
de la Chaîne alimentaire

Politique de Contrôle
Direction Santé des
Animaux et Sécurité des
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Votre lettre du	Vos références	Nos références PCCB/S2/CRR 114544	Annexes	Date 15 DEC. 2005
Objet :	Après-midi d'étude WAVFH : réponses aux questions			

Monsieur Korsak,

Au cours de la table ronde qui a été organisée avec les intervenants et les représentants des secteurs à la fin de l'après-midi d'étude consacrée au thème suivant « Le transport, un sujet chaud », quelques questions n'ont pu obtenir de réponses immédiatement. Vous trouverez ci-dessous les réponses des différents experts concernés aux questions posées :

1. Concernant les difficultés de la nouvelle législation pour les opérateurs : la législation demande que lors du transport de viandes surgelées, des documents de transport soient présents, documents qui doivent comporter le numéro de lot et la date de surgélation des produits. Un camion pouvant transporter des centaines de produits différents, cela représente une quantité très importante de documents à transporter. D'autre part, pour quelle raison la date de surgélation est-elle nécessaire ?
   RE : L'obligation concernant la présence de documents d'accompagnement est prévue par l'AR du 30 décembre 1992 relatif au transport des viandes fraîches, des produits à base de viande et des préparations de viandes. Diverses informations doivent y être reprises, notamment les indications de la marque de salubrité ou de la marque d'identification dont la viande, ou l'emballage, est muni, et pour les viandes surgelées la mention en clair du mois et de l'année de surgélation. Elle répond aux exigences de la directive 64/433/CEE relative aux conditions sanitaires de production et de mise sur le marché de viandes fraîches. Cette obligation s'ajoute aux exigences d'étiquetage telles que mentionnées dans les règlements européens 1760/2000 et 1825/2000 concernant l'étiquetage de la viande bovine et des produits à base de viande. Toutes ces mesures visent à assurer la traçabilité des produits.

2. Les exigences d'enregistrement de la température durant le transport ne tiennent pas compte des cas où plusieurs arrêts sont réalisés pour décharger. La température peut alors augmenter et ne plus être conforme aux exigences légales. Néanmoins, les équipements de refroidissement performants peuvent rapidement diminuer la température et donc plus aucune trace de ce dépassement ne sera observée lors de l'enregistrement de la température. L'Afsca va t'elle rendre l'enregistrement de la température en continu obligatoire ? Quid d'un contrôle réalisé au moment du déchargement qui mettrait en évidence un dépassement des prescriptions légales alors que l'enregistrement des températures réalisé par

Notre mission est de veiller à la sécurité de la chaîne alimentaire et à la qualité de nos aliments, afin de protéger la santé des hommes, des animaux et des plantes.

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l'opérateur est normal ? Quid des transport de nuit pour lesquels il n'y a pas de contrôles ?
RE : La fréquence des enregistrements de la température n'est pas définie dans la législation, c'est aux opérateurs de la déterminer sur base de leur HACCP. Les guides doivent d'ailleurs décrire les actions à mettre en place lorsqu'une augmentation de la température est constatée. Si les opérateurs suspectent qu'une succession de déchargements est susceptible d'entraîner un accroissement de la température au-delà des limites légales, ils doivent prendre les mesures adéquates pour contrôler ce danger (diminution de la température dans le camion, limiter le temps d'ouverture des portes, cloisonnement du camion, ...). Quoiqu'il en soit, si lors d'un contrôle officiel, un dépassement de la température légale des denrées était observé, cela serait considéré comme une non conformité et les actions nécessaires seraient prises afin d'assurer la sécurité du consommateur.

3. Pourquoi avoir inclus une limite de 50 km dans les exigences relatives au transport à chaud ?
RE : Il s'agit de la concrétisation nationale de la législation européenne. En effet, la directive 64/433/CEE relative aux conditions sanitaires de production et de mise sur le marché de viandes fraîches offre, aux Etats membres, la possibilité d'accorder une dérogation à l'exigence de maintien des viandes à une température de 7°C si le transport est réalisé vers des ateliers ou boucheries situés aux abords immédiats de l'abattoir, pour autant que la durée du transport n'excède pas 2 heures. Afin d'éviter une interprétation variable d'un opérateur à un autre (d'autant plus que le critère des 2 heures pour le chargement, le déplacement et le déchargement est difficile à garantir et à contrôler), une distance maximale de 50 km a donc été déterminée en prenant en compte la situation de la circulation routière en Belgique.

4. Pourquoi n'y a t'il pas de définition précise de ce qu'est le refroidissement rapide, alors que la législation belge exige que les abattoirs voulant appliquer le transport à chaud doivent être pourvu d'un tel équipement ?
RE : L'étape de refroidissement rapide et les critères techniques pour y parvenir varient d'un opérateur à l'autre en fonction de ses conditions de production et d'équipement. Inclure une définition précise dans la législation serait donc trop contraignant pour les opérateurs. Plusieurs combinaisons de température, d'humidité et de temps nécessaires sont possibles et décrites dans la littérature. On peut notamment utiliser une phase initiale de refroidissement à une température de l'air < - 6 °C (4 heures maximum) avec une vitesse de circulation d'air d'au moins 2 m/sec, durant laquelle la congélation des viandes doit être évitée, suivie d'une phase où le refroidissement est prolongé à une température de l'air de – 1°C à + 2 °C jusqu'à obtention d'une température à cœur de + 16 °C et d'une température superficielle de + 9 °C.

Veuillez agréer, Monsieur Korsak, l'expression de mes salutations sincères.

Ir. Gil Houins
Directeur général

2

3. EVENEMENTS

5 avril 2006 : journée d'étude BAMST : Microbiologie et viande : que faire demain ? »
Lieu : Gembloux
Horaire : 9h à 17h
Renseignements :
http://www.vvv.ugent.be/BAMST.html

4 mai 2006 : AG de la WAVFH Wallonie-Bruxelles + Présentation et démonstration des nouvelles potentialités du site web
Lieu : Sart-Tilman, Bât B43bis
Horaire : 19h30

10-12 mai 2006 : colloque international « International symposium Salmonella and Salmonellosis »
Lieu : Saint-malo
Renseignements :
http://www.zoopole.com/ispaia/i3s2006

14-15 septembre 2006 : 11ème colloque de microbiologie des aliments
Lieu : Université de Gand
Renseignements : auprès du Prof. Daube

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Hazard and control of group II (non-proteolytic) *Clostridium botulinum* in modern food processing

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Abstract

Group II (non-proteolytic) *Clostridium botulinum* poses a safety hazard in modern food processing, which consists of mild pasteurization treatments, anaerobic packaging, extended shelf lives and chilled storage. The high risk is reflected in the relatively large number of botulism cases due to group II *C. botulinum* in commercially produced foods during the past decades. Because of the high prevalence of group II *C. botulinum* in the environment, food raw materials may carry spores. Although group II spores are less heat-resistant than group I (proteolytic) spores, they can tolerate the heat treatments employed in the chilled food industry. Some food components may actually provide spores with protection from heat. Spore heat resistance should therefore be investigated for each food in order to determine the efficiency of industrial heat treatments. Group II strains are psychrotrophic and thus they are able to grow at refrigeration temperatures. Anaerobic packages and extended shelf lives provide *C. botulinum* with favourable conditions for growth and toxin formation. As the use of salt and other preservatives in these foods is limited, microbiological safety relies mainly on refrigerated storage. This sets great challenges on the production of chilled packaged foods. To ensure the safety of these foods, more than one factor should safeguard against botulinal growth and toxin production.

Keywords: *Clostridium botulinum*; Botulism; Botulinum neurotoxin; Spore heat resistance; Food safety

1. Introduction

Clostridium botulinum is an anaerobic bacterium that under non-optimal growth conditions can form heat-resistant endospores. During their late-logarithmic growth *C. botulinum* strains produce highly potent neurotoxins that cause a neuroparalytic disease known as botulism in humans and animals. Botulism may lead to death due to respiratory muscle paralysis unless treated appropriately. The most common forms of human botulism include foodborne botulism, an intoxication due to ingestion of preformed neurotoxin in foods; infant botulism, an infection due to *C. botulinum* spores germinating, outgrowing and producing neurotoxin in the infant's gastrointestinal tract, where the protective, competitive microflora is poorly developed; and wound botulism, an infection with *C. botulinum* spores growing and producing toxin in deep anaerobic wounds. Other rare forms of botulism consist of adult infectious botulism, which resembles infant botulism, inhalational botulism and iatrogenic botulism, a consequence of botulinum toxin treatment.

C. botulinum is ubiquitous in nature and its spores are naturally present in soil and water. Based on the serological properties of the toxins they produce, *C. botulinum* strains are divided into types A through G. These strains form a diverse group of organisms possessing various genetic (Lee and Riemann, 1970; Hielm et al., 1999; Keto-Timonen et al., 2005; Nevas et al., 2005) and phenotypic characteristics, and thus, have been further divided into four subgroups I to IV (Holdeman and Brooks, 1970; Lee and Riemann, 1970; Suen et al., 1988). The strains causing human botulism belong to groups I and II. Group I consists of proteolytic organisms producing type A, B, and F neurotoxins. These strains are mesophilic and grow optimally at 35–37 °C, but not at all below 10 °C (Lynt et al., 1982). Their growth-limiting pH is 4.3–4.5 (Smelt et al., 1982), and they can tolerate NaCl concentrations as high as 10% in brine. Their spores are highly resistant to heat. Group II consists of strains with non-proteolytic metabolism that produce type B, E and F toxins.

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These strains are psychrotrophic, with an optimum growth temperature of 26–30 °C. However, they may grow at temperatures as low as 3 °C (Schmidt et al., 1961; Eklund et al., 1967a,b; Graham et al., 1997). Their growth-limiting pH is 5 (Segner et al., 1966), and inhibitory NaCl concentration in brine is 5%. While group II spores are less heat-resistant than group I spores, they can still survive the heat processes employed in the food industry (Hyytiä et al., 1998; Hyytiä-Trees et al., 2000; Lindström et al., 2003).

The strains belonging to groups I and II possess different types of risks in food processing. Group I strains, the spores of which are highly heat-resistant, are frequently related to insufficiently processed home-preserved foods such as canned vegetables and cured meats. Group II strains, owing to their ability to grow at refrigerated temperatures, are a safety risk in modern industrially processed foods. These foods are processed with mild heat treatments that may allow the survival of group II spores. Hermetic sealing yields anaerobic conditions and ensures extended shelf lives, providing botulinal spores with conditions favourable for growth. Due to limited use of salt and other preservatives, the microbiological safety of refrigerated processed foods of extended durability (REPFED) relies mainly upon refrigerated storage. However, the storage temperatures commonly used at the retail level and in home refrigerators may reach 10 °C (Evans, 1998), a temperature that enables the growth and toxin formation of group II strains. This paper focuses on the safety risks posed by group II *C. botulinum* in REPFED products.

2. Foodborne botulism due to group II *C. botulinum* in industrially processed foods

Independent of the type of toxin, the clinical manifestation of foodborne botulism is always similar. Through binding to presynaptic nerve endings, the toxin blocks acetylcholine secretion to the synaptic cleft and inhibits muscle contraction, causing flaccid paralysis. The incubation period is 12–72 h, and typically the symptoms start in the cranial parts of the body causing double vision, uncontrolled salivation, blurred speech and difficulty in swallowing. This is followed by paralysis of the caudal muscles. The foodborne form may also cause gastrointestinal signs such as nausea and constipation. The condition requires urgent therapy as it may lead to death when respiratory musculature fails. The therapy consists of intravenous delivery of trivalent (types A, B and E) antitoxin and intensive supportive care including mechanical ventilation

Table 1
Human botulism due to group II *Clostridium botulinum* in commercial food products in 1980–2004

Country	Year	Number of cases	*C. botulinum* type	Vehicle food item (country of origin if known)	Reference
Algeria	1998	1400	ND[a]	Rotten poultry, processed meat, 'kashir' (Algeria)	Anonymous, 1998
Denmark	2002	1	B[b]	Ready-to-eat garlic in chilli oil dressing (Germany)	Krusell, 2003
Egypt	1991	91	E	Salted uneviscerated mullet fish, 'faseikh'	Weber et al., 1993
France	1997	1	E	Scallops	Therre, 1999
France	1999	1	E	Vacuum-packaged frozen scallops	Boyer et al. 2001
France	1999	1	E	Vacuum-packaged frozen prawns	Boyer et al. 2001
France	2003	4	B[b]	Beef and poultry sausages, 'halal'	Espié et al., 2003
Germany	1997	2	E	Smoked fish	Therre, 1999
Germany	1997	1	E	Deep-frozen fish	Therre, 1999
Germany	1997	2	E	Vacuum-packaged hot-smoked whitefish (Finland)	Korkeala et al., 1998
Italy	1997	1	B[b]	Canned truffle cream (Italy)	Therre, 1999
Italy	ND[b]	ND	ND	Ethnic cheese product (Italy)	Aureli et al., 1999
Italy	ND	ND	B[b]	Pasteurized vegetables in oil (Italy)	Aureli et al., 1999
Italy	1997	1	ND	Roast mushrooms in oil (Italy)	Therre, 1999
Italy	1983	1	E	Canned tuna fish in oil	Mongiardo et al., 1985
Japan	1998	6	B[b]	Salted olives (Italy)	Matsuki, 1998
Kyrgyzstan	2004	5	ND	Canned eggplant	Peredkov, 2004
Morocco	1999	78	B[b]	Mortadella sausage (Morocco)	Ouagari et al., 2002
Norway	1997–2003	9	E	Fermented fish product, 'rakfisk' (Norway)	Kuusi et al., 1998, 1999
Poland	2000	9	ND	Sausages	Przybylska, 2002
Poland	2001	7	ND	Canned fish	Przybylska, 2003
Republic of Georgia	1980–2002	85[c]	E	Smoked fish (Georgia)	Varma et al., 2004
Russia, Buryatia	1999	72	ND[d]	Fish	Pollack, 1999
	2004	6	ND[d]	Smoked fish, 'omul'	Anonymous, 2004a
Russia, Volgograd	2004	4	ND[d]	Dried fish	Anonymous, 2004b
Spain	1997	3	B[b]	Canned asparagus	Therre, 1999
Switzerland	1993–1994	12	B[b]	Cured ham	Troillet and Praz, 1995
Ukraine	2004	6	ND[d]	Dried fish	Melnik, 2004
USA, Hawaii	1990	3	B[b]	Sturgeon fish, 'palani' (USA)	CDC, 1991
USA, New Jersey	1992	3	E	Salted uneviscerated fish, 'moloha' (USA)	French et al., 1992
USA, Oregon	1997	1	B[b]	Burrito (USA)	Sobel et al., 2004

[a] ND, no data available.
[b] The physiological group of type B toxin-producing *C. botulinum* has not been reported.
[c] Estimated from smoked fish, accounting for 12% of the events and involving 706 persons.
[d] ND, no data available; however, botulism related to fish products is frequently due to type E toxin.

(Robinson and Nahata, 2003). As the toxin binding is irreversible, recovery follows the development of sprouting nerve endings. Depending on the toxin type and dose, recovery may take a couple of weeks to several months.

Traditionally, foodborne botulism has been associated with such home-preserved foods as cured meat and canned vegetables, where group I *C. botulinum* prevails. Disease due to commercial products has been less frequent. However, with an accelerating trend in ready-to-eat food consumption, the number of botulism cases caused by these foods has increased. Concern of botulism as a result of consumption of REPFED products first emerged in the 1960s, when large outbreaks of type E botulism from group II strains in commercial vacuum-packaged hot-smoked fish occurred in the Great Lakes district of the United States. The risk has since been established, and cases due to group II *C. botulinum* have been reported in the last 25 years all over the world (Table 1). A pitfall in the laboratory investigation of botulism outbreaks triggered by type B toxin, and more rarely also by type F toxin, is that the physiological group of the causative agent often remains unclear. As the risks possessed by the two groups of *C. botulinum* are distinct because of very different physiologies, information on the causative agent would aid in designing prevention strategies and tools against foodborne botulism in the food industry. Surveillance systems and reporting of botulism outbreaks should thus be improved to include more information on the causative organisms and vehicle foods of botulism outbreaks.

3. Prevalence of group II *C. botulinum* in foods

Because of the high prevalence of *C. botulinum* in the environment (Johannsen, 1962, 1963; Cann et al., 1965,1968; Eklund and Poysky, 1965; Bott et al., 1967; Kravchenko and Shishulina, 1967; Laycock and Loring, 1971; Smith, 1978; Notermans et al., 1979; Huss, 1980; Smith and Young, 1980; Yamakawa and Nakamura, 1992; Hielm et al., 1996,1998a,b; Dhaked et al., 2002), food raw materials may carry spores, challenging the heat processes employed in the food industry. While the prevalence of group II spores in fish and other seafoods has been studied extensively, only a few reports on

Table 2
Prevalence of group II *Clostridium botulinum* in raw foods

Sample	Country	Positive samples (%)	*C. botulinum* type	Reference
Meat	Germany	36	E	Klarmann, 1989
Fish	Denmark	65	E	Huss et al., 1974
	Finland	7.1	E	Ala-Huikku et al., 1977
	Finland	19	E	Hyytiä et al., 1998
	Indonesia	5.1	B[a], E, F[a]	Haq and Suhadi, 1981
	Japan	4.5	E, F[a]	Yamamoto et al., 1970
	Norway	11	E	Tjaberg and Håstein, 1975
	Russia	35	E	Rouhbakhsh-Khaleghdoust, 1975
	Sweden	46	E	Johannsen, 1963
	Sweden and Norway	4.8	E	Cann et al., 1966, 1967
	UK	10	B, E, F	Cann et al., 1975
	UK	1.4	B	Burns and Williams, 1975
	USA	6.3	E	Chapman and Naylor, 1966
	USA, Milwaukee	8.7	B[a], E	Pace et al., 1967a,b
Fish and seafood	USA	43	B, E, F	Baker et al., 1990
	USA	3.6	B, E, F	Baker et al., 1990
Fish viscera, roe and flesh	USA, Alaska	4.9	E	Miller, 1975
Fish gills and viscera	USA, Alaska	1.2	E	Houghtby and Kaysner, 1969
	USA, West coast	9.5	B[a], E	Craig and Pilcher, 1967
Fish skin and intestines	Finland	10	E	Hielm et al., 1998b
Fish intestines, gills and skin	Germany	30	E	Hyytiä-Trees et al., 1999
Fish intestines	Nordic countries	15	E	Huss and Pedersen, 1979
	Poland	18	E	Zaleski et al., 1978
	Thailand	2.3	E	Tanasugarn, 1979
	USA, East coast	4.5	E	Nickerson et al., 1967
	USA, Great Lakes	11	E	Bott et al., 1966
	USA, Great Lakes	17	E	Bott et al., 1968
Fish roe	Finland	7.7	E	Hyytiä et al., 1998
Crab	USA, West coast	53	B[a], E	Eklund and Poysky, 1967
Shellfish	Nordic countries	14	E	Huss and Pedersen, 1979
	USA, West coast	23	B[a], E	Craig et al., 1968
Oysters	USA, California	25	E	Taclindo et al., 1967
Vegetables	Italy	4.3	B[a]	Quarto et al., 1983
Mushrooms	Canada	NR[b]	B[a]	Hauschild et al., 1975
Potato peels	Sweden	68	E	Johannsen, 1963

[a] The physiological group of *C. botulinum* types B and F was not indicated in the original report; however, a low incubation temperature (26–30 °C) was used and/or trypsin activation was required in the detection of types B and F toxins, thus, the presence of group II organisms can not be excluded.
[b] NR, not reported.

Table 3
Heat resistance of group II *Clostridium botulinum* spores in various media

Heating medium	Group II *C. botulinum* type	Temperature (°C) for *D*-value	*D*-value (min)	Temperature range (°C) for *z*-value	*z*-value (°C)	Reference
Phosphate buffer	B	77.5	4.0–103			Smelt, 1980
		80.0	0.6–2.3[a]			Juneja et al., 1995
		85.0	2.5–51.0	85.0–90.0	6.3[a]	Smelt, 1980
		87.5	1.5–24.0			Smelt, 1980
		90.0	0.4–8.3			Smelt, 1980
	E	77.5	1.5–38.0			Smelt, 1980
		80.0	0.4–3.9[a]			Juneja et al., 1995
		80.0	1.2–36.0	80.0–87.5	8.3–9.4[a]	Smelt, 1980
		82.5	0.5–23.6			Smelt, 1980
		85.0	0.3–10.4			Smelt, 1980
		87.5	0.2–6.1			Smelt, 1980
	F	73.9	9.1–12.7	71.1–85.0	5.2–6.7[a]	Lynt et al., 1983
		76.6	1.7–6.6			Lynt et al., 1983
		79.4	0.9–2.1			Lynt et al., 1983
		82.2	0.3–0.8			Lynt et al., 1983
		85.0	0.4			Lynt et al., 1983
Pork and pea broth	B	82.2	1.5–32.3	75.0–100.0	6.5–16.5	Scott and Bernard, 1982
	E	82.2	0.3	75.0–100.0	8.7	Scott and Bernard, 1982
Phosphate buffer, L[b]	B	75.0	283			Peck et al., 1993
		80.0	2.5–4.3[a]			Juneja et al., 1995
		85.0	73.6–90	85.0–95.0	7.6	Peck et al., 1993
		90.0	18.1			Peck et al., 1993
		95.0	4.6			Peck et al., 1993
	E	80.0	1.0–4.5[a]			Juneja et al., 1995
		85.0	48.3	85.0–95.0	8.3	Peck et al., 1993
		90.0	12.6			Peck et al., 1993
		90.6	13.5			Alderton et al., 1974
		95.0	3.2			Peck et al., 1993
		93.3	3.8			Alderton et al., 1974
Distilled water, L		90.6	5.0			Alderton et al., 1974
Pork and pea broth, L	B	82.2	28.2–2224			Scott and Bernard, 1985
	E	82.2	24.2			Scott and Bernard, 1985
Crabmeat	B	88.9	12.9	88.9–94.4	8.5	Peterson et al., 1997
		90.6	8.2			Peterson et al., 1997
		92.2	5.3			Peterson et al., 1997
		94.4	2.9			Peterson et al., 1997
	E	73.9	6.2–13	73.9–85.0	6.4–8.1[a]	Lynt et al., 1977, 1983
		76.6	1.7–4.1			Lynt et al., 1977, 1983
		79.4	1.1–1.7			Lynt et al., 1977, 1983
		82.2	0.5–0.7			Lynt et al., 1977
		82.2	0.5–0.8			Lynt et al., 1983
		85.0	0.2[a]			Cockey and Tatro, 1974
		85.0	0.3			Lynt et al., 1977, 1983
	F	76.6	9.5	76.6–85.0	6.6[a]	Lynt et al., 1983
		79.4	3.6			Lynt et al., 1983
		82.2	1.2			Lynt et al., 1983
		85.0	0.5			Lynt et al., 1983
Crawfish	E	80.0	4.9–7.0	80.0–95.0	8.0–14.5	De Pantoja, 1986
		85.0	6.7–8.8			DePantoja, 1986
		90.0	2.5–3.1			DePantoja, 1986
Oyster homogenate	E	73.9	2.0–9.0	73.9–82.2	4.2–6.2	Chai and Liang, 1992
		75.0	1.3–5.3			Chai and Liang, 1992
		76.7	0.7–2.7			Chai and Liang, 1992
		79.4	0.3–1.0			Chai and Liang, 1992
		80.0	0.8	70.0–80.0	7.6	Bucknavage et al., 1990
		82.2	0.1–0.4			Chai and Liang, 1992
Cod homogenate	B	75.0	53.9	75.0–92.0	8.6	Gaze and Brown, 1990
		80.0	18.3			Gaze and Brown, 1990
		85.0	4.0			Gaze and Brown, 1990
		90.0	1.1			Gaze and Brown, 1990
		92.0	0.6			Gaze and Brown, 1990

Table 3 (*continued*)

Heating medium	Group II *C. botulinum* type	Temperature (°C) for *D*-value	*D*-value (min)	Temperature range (°C) for *z*-value	*z*-value (°C)	Reference
	E	75.0	58.5	75.0–92.0	8.3	Gaze and Brown, 1990
		80.0	15.1			Gaze and Brown, 1990
		85.0	4.8			Gaze and Brown, 1990
		90.0	0.8			Gaze and Brown, 1990
		92.0	0.6			Gaze and Brown, 1990
Whitefish chubs	E	80.0	1.6–4.3	80.0–90.0	5.7–7.6[a]	Crisley et al., 1968
Rainbow trout	E	75.0	4.6	75.0–93.0	10.4	Lindström et al., 2003
Rainbow trout, L	E	75.0	255			Lindström et al., 2003
		85.0	98			Lindström et al., 2003
		93.0	4.2			Lindström et al., 2003
Rainbow trout	E	85.0	2.0			Lindström et al., 2003
		93.0	0.4			Lindström et al., 2003
Whitefish	E	81.0	1.9	81.0–90.0	10.1	Lindström et al., 2003
		90.0	1.0			Lindström et al., 2003
Whitefish, L	E	81.9	55			Lindström et al., 2003
		90.0	7.1			Lindström et al., 2003
Turkey slurry, L	B	75.0	32.5	70.0–90.0	9.4	Juneja et al., 1995; Juneja, 1998
		80.0	15.2			Juneja et al., 1995; Juneja, 1998
		85.0	4.9			Juneja et al., 1995; Juneja, 1998
		85.0	7.8	80.0–90.0	10.8	Juneja and Eblen, 1995
		90.0	0.8			Juneja et al., 1995; Juneja, 1998
		90.0	1.1			Juneja and Eblen, 1995
	E	75.0	18.1	70.0–90.0	9.9	Juneja et al., 1995; Juneja, 1998
		80.0	13.4			Juneja et al., 1995; Juneja, 1998
		85.0	1.2			Juneja et al., 1995; Juneja, 1998
Carrot homogenate	B	75.0	19.4	75.0–92.0	9.8	Gaze and Brown, 1990
		80.0	4.2			Gaze and Brown, 1990
		85.0	1.6			Gaze and Brown, 1990
		90.0	0.4			Gaze and Brown, 1990
		92.0	0.4			Gaze and Brown, 1990
	E	75.0	18.1	70.0–90.0	9.8	Gaze and Brown, 1990
		80.0	4.3			Gaze and Brown, 1990
		85.0	0.7			Gaze and Brown, 1990
		90.0	0.5			Gaze and Brown, 1990

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

these strains in raw meat and vegetables are available (Table 2). Furthermore, as detection and identification of *C. botulinum* have traditionally been based merely on the ability of a strain to produce botulinum neurotoxin, many earlier studies of raw foods do not indicate the physiological group of the strains. However, based on some laboratory tests employed to demonstrate the toxin formation ability of *C. botulinum* strains at low incubation temperatures and possible trypsin activation of toxin, both indicative of the isolation of group II strains, some type B and F strains included in Table 2 are assumed to belong to group II.

The prevalence of group II *C. botulinum* in food raw materials varies by food stuff and geographical location, with the highest prevalence reported in fish caught in Scandinavia, particularly in the Baltic Sea region (Johannsen, 1963; Huss et al., 1974, Zaleski et al., 1978; Hyytiä et al., 1998; Hyytiä-Trees et al., 1999), in Russia (Rouhbakhsh-Khaleghdoust, 1975) and in the United States (Eklund and Poysky, 1967; Baker et al., 1990). The prevalence in fish and other seafood may be as high as 40–70% (Johannsen, 1963; Huss et al., 1974; Baker et al., 1990). Apart from fish, a high prevalence (36%) of type E spores has been found in meat (Klarmann, 1989) and in potato peels (Johannsen, 1963) (Table 2). The latter reflects not only the high spore contamination level in soil but also the risk of natural contamination of food raw materials with spores. Group II *C. botulinum* counts in raw foods are typically not very high, varying from less than 1 spore/kg (Tanasugarn, 1979; Baker et al., 1990) to 10^2 spores/kg (Hielm et al., 1998a).

The presence of *C. botulinum* spores in food raw materials sets great challenges on the food industry. Consumers are increasingly demanding fresher foods with high nutritive and sensory qualities, and thus, minimal heat processing and limited use of preservatives are desired. These food processing practices do not, however, take into account the microbiological hazards posed by psychrotrophic spore-forming bacteria. Group II *C. botulinum* has been shown to be present in heat-processed foods such as hot-smoked fish (Pace et al. 1967a; Christiansen et al. 1968; Hayes et al., 1970; Hyytiä et al., 1998; Korkeala et al. 1998) and vegetable sausages (Lindström et al., 2001a). While a recent survey suggested a low prevalence of *C. botulinum* in chilled foods (Braconnier et al., 2001), there is evidence that vacuum-packaged frankfurters (Insalata et al., 1969), cured luncheon meat (Taclindo et al., 1967) and smoked turkey products (Abrahamsson and Riemann, 1971) may

contain group II spores. However, as stated earlier, many of these reports do not indicate the physiological group of *C. botulinum*. More extensive studies on the prevalence of group II *C. botulinum* in processed foods are therefore required for thorough risk assessment of this toxin-producing pathogen in REPFED products.

4. Thermal resistance of group II *C. botulinum* spores

Bacterial endospores are known to tolerate different types of stress such as starvation, drying and extreme temperatures. Heat resistance of bacterial spores has been widely studied using *Bacillus* sp. and *Clostridium* sp. as model organisms. Due to the fatal consequences of botulism, particular attention has been paid to the thermal resistance of *C. botulinum*. Spore destruction of group II *C. botulinum* has been researched in a range of media, including laboratory broths, meat, fish and vegetables (Table 3). The heating medium and its pH, water activity (a_w) and protein and fat content, and the natural physiological variation between bacterial strains all have a marked effect on the heat resistance of group II *C. botulinum* spores. *D*-values (decimal reduction time, the time [min] required to reduce the bacterial number by one log-cycle) in different types of foods vary considerably (Table 3).

Because of the high risk of botulism in fish and other seafoods (Table 2), these foods have frequently been used as model matrices for estimating the heat resistance of group II *C. botulinum* spores. *D*-values in oyster homogenate (Bucknavage et al., 1990; Chai and Liang, 1992) have been reported to be lower than those measured in cod homogenate (Gaze and Brown, 1990), crawfish (De Pantoja, 1986) and crabmeat (Cockey and Tatro, 1974; Lynt et al., 1977, 1983; Peterson et al., 1997). Interestingly, in crabmeat, the *D*-values measured by Peterson et al. (1997) were generally higher than those reported elsewhere (Cockey and Tatro, 1974; Lynt et al., 1977, 1983). Methodological differences certainly affect the results of different studies. However, another potential explanation for differences in heat resistance of spores in different foods is the presence or absence of such lytic enzymes as lysozymes.

When present in the recovery medium of heat-injured spores, enzymes with lytic activities increase the apparent heat resistance of group II *C. botulinum* spores (Alderton et al., 1974; Peck et al., 1992a,b,1993; Peck and Fernández, 1995). These enzymes have been postulated to permeate the heat-injured spore coat and induce germination by hydrolysing peptidoglycan in the spore cortex (Gould, 1989). From 0.1% to 20% of the group II *C. botulinum* spore population have been reported to be naturally permeable to lysozyme, possessing a higher measured heat resistance than spores not permeable to lysozyme (Peck et al., 1992a, b, 1993; Lindström et al., 2003). This explains the biphasic thermal destruction curve, with spores non-permeable to lysozyme being destroyed more often than those permeable to lysozyme (Peck et al., 1992a,b; Lindström et al., 2003). Concerns regarding the safety of minimally heat-treated foods have arisen in the food industry since lysozymes and other lytic enzymes are present in many food stuffs (Scott and Bernard, 1985; Proctor and Cunningham, 1988; Lie et al., 1989; Peck and Stringer, 1996; Stringer and Peck, 1996; Stringer et al., 1999). The impact of lysozyme on the heat resistance of spores in various media is clearly illustrated in Table 4. *C. botulinum* type E spores were shown to possess a greater heat resistance in raw fish mince than in autoclaved fish (Alderman et al., 1972), probably indicating higher activity of lytic enzymes in raw foods than in processed foods (Lund and Peck, 1994). A considerable difference in the heat resistance of type E spores in rainbow trout and whitefish was observed between samples incubated in the presence and in the absence of lysozymes (Lindström et al., 2003).

Because the *D*-values of *C. botulinum* group II spores are greatly affected by the heating medium, it is obvious that *z*-values, which are extrapolated from the thermal death-time curves formed by *D*-values measured at different temperatures, are also influenced by the matrix. This is demonstrated in Table 3, where the reported *z*-values vary from 4.2 °C in oyster homogenate to 16.5 °C in pork and pea broth. The *z*-values have been used to create mathematical predictions of process lethality of certain microorganisms, and the *z*-value chosen has a marked effect on the estimate of a safe process time–temperature combination. For example, the recommended heat treatments for processing of group II *C. botulinum* spores vary from 36 min (ACMSF, 1992) to 52 min (ECFF, 1996) at 85 °C based on reference *z*-values of 7 °C (ECFF, 1996) and 9 °C (ACMSF, 1992), respectively. Thus, it is not difficult to see how a fatal underestimation of sufficient heat processes in the food industry might occur. Toxin formation at 8 °C from group II spores has been reported to occur in fish processed at 85 °C for 44 min within 35 days, whereas meat processed for 52 min at the same temperature remained non-toxic for longer than 90 days (Table 4). Therefore, before making mathematical predictions for safe processing of foods, it is of the utmost importance that the *D*- and *z*-values used in the calculations are for the food type in question instead of averages from the literature.

The a_w of the heating medium seems to have a considerable impact on the thermal destruction of *C. botulinum* spores. In an early study by Murrell and Scott (1957) the greatest heat resistance of *C. botulinum* type E spores at 110 °C was observed at an a_w of 0.2–0.9; at an a_w of 0.998, the heat resistance of type E spores decreased drastically by a factor of 30 000. 'Moist' heat has since been employed to facilitate spore destruction in the hot-smoking processing of fish (Pace et al., 1967a; Lindström et al., 2003). A relative humidity (RH) of 70% combined with heat processing at 82 °C for 30 min, the heat treatment officially recommended for commercial hot-smoking of fish in the US in the 1960s (Anonymous, 1964; City of Milwaukee, 1964) were sufficient to eliminate *C. botulinum* type E spores in whitefish chubs by a factor of 10^5 (Pace et al., 1972). When the same heat treatment was employed in the presence of a lower RH, growth and toxin production of 10^5 to 10^6 type E spores were observed (Christiansen et al., 1968; Alderman et al., 1972; Pace et al., 1972). In a later study, RH greater than 70% combined with a heat treatment of 85 °C for 42 min inhibited growth and toxin production from 10^6 type E spores in vacuum-packaged hot-

Table 4
Time to toxin production from 10^3 to 10^6 spores of group II *Clostridium botulinum* types B, E and F in unprocessed and processed laboratory media and foods stored at 5–10 °C

Medium	Heat process		Storage temperature (°C)	Time to growth or toxicity (days)	pH	NaCl content (% v/v)	Type and number of spores	Reference
	Process temperature (°C)	Process time (min)						
Meat	–	–	8	5–7	6.5	0.6–1.5	BEF 10^6	Graham et al., 1996; Fernández et al., 2001
Peptone-yeast extract-glucose-starch (PYGS)	–	–	5	14	6.5	1.5	BEF 10^6	Fernández et al., 2001
Vacuum-packaged salmon	–	–	8	9	6.4	NR[a]	BEF 10^4	Baker and Genigeorgis, 1990
Mushroom	–	–	10	5	6.4	NR	BEF 10^3	Carlin and Peck, 1996
Asparagus	–	–	10	8	5.3	NR	BEF 10^3	Carlin and Peck, 1996
Broccoli	–	–	10	19	5.5	NR	BEF 10^3	Carlin and Peck, 1996
Meat	85	11.4	5	58	6.5	NR	BEF 10^6	Fernández and Peck, 1997
		18.1	5	104	6.5	0.6	BEF 10^6	Graham et al., 1996
		11.4	8	24	6.5	NR	BEF 10^6	Fernández and Peck, 1997
		19.2	8	53	6.1–6.3	NR	BEF 10^6	Peck et al., 1995
		17.3	8	>60	6.1–6.3	NR	BEF 10^6	Peck et al., 1995
		23.3	8	>90	6.5	NR	BEF 10^6	Fernández and Peck, 1997
		17.5	8	>91	6.5	0.6	BEF 10^6	Graham et al., 1996
Meat, L[b]	85	23.3	5	>90	6.4–6.6	NR	BEF 10^6	Fernández and Peck, 1999
		35.7	5	>90	6.4–6.6	NR	BEF 10^6	Fernández and Peck, 1999
		18.1	8	43	6.5	2.5	BEF 10^6	Graham et al., 1996
		18.1	8	64	6.5	0.6	BEF 10^6	Graham et al., 1996
		23.3	8	61	6.4–6.6	NR	BEF 10^6	Fernández and Peck, 1999
		35.7	8	48	6.4–6.6	NR	BEF 10^6	Fernández and Peck, 1999
		52.0	8	>90	6.4–6.6	NR	BEF 10^6	Fernández and Peck, 1999
	90	10.3	5	>90	6.4–6.6	NR	BEF 10^6	Fernández and Peck, 1999
		10.3	8	54	6.4–6.6	NR	BEF 10^6	Fernández and Peck, 1999
		10.9	8	58	6.4–6.6	NR	BEF 10^6	Fernández and Peck, 1999
		15.3	8	68	6.4–6.6	NR	BEF 10^6	Fernández and Peck, 1999
PYGS, L	90	1.0	5	>161	6.8	NR	BEF 10^6	Stringer et al., 1997
		15.0	10	7	6.8	1.5	B $10^{5.1}$	Stringer and Peck, 1997
		15.0	10	14	6.8	3.0	B $10^{5.1}$	Stringer and Peck, 1997
		60.0	10	>161	6.8	NR	BEF 10^6	Stringer et al., 1997
Crab analogue	85	15.0[c]	10	>120	7.2	2.1	B 10^4	Peterson et al., 2002
Hot-smoked salmon	92.2	45.0[d]	10	>120	7.2–7.4	1.0–2.0	BE 10^6	Eklund et al., 1988
Vacuum-packaged hot-smoked rainbow trout, L	85[e]	26–34[e]	8	>35	6.4–6.9	<0.5%	E 10^6	Lindström et al., 2003
Vacuum-packaged hot-smoked whitefish, L	85[e]	44[e]	8	35	6.7–7.6	<0.5%	E 10^6	Lindström et al., 2003
Sous vide beef, L	85[e]	<0.1[e]	8	21	6.2	<0.5%	B $10^{5.3}$	Lindström et al., 2001b
Sous vide pork, L	85[e]	15[e]	8	21	6.0–6.3	0.7%	B $10^{5.3}$	Hyytiä-Trees et al., 2000

[a] NR, not reported.
[b] L, lysozyme added to the recovery medium of heated spores.
[c] Process time does not include the effect of the come-up time of 12 min to the target temperature of 85 °C.
[d] Process time includes a come-up time of 27.7 min to the target temperature of 92.2 °C.
[e] These time–temperature combinations correspond to processes employed in chilled food industry.

smoked fish stored at 8 °C for 5 weeks, whereas the same heat treatment at a low RH resulted in type E toxin production at 8 °C (Lindström et al., 2003). High RH in processing of unpackaged foods should thus be considered another tool for preventing botulism.

5. Risk of group II *C. botulinum* in modern food processing

Increased consumer demand for convenient and fresher foods with minimal preservatives and low thermal processing has led to a tremendous increase in the sales of REPFED products worldwide. These foods are treated with mild heat processes, with maximum temperatures typically reaching 65–95 °C. Whereas these heat treatments eliminate vegetative bacteria, they do not necessarily destroy bacterial spores (Hyytiä et al., 1999; Hyytiä-Trees et al., 2000). Heat treatments are followed by rapid cooling and chilled storage at 1–8 °C. The microbiological quality of REPFED foods thus relies mainly upon the heat treatment and the refrigerated storage temperature. As REPFED products are generally packaged under vacuum or in modified atmospheres (MA) to ensure anaerobic conditions, the shelf lives may be several weeks. This has raised food safety concerns with regard to anaerobic, psychrotrophic spore-forming bacteria (Genigeorgis, 1985; Del Torre et al., 1998, 2001). The three main types of REPFED products are (1) foods that are first processed and then

packaged, (2) foods that are first packaged and then processed and (3) foods that are first cooked, then packaged and pasteurized. Ingredients used in the production of REPFED foods are abundant.

As highlighted above, several factors increase the risk of group II *C. botulinum* in REPFED foods. These include (1) the raw materials applied in REPFED technology containing spores; (2) heat treatments generally being too low to eliminate spores but sufficiently high to destroy competing vegetative bacterial flora; (3) vacuum and MA packaging result in extended shelf lives, thereby allowing multiplication of facultative anaerobic and anaerobic bacteria; (4) group II *C. botulinum* growing at temperatures as low as 3 °C (Schmidt et al., 1961; Eklund et al., 1967a,b; Graham et al., 1997), while typical storage temperatures measured at retail and consumer levels often exceed 10 °C (Evans, 1998); (5) the limited use of NaCl and other preservatives in REPFED foods; and (6) the products not always being further heated, and toxin production by group II *C. botulinum* sometimes preceding the sensory spoilage of the product (Post et al., 1985; Garcia et al., 1987; Ikawa and Genigeorgis, 1987; Gorris and Peck, 1998; Reddy et al., 1999; Lawlor et al., 2000).

6. Measures to control the risk of group II *C. botulinum* in modern food processing

Ideally, the control of group II *C. botulinum* in REPFED products should employ the parallel use of multiple inhibitory factors (Peck and Stringer, 2004) such as sporicidal heat processes and factors inhibiting the germination, growth and toxin production from spores potentially surviving heat treatment (Conner et al., 1989; Gorris and Peck, 1998). According to European guidelines (ACMSF, 1992; ECFF, 1996), the safety of REPFED foods with respect to group II *C. botulinum* should be ensured by a 6D heat treatment, reducing the initial number of group II *C. botulinum* spores by a factor of 10^6. This is analogous to the 'botulinum cook' or 12D concept known in the canning industry. For products to be stored longer than 10 days, time–temperature combinations of 10 min at 90 °C, 36 min at 85 °C, and 129 min at 80 °C (z-value of 9 °C; ACMSF, 1992), or 10 min at 90 °C, 52 min at 85 °C, and 270 min at 80 °C (z-value of 7 °C; ECFF, 1996) have been proposed to ensure a 6D reduction. Equivalent time–temperature combinations with respect to spore elimination can be extrapolated from the two regression lines (ACMSF, 1992; ECFF, 1996). However, many of these time–temperature combinations have been shown to be insufficient to cause a significant number of decimal reductions for the elimination of spores existing in the product and thus preventing the potential germination growth and toxigenesis (Table 4). Furthermore, factors like a high protein and fat content or the presence of lytic enzymes in the raw material may provide spores with a higher heat resistance (Lindström et al., 2003). The use of high RH together with moderate heat processing could increase the safety of unpackaged foods (Pace et al., 1972; Lindström et al., 2003). As creating universally applicable guidelines for safe processing of REPFEDs is impossible due to the wide variation of different types of ingredients, it is essential that before being launched on the market all products and processes are tested for safety with inoculated pack studies.

If a 6-log reduction in group II spore number can not be guaranteed, the germination and outgrowth of spores must be inhibited. NaCl content of 5% (w/v), a_w below 0.97 (Ohye and Christian, 1966; Baird-Parker and Freame, 1967; Emodi and Lechowich, 1969) or pH below 5.0 (Segner et al., 1966; Lund et al., 1990) will inhibit the growth of group II strains. Alternatively, a storage temperature of under 3 °C throughout the entire storage period will effectively control the risk. However, as the germination of group II *C. botulinum* occurs at a wider temperature range than does growth, i.e. 1–50 °C, with the optimum being 9–25 °C (Strasdine, 1967; Ando and Iida, 1970; Grecz and Arvay, 1982; Evans et al., 1997; Plowman and Peck, 2002), even a slight fluctuation in storage temperature may be dangerous.

Various gas mixtures in MA packages have been applied to control botulinal growth in REPFED foods. A good antitoxigenic effect has been demonstrated with 65–100% CO_2 with or without O_2 and N_2 (Post et al., 1985; Baker and Genigeorgis, 1990; Reddy et al., 1997; Lawlor et al., 2000). Preservatives, although not extensively used in REPFED technology, have been reported to effectively control the growth of group II *C. botulinum*. These include sodium lactate (Meng and Genigeorgis, 1993, 1994) and bacteriocins (Okereke and Montville, 1991) such as subtilin (LeBlanc et al., 1953) and nisin (Scott and Taylor, 1981a,b; Taylor et al., 1985; Somers and Taylor, 1987). Nisin is added to such commercial milk products as yoghurt and cheese. However, as it is most active at an acidic pH, its usefulness in neutral pH foods, like meat, is limited (Lindström et al., 2001b). Nisin has also been reported to decrease the heat resistance of bacterial spores (Penna and Moraes, 2002). In addition, competitive microflora, e.g. lactic acid bacteria, have been shown to inhibit the growth of *C. botulinum* (Lyver et al., 1998a,b; Skinner et al., 1999).

While inhibition of group I *C. botulinum* by sodium nitrite has been extensively studied (Roberts and Ingram, 1973; Christiansen et al., 1974; Roberts, 1975; Tompkin et al., 1978; Sofos et al., 1979), reports on its effects on group II strains are scarce (Cuppett et al., 1987; Hyytiä et al., 1997; Keto-Timonen et al., 2002). In heat-processed foods, nitrite forms the Perigo factor, which is inhibitory to *C. botulinum* (Perigo et al., 1967; Christiansen et al., 1973). The use of nitrite is, however, limited in European countries due to its possible adverse health effects, and in fish products produced in the EU region its use is banned entirely. A large number of other compounds with moderate inhibitory actions against group II *C. botulinum* have been reviewed (Roberts and Gibson, 1982; Rhodehamel et al., 1992).

Mathematical models predicting the lag time to growth and toxin production from unheated and heated spores of group II *C. botulinum* in foods have been developed based on large data series obtained in laboratory media and model food media (Lindroth and Genigeorgis, 1986; Baker and Genigeorgis, 1990; Genigeorgis et al., 1991; Meng and Genigeorgis, 1993; Graham et al., 1996; Fernández and Peck, 1997, 1999; Skinner

and Larkin, 1998; Fernández et al., 2001) (Table 4). The models typically describe the estimated reduction in spore numbers and/or the probability of growth from a single spore when various spore loads are treated by different time–temperature combinations and then incubated under a range of conditions. Theoretically, these models provide a convenient tool to estimate safe shelf lives and minimum heat treatments required in the food industry. However, the commercially available microbiological models do not take into account the possible effect of lysozymes present in the foods, or the effect of other process parameters, such as RH. Moreover, as the data employed in the development of the models are often derived from studies using laboratory media that provide optimal conditions for botulinal growth, the models may generate false predictions (Meng and Genigeorgis, 1993; Gould, 1999; Hyytiä et al., 1999). Safety evaluations by inoculated pack studies are therefore essential (Hyytiä et al., 1999).

7. Conclusions

Several factors contribute to the health hazard posed by group II *C. botulinum* in modern food production. The presence of spores in REPFED foods challenges the chilled storage of foods and questions the safety of packaging minimally heat-treated foods. Botulism arising from REPFED products has been reported since the 1960s and cases continue to emerge. Fish products are a common vehicle for human botulism due to group II *C. botulinum*, but the spectrum of potential causative food items is broad.

The high concentration of lytic enzymes in some foods may enhance the apparent heat resistance of group II *C. botulinum*. This should be borne in mind when evaluating the safety of foods with ingredients that may potentially contain these enzymes. As the heat resistance of group II spores varies greatly depending on the food, parameters (*D*- and *z*-values) employed in predicting safe time–temperature combinations for processing should be individually determined for each food. Production of universal guidelines for different types of food products should be avoided, and the safety of each product should be challenge-tested by inoculated pack studies. Maximum storage temperatures of 3 °C should be enforced for all REPFED products throughout Europe.

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Review

Bacillus cereus Food Poisoning and Its Toxins

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ABSTRACT

The genus *Bacillus* includes members that demonstrate a wide range of diversity from physiology and ecological niche to DNA sequence and gene regulation. The species of most interest tend to be known for their pathogenicity and are closely linked genetically. *Bacillus anthracis* causes anthrax, and *Bacillus thuringiensis* is widely used for its insecticidal properties but has also been associated with foodborne disease. *Bacillus cereus* causes two types of food poisoning, the emetic and diarrheal syndromes, and a variety of local and systemic infections. Although in this review we provide information on the genus and a variety of species, the primary focus is on the *B. cereus* strains and toxins that are involved in foodborne illness. *B. cereus* produces a large number of potential virulence factors, but for the majority of these factors their roles in specific infections have not been established. To date, only cereulide and the tripartite hemolysin BL have been identified specifically as emetic and diarrheal toxins, respectively. Nonhemolytic enterotoxin, a homolog of hemolysin BL, also has been associated with the diarrheal syndrome. Recent findings regarding these and other putative enterotoxins are discussed.

The genus *Bacillus* includes gram-positive and gram-variable rod-shaped bacteria that sporulate under aerobic conditions. The vegetative cells are 0.5 by 1.2 to 2.5 by 10 μm and occur singly or in chains. Most are motile via peritrichous flagella and produce catalase *(40, 46, 82, 94, 149, 162).* Aerobic sporulation and production of catalase distinguish members of this genus from those of *Clostridium. Bacillus* possesses aerobic or facultatively anaerobic modes of energy metabolism *(40, 46, 82, 149, 162).* Members of the genus survive in a wide variety of environmental conditions because of the ability to form endospores, which are resistant to heat, dehydration, and other physical stresses *(40, 149).* Optimal growth temperatures range from 25 to 37°C, although some psychrotrophic strains can grow at temperatures as low as 3°C and some thermophilic strains can grow at 75°C. Species that grow at extremes of acidity and alkalinity, ranging from pH 2 to pH 10, have been identified *(46).* As a result of this physiological diversity, *Bacillus* is found in a wide variety of habitats, from soil to thermal springs *(162).*

Several *Bacillus* species are known for their pathogenicity. *Bacillus anthracis* causes anthrax in mammals. *Bacillus thuringiensis* is widely used for its insecticidal properties; however, it has also been associated with foodborne disease *(86)* and infections in humans *(42, 78). Bacillus cereus* causes two types of food poisoning (the emetic and diarrheal syndromes) and a variety of local and systemic infections such as endophthalmitis, endocarditis, meningitis, periodontitis, osteomyelitis, wound infections, and septicemia *(46).* The pathogenesis of *B. cereus* is still largely undefined. The organism produces a large number of potential virulence factors, including multiple hemolysins, phospholipases, and proteases *(14, 46).* However, the roles of these factors in specific infections have not been established. The emetic toxin has been identified as cereulide *(3, 4)* and the tripartite hemolysin BL (HBL) has been established as a diarrheal enterotoxin *(19).* A homolog of HBL, nonhemolytic enterotoxin (Nhe), also has been associated with the diarrheal syndrome *(61, 101).* Many other enterotoxin candidates have been reported; however, their biological activity and involvement in *B. cereus* food poisoning have not been defined. In this review, we provide a historical perspective and evaluate and summarize recent findings regarding the toxins involved in *B. cereus*–associated foodborne illness.

TAXONOMY

The guanine plus cytosine content in chromosomal DNA from *Bacillus* species was reported to range between 32 and 69% *(125, 127),* indicating substantial genetic diversity. The genus includes 51 recognized species *(162)* and is divided into three groups based on the morphology of the spore and sporangium *(58, 59, 148, 162).* The species of group 1 have ellipsoidal or cylindrical spores that are centrally or terminally located and do not distend the sporangium. Group 1 is further subdivided into subgroups 1A and 1B based on cell size and presence of poly-beta-hydroxybutyrate globules in the protoplasm. The *B. cereus* group, consisting of *B. cereus, Bacillus mycoides, B. thuringiensis,* and *B. anthracis,* belongs to subgroup 1A, mem-

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bers of which have bacillary body widths of ≥1 μm and protoplasmic globules. Recently, two additional species, *Bacillus pseudomycoides (114)* and psychrotolerant *Bacillus weihenstephanensis (96)* were described and proposed for inclusion in the *B. cereus* group.

Results from DNA-DNA hybridization studies revealed a high degree of similarity among *B. cereus, B. thuringiensis, B. anthracis,* and *B. mycoides (89, 137),* and their 16S and 23S rRNA sequences are almost identical *(9, 10).* Some workers have suggested that *B. anthracis, B. cereus,* and *B. thuringiensis* should be considered one species based on the high degree of relatedness indicated by multilocus enzyme electrophoresis and sequence analysis of genes *(75, 76).* Comparison of the recently published genome sequences of *B. anthracis* Ames *(130), B. cereus* ATCC 14579 *(85),* and *B. cereus* ATCC 10987 *(129)* revealed that *B. anthracis* Ames and *B. cereus* ATCC 10987 are 93.94% identical, and *B. cereus* ATCC 14579 and *B. cereus* ATCC 10987 are 90.94% identical. Antigenic cross-reactions are also common among these species. Cross-agglutination between the flagellar antigens of *B. cereus* and *B. thuringiensis* and between spore antigens of *B. thuringiensis, B. anthracis,* and *B. cereus* has been observed *(46, 95).*

Use of pulsed-field gel electrophoresis, multilocus enzyme electrophoresis, and multilocus sequence typing *(32, 74–76)* to compare multichromosomal factors revealed a high level of diversity among members of the *B. cereus* group, but isolates assigned to the same species using currently accepted criteria could not be differentiated with these methods. Efforts to differentiate species in this group by genome mapping were also unsuccessful. Although considerable variation was observed between isolates, the genomic maps of *B. thuringiensis* subsp. *canadiensis* HD224 and *B. cereus* ATCC 14579 did not suggest that they were different species *(34).* Two recent studies using fluorescent amplified fragment length polymorphism (AFLP) analysis *(79, 158)* demonstrated a high degree of diversity among isolates of *B. cereus* and *B. thuringiensis,* with different isolates interspersed over all branches of the AFLP-based phylogenetic tree. In contrast, *B. anthracis* was very monomorphic.

The differentiation of *B. anthracis* is important because of its clinical significance. *B. anthracis* is the causative agent of anthrax, which is potentially fatal to animals and humans. Hemolysis, motility, penicillin resistance, tyrosine degradation, and phosphatase production have been used traditionally to distinguish *B. cereus* and *B. thuringiensis* from *B. anthracis*; *B. cereus* and *B. thuringiensis* are positive and *B. anthracis* is generally negative for all five characteristics *(40, 94, 162).* However, Slamti et al. *(147)* reported recently that distinct mutations in the pleiotropic regulator PlcR caused some strains of *B. cereus* and *B. thuringiensis* to be nonhemolytic, and Klichko et al. *(90)* found that *B. anthracis* was hemolytic when grown anaerobically. Mignot et al. *(110)* observed that *B. anthracis* was not hemolytic against sheep erythrocytes but was weakly hemolytic against human erythrocytes. Key virulence genes, including those that encode for the anthrax toxin and capsule, are harbored by two virulence plasmids, pXO1 and pXO2, respectively, that are absent in *B. cereus* and *B. thuringiensis (46, 82, 123, 126).* However, a recent report indicated that anthrax toxin genes were present in a *B. cereus* isolate from a patient with life-threatening pneumonia resembling inhalation anthrax *(80).*

The presence of diamond-shaped parasporal crystals is critical in the differentiation of *B. thuringiensis* from *B. cereus. B. thuringiensis* produces a wide variety of crystal proteins known as delta endotoxins. These highly specific insecticidal toxins affect certain members of *Coleoptera* (beetles), *Diptera* (mosquitoes), and *Lepidoptera* (butterfly and moth larvae) *(81, 126).* The delta endotoxins are plasmid encoded. Thus, mutants that have lost the plasmid cannot be differentiated easily from *B. cereus.*

ISOLATION AND IDENTIFICATION OF *B. CEREUS*

Methods for isolation and identification of *B. cereus* in foods and from clinical samples obtained from food poisoning cases have been described in detail by Bennett and Belay *(25)* and Kramer and Gilbert *(94).* Two differential media in common use are mannitol–egg yolk–polymyxin agar and the same agar with pyruvate and bromothymol blue added. These formulations take advantage of the fact that unlike many *Bacillus* species, *B. cereus* does not ferment mannitol but does produce phosphatidylcholine-preferring phospholipase C (lecithinase). Polymyxin B is used as the primary selective agent for the *B. cereus* group.

Classical identification schemes for members of the *Bacillus* genus have been described extensively *(40, 115, 123, 162).* Generally, *B. cereus* colonies on solid media are 5 to 6 mm in diameter and have a ground glass or matte appearance, with edges that range from circular and entire to irregular and fimbriate. Colonies often appear greenish on blood agar. The spores are ellipsoidal or cylindrical and do not swell the sporangia.

Confirmation of *B. cereus* involves a battery of biochemical tests. These tests can be performed using conventional methods *(25, 40, 71, 123, 162)* or using miniaturized commercial systems that combine biochemical profiles with information in large databases. Serotyping has been a useful tool in epidemiological studies. Forty-two serotypes have been identified based on serological classification of spore, somatic, and flagellar antigens. Twenty-three of these 42 serotypes are associated with *B. cereus*–related disease *(46).* The flagellar (H) serotypes most commonly associated with diarrheal food poisoning are 1, 2, 6, 8, 10, 12, and 19. The H serotypes 1, 5, 8, 12, and 19 are commonly associated with the emetic food poisoning syndrome. Some *B. cereus* strains may cause both forms of food poisoning *(60, 94).*

Phage typing as a means of *B. cereus* identification was explored because of the specificity of *Bacillus*-associated bacteriophages for their host strain *(154, 165).* In a study by Väisänen et al. *(165),* 21 different lysotypes were identified among the dairy isolates studied. Only 10% of the isolates could not be typed.

NONGASTROINTESTINAL ILLNESS

B. cereus has been associated with illness other than food poisoning, although these infections are not common.

The bacterium has been found contaminating postsurgical or traumatic wounds and burns and causes a variety of opportunistic infections, especially in immunocompromised patients, including bacteremia, septicemia, endocarditis, meningitis, pneumonia, pleuritis, osteomyelitis, and endophthalmitis *(46).* These infections can be highly fulminant and sometimes fatal. *B. cereus* can also play a role in sepsis caused by use of contaminated needles by intravenous drug abusers or by following penetrating wounds *(14, 46, 160).* In both instances, the outcome is usually poor.

Involvement of *B. cereus* in pneumonia is rare and is usually associated with other risk factors such as leukemia. However, two unusual cases were reported that involved two previously healthy middle-aged individuals who experienced fulminant bacteremia and pneumonia caused by *B. cereus,* with symptoms similar to those of *B. anthracis* pulmonary infection *(113).* Both individuals experienced chills for three to five days, fever, cough, and bloody expectoration before hospitalization. Their conditions deteriorated in the hospital, and both patients died.

B. cereus is a common cause of eye infections, often causing irreversible tissue damage in a short time (within 24 h). It is one of the most common causes of posttraumatic endophthalmitis, where the organism is introduced into the eye by foreign bodies as a consequence of traumatic injury *(29, 43, 77, 164).* It also causes metastatic endophthalmitis from hematogenous spread of the organism to the eye from other sites. Metastatic endophthalmitis is often highly fulminant and sometimes fatal. The rapid progression and fulminance of *B. cereus* endophthalmitis is attributed to multiple toxins elaborated by the organism, including the diarrheal enterotoxin HBL *(17, 18, 43).*

FOODBORNE ILLNESS

***B. cereus* in foods.** *B. cereus* is a primary inhabitant of the rhizosphere and often is the most frequently isolated soil bacterium *(115, 148).* Because of its abundance and the resistance of its spores, *B. cereus* contaminates nearly all agricultural products and plays a major role in the contamination and spoilage of food products *(94).* It can be commonly isolated from a variety of food products, including spaghetti, other pasta, rice, dairy and dried milk products, spices, dried foodstuffs, meat, chicken, vegetables, fruits, grains, and seafood *(62, 88, 120, 132, 155, 165). B. cereus* is a common spoilage organism in pasteurized milk and milk products *(107).* The optimum heat-activation temperature for spore germination is 65 to 75°C. This range is similar to the temperature used in high-temperature short-time pasteurization of milk. Some *B. cereus* strains are psychrotrophic *(52, 107, 166).* The combination of abundance, heat resistance, and psychrotrophy makes it difficult to control this organism in the food processing environment *(92). B. cereus* can form biofilms on stainless steel *(5, 98, 124)* that are more resistant to sanitizers than are planktonic and attached single cells *(124).* Significant increases in heat resistance were also observed in *B. cereus* spores attached to stainless steel surfaces *(144).*

Epidemiology. The percentage of food poisoning outbreaks associated with *B. cereus* varies from country to country and is dependent on the reporting system. In The Netherlands, from 1991 to 1994 *B. cereus* was identified as the most common cause (19%) of food poisoning outbreaks *(145).* In Taiwan, from 1986 to 1995 *B. cereus* outbreaks ranked third, behind those caused by *Vibrio parahaemolyticus* and *Staphylococcus aureus (121).* Between 1973 and 1985, *B. cereus* caused 17.8% of the total bacterial food poisoning outbreaks in Finland, 11.5% in The Netherlands, 2.2% in Canada, 0.8% in Scotland, 0.7% in England and Wales, and 0.7% in Japan *(92).* Mead et al. *(106)* estimated that more than 27,000 foodborne illnesses annually in the United States are caused by *B. cereus,* which from 1993 to 1997 ranked seventh among the etiological agents causing reported bacterial foodborne outbreaks (14 of 655; 0.5%) and sixth as the causative agent for reported cases (691 of 43,821; 0.8%) *(118).*

Emetic food poisoning. The emetic syndrome was first identified in the United Kingdom in the early 1970s and was associated with the consumption of cooked (usually fried) rice from Chinese restaurants. This syndrome is characterized by nausea, vomiting, and abdominal cramping, which occur 1 to 5 h after ingestion of the contaminated food *(161).* The symptoms are similar to those of *S. aureus* food poisoning, and the rapid onset indicates presence of preformed toxin. The illness is self-limiting, and recovery usually occurs within 6 to 24 h. On occasion, hospitalization is required because of excessive vomiting. Fatality is rare.

Kramer and Gilbert *(94)* evaluated the distribution of serotypes from 200 emetic outbreaks that occurred in 11 countries. The serotypes most frequently isolated were H1 (63.5%) and H8 (8%). H1 spores are more heat resistant than the other spore types and may more frequently survive cooking *(122, 161).*

Foods implicated in emetic food poisonings include beef, poultry, vanilla sauce, pasteurized cream, milk pudding, pasta, and infant formulas *(54, 94).* However, the majority of cases have been associated with cooked rice dishes. Various studies reported that 10 to 100% of raw and cooked rice samples were contaminated with *B. cereus (94).* Contaminating organisms can multiply to large numbers after the rice is boiled, cooled, and held at room temperature. Temperatures achieved during reheating are usually not sufficient to inactivate the heat-stable emetic toxin *(49).* Improper holding temperature of cooked foods is the most common factor associated with the emetic syndrome. An example of an emetic outbreak occurred in 1993 at two Virginia day care centers owned by the same provider. Rice was cooked and then cooled at room temperature before being refrigerated. The next morning, the rice was pan-fried with cooked chicken pieces, delivered to the day care centers, held without refrigeration, and served without reheating. *B. cereus* was isolated from leftover chicken fried rice and from the vomitus of one child *(7).*

Emetic toxin. Melling et al. *(109)* first demonstrated that a toxin was associated with the emetic syndrome based

on rhesus monkey feeding studies using *B. cereus* strains isolated from emetic outbreaks. Further studies allowed Melling and Capel *(108)* to characterize the toxin as extracellular, heat stable, smaller than 10,000 daltons, and poorly antigenic.

The expense and impracticality of the monkey-feeding assay hampered research on the emetic toxin until Hughes et al. *(83)* noted that culture filtrates from emetic food poisoning samples produced vacuoles in HEp-2 cells. Using this detection assay, Agata et al. *(4)* isolated and identified the emetic toxin as cereulide, which is a dodecadepsipeptide produced primarily by *B. cereus* serotype H1 *(3)*. The structure, (D-*O*-Leu-D-Ala-L-*O*-Val-L-Val)$_3$, is similar to that of the potassium ionophore valinomycin, consisting of a 36-member ring with alternating ester and amide bonds *(2, 84)*. The peptide is 1,165 Da with a predicted pI of 5.52. Cereulide is hydrophobic and not easily solubilized in aqueous solutions and may be delivered to its target cells bound to or dissolved in carriers found in food. Therefore, the cereulide activity of a sample may be underestimated if particulates are removed by filtration or centrifugation *(153, 163)*.

Two animal models, rhesus monkey (*Macaca mulatta*) and Asian musk shrew (*Suncus murinus*), have been used for cereulide assays. In one trial with three rhesus monkeys, Shinagawa et al. *(140)* found that 9 to 12 μg/kg induced emesis in 2 to 4 h in all three animals. The 50% emetic dose in the musk shrew was estimated to be 12.9 μg/kg by oral administration and 9.8 μg/kg by intraperitoneal injection *(4)*. These assays have received limited use because they can be difficult and expensive and because cell culture assays have been developed and improved. The HEp-2 vacuolation assay introduced by Hughes et al. *(83)* with colorimetric modifications *(50, 111, 153)* is commonly used to test for the emetic toxin. In this assay, the mitochondrial swelling caused by cereulide appears as cytoplasmic vacuoles in HEp-2 cells *(134)*. Paralysis of boar spermatozoa *(6)* and changes in oxidation rates in isolated rat liver mitochondria *(103, 112, 134)* have also been used as indicators of cereulide-induced toxicity. Measurement of oxygen consumption in these assays indicates that cereulide acts by uncoupling mitochondrial oxidative phosphorylation. Häggblom et al. *(68)* described a quantitative chemical assay based on high-performance liquid chromatography and ion trap mass spectrometry for cereulide. The assay has been calibrated to the boar sperm bioassay and can be used to detect concentrations ranging from 0.02 to 230 μg/ml. Recently, a PCR fragment of unknown function was identified as specific for emetic toxin–producing *B. cereus* strains and was used to develop a PCR assay for rapid detection *(47)*.

Cereulide was involved in a fatal case of *B. cereus* food poisoning where the patient died of fulminant liver failure *(103)*. High concentrations of the emetic toxin were found in the small intestine, liver, bile, and plasma of the patient and in the pan used to reheat food. Diffuse microvascular steatosis and midzonal necrosis were observed in the patient's liver and were attributed to inhibition of mitochondrial fatty acid metabolism by the toxin. When synthetic cereulide was injected intraperitoneally into mice, similar histopathological changes in the liver were observed *(168)*. Paananen et al. *(119)* reported that cereulide inhibited the activity of human natural killer cells, caused mitochondrial swelling, and eventually induced apoptosis, suggesting that this toxin might also have immunomodulating properties.

Diarrheal food poisoning. Hauge *(72)* provided the first and most comprehensive description of *B. cereus* diarrheal syndrome in the 1950s after investigating four Norwegian hospital outbreaks involving about 600 patients. High numbers of *B. cereus* were recovered from the vanilla sauce that was incriminated as the vehicle in the outbreaks. Hauge found that the cornstarch used to prepare the sauce contained about 10^4 *B. cereus* spores per g. To establish the toxicity of the suspected organism and vehicle of transmission he inoculated sterile vanilla sauce with the isolate (approximately 10^4 cells per ml) and incubated it for 24 h at room temperature. Hauge consumed 200 ml of vanilla sauce that contained 9.2×10^7 cells per ml, and within 13 h he experienced abdominal pain, diarrhea, and rectal tenesmus that lasted 8 h.

The onset time of the diarrheal syndrome generally ranges from 8 to 16 h, and the symptoms resolve in 12 to 14 h. Occasionally, nausea and vomiting can be experienced in addition to the symptoms described by Hauge *(72)*. This symptom profile is similar to that of food poisoning caused by *Clostridium perfringens*. Outbreaks with unusual onset and duration have also been reported. In an outbreak caused by consumption of barbecued pork that had not been refrigerated for the previous 18 h, 34% of the ill persons noted an onset time shorter or longer than the normal range of 6 to 24 h and 23% noted fever *(99)*. In a separate outbreak, Andersson et al. *(5)* noted prolonged diarrhea. Three of the 17 people affected were hospitalized, and one person was hospitalized for 5 weeks.

Meats, fish, vegetables, soups, sauces, and dairy products have been associated with the diarrheal syndrome, and a large number of cases are attributed to proteinaceous dishes. *B. cereus* concentrations are usually $>10^5$ cells per g of implicated food, suggesting that a high dose is required to cause illness *(94)*. Reports range from 200 to 10^9 cells per g of food, with calculated infective doses ranging from 5×10^4 to 10^{11} cells *(63, 72, 94)*.

History of diarrheal enterotoxin detection. Animal models that have been used in the detection of *B. cereus* diarrheal enterotoxin include the ileal loop, vascular permeability, and monkey feeding assays. At present, the rabbit ileal loop assay is considered the best in vivo model for assaying enterotoxigenic activity *(26)*. Mice *(141)* and rats *(159)* have also been used. However, the size of the ileum in these animals limits the number of samples that can be evaluated per animal. For assaying activity of the *B. cereus* enterotoxin, Spira and Goepfert *(150)* found that the age and weight of the rabbit are important; the rabbits should be ≤8 weeks old and weigh <1 kg. The volume of fluid accumulated (V) is expressed as a ratio of the length of each loop (L). Samples causing a V/L ratio of ≥0.5 in at least 50% of the test animals are considered positive *(157)*.

Glatz et al. *(55)* examined the correlation of the results from the vascular permeability assay with those of the rabbit ileal loop assay. In the vascular permeability assay, culture filtrates or protein samples (50 μl) are injected intradermally into the shaved backs of rabbits. Visualization of leakage of serum proteins due to increase in vascular permeability is aided with injection of Evan's blue dye into the ear vein. Necrosis surrounding the sample injection site can also be observed. Compared with the rabbit ileal loop, the vascular permeability assay is easier to perform and requires less protein. Occasionally, the results can be difficult to interpret. Glatz et al. *(55)* noted that the bluing response was transient; thus, a standard interval for reading the reactions had to be determined.

In the monkey feeding assays, 1.5- to 3.0-kg rhesus monkeys are fed toxin preparations, culture supernatants, or contaminated food extracts. They are then observed for 6 h for signs of diarrhea. However, this assay was invalidated when Thompson et al. *(157)* found that the combination of disodium phosphate from the growth medium and sodium bicarbonate in the neutralizing buffer administered prior to the culture supernatant could cause diarrhea in these animals.

Cytotoxicity in cell culture assays has been used as an indication of the presence of *B. cereus* diarrheal toxin in crude samples such as food extracts or culture supernatants *(11, 30, 31, 38, 51, 66, 167)*. However, the validity of these assays must be confirmed. Although the enterotoxin is considered cytotoxic, a variety of enzymes and toxins produced by *B. cereus* can act on cell membranes and therefore also produce a cytotoxic effect. As yet, no cell culture system specific for *B. cereus* diarrheal toxin has been established.

Two commercial kits to detect *B. cereus* enterotoxin were independently developed. Oxoid distributes a *B. cereus* enterotoxin reverse passive latex agglutination kit (BCET-RPLA kit, Denka Seiken Ltd., Tokyo, Japan). BCET-RPLA is a semiquantitative assay. Beecher and Wong *(20)* found that this kit allows detection of the L_2 component of the tripartite toxin HBL. The second kit, *Bacillus* Diarrhoeal Enterotoxin Visual Immunoassay (Tecra Bioenterprises, Pty., Ltd., Roseville, Australia), allows detection of toxin using a double-sandwich enzyme immunoassay. The antigen detected by this kit is a 45-kDa protein *(20)*, which was later identified as NheA from the Nhe complex *(65)*. Both kits have been used extensively *(38, 44, 62, 116, 151, 152)*, and kit results have been compared with each other and with results of cytotoxicity tests in tissue culture *(30, 31, 132)*. Some of the discrepancies that arose in these studies can be explained by the fact that each kit detects a different enterotoxin component. Because the Oxoid kit detects L_2 from HBL and the Tecra kit detects NheA from Nhe, the kits may be useful as indicators of which samples contain a specific toxin complex, but they cannot be used to confirm the presence of biologically active toxin because both complexes require all three components for biological activity.

Identification of the diarrheal enterotoxin. The identification of *B. cereus* diarrheal enterotoxin(s) has long been a controversial topic. The earliest study demonstrating the toxigenicity of *B. cereus* was published by Chu *(39)*, who found that cell-free filtrates injected intravenously killed mice. This effect was thought to be caused by a single protein, the lethal toxin, that also possessed hemolytic and lecithinase activity. This work was performed using crude culture filtrates, and the claim that one protein produced multiple biological activities was never substantiated.

Using gel filtration chromatography, Johnson and Bonventre *(87)* separated the lethal, phospholipolytic (lecithinase), and hemolytic activities. There was considerable overlap in the activity of the fractions, which led the authors to suggest that more than one protein was responsible for the three activities. Ezepchuk and Fluer *(48)* purified a nonhemolytic, nonphospholipolytic protein (55 to 65 kDa) that they claimed was the *B. cereus* lethal toxin. This assertion contradicted that of Bernheimer and Grushoff *(27)*, who reported that purified cereolysin, a hemolysin produced by *B. cereus*, was lethal to mice.

In a study by Turnbull et al. *(163)*, two chromatographic fractions individually exhibited lethal activity. One fraction caused mouse death, was hemolytic, and contained cereolysin. The other fraction was enterotoxigenic, as determined by rabbit ileal loop assays, but the protein(s) responsible for this activity was not determined. Bernheimer and Grushoff *(27)* found that the toxin was produced during the transition from vegetative growth to sporulation and that biological activities included mouse death, vascular permeability in rabbits, fluid accumulation in rabbit ileal loops, necrosis, and cytotoxicity *(161, 163)*.

In the late 1970s and early 1980s, researchers began to elucidate the identity of the enterotoxin. An unstable protein (approximately 50 kDa) with a pI of 4.85 was isolated originally *(93)*. The protein was inactivated in 30 min at 56°C, induced fluid accumulation in rabbit ileal loops, increased vascular permeability in rabbit skin, and was lethal to mice. Later, it was found that the enterotoxin dissociated into two moieties with pI values of 5.1 and 5.6, suggesting a bicomponent toxin.

Thompson et al. *(157)* reported that diarrheal activity was caused by a complex of two or three proteins. A combination of chromatographic procedures yielded two fractions eluted from a hydroxylapatite column that contained three proteins of approximately 43, 39.5, and 38 kDa with pI values of about 5.3. Individually, the fractions were not biologically active; however, when combined they had all activities associated with *B. cereus* enterotoxin, including fluid accumulation in rabbit ileal loops, vascular permeability, hemolysis, cytotoxicity, and mouse lethality. Bitsaev and Ezepchuk *(28)* supported the idea of the diarrheal toxin being composed of two or more proteins. They had isolated a tricomponent complex, diarrheagenic-lethal toxin. The complex consisted of proteins A, B, and C with molecular masses of 37.5, 42, and 36 kDa, respectively. The combination of A and B produced fluid accumulation in rabbit ileal loops and edema in mouse paws, and B and C together were lethal to mice. The B protein bound a cellular receptor, which then allowed the A or C component to bind and produce biological activity.

Granum and Nissen *(64)* reported the purification of three proteins (34, 40, and 48 kDa) that were reactive to enterotoxin-specific antibodies (provided by J. M. Kramer, Public Heath Laboratory Service, London, UK). The 34-kDa protein, whose N-terminal sequence was nearly identical to that of *B. cereus* sphingomyelinase, was hemolytic, and the 40-kDa was cytotoxic to Vero cells. However, the proteins were only partially purified, and therefore the function of the individual proteins could not be defined.

Shinagawa *(139)* and Shinagawa et al. *(141, 142)* described a 45-kDa protein with a pI of 5.5 isolated from *B. cereus* strain FM-1. The protein induced vascular permeability, mouse lethality, and fluid accumulation and was cytotoxic but nonhemolytic. However, the reported minimum amount of protein required to elicit these activities was high, raising concerns about protein purity. The amounts were 50 and 200 ng for rabbit and mouse vascular permeability, respectively, 12 μg for mouse lethality, and 30 to 50 μg or 500 μg/mg for fluid accumulation in mice and rabbits, respectively *(142)*.

Asano et al. *(8)* cloned and sequenced a gene (*entFM*) from *B. cereus* strain FM1, *B. thuringiensis* subsp. *sotto*, and *B. thuringiensis* subsp. *israelensis*, which they identified as the gene for the 45-kDa protein described by Shinagawa et al. *(143)*. However, as pointed out by Beecher *(14)*, the predicted pI (9.6) of the entFM protein is quite different than that for the 45-kDa protein of Shinagawa et al. *(143)*; in addition, it is similar to a phosphatase-associated protein with cell-wall hydrolase activity from *Bacillus subtilis* described by Margot et al. *(105)*.

The *bceT* gene was identified in a clone from a genomic library of *B. cereus* strain B-4ac *(4)*. The bceT protein has a predicted size of 41 kDa. Lysates from *Escherichia coli* containing the *bceT* gene were cytotoxic to Vero cells, caused fluid accumulation in the mouse ileal loop assay, and increased vascular permeability in rabbit skin. However, the protein has not yet been demonstrated to be secreted by *B. cereus*. Hansen et al. *(69)* reappraised the *bceT* sequence and suggested that the cloned *bceT* gene did not exist as a single gene in *B. cereus* B-4ac but as four independent DNA fragments that were joined during ligation. One of the fragments had 93% homology to an open reading frame (ORF 101) in the pathogenicity island of the virulence plasmid pXO1 in *B. anthracis*. Hansen et al. suggested that the enterotoxic activity observed by Agata et al. *(4)* was due to either the fusion gene or to the fragment with homology to *B. anthracis* ORF 101 in pX01.

Lund et al. *(100)* isolated a 34-kDa cytotoxin (CytK) from a *B. cereus* strain associated with a food poisoning outbreak that resulted in three deaths in France. CytK is identical to hemolysin IV, which was identified independently by Beecher et al. *(17)* and shown to be toxic to retinal tissue in vitro and hemolytic. The deduced amino acid sequence of CytK has about 30% identity to proteins belonging to the family of β-barrel channel–forming toxins *(100)*. Hardy et al. *(70)* found that CytK could form pores in planar lipid bilayers and was cytotoxic to human colon cancer Caco-2 cells. These authors suggested that CytK possessed potential enterotoxic activity and could be a cause of necrotic enteritis in humans and animals *(70, 100)*.

HBL. Beecher and Macmillan *(15, 16)* isolated HBL, a tripartite toxin produced by *B. cereus*, from strain F837/76. HBL is the only factor that has been highly purified *(15, 16, 21)* and established to be a diarrheal toxin by the ligated rabbit ileal loop assay *(19)*. In addition to demonstrating enterotoxigenic activity, Beecher et al. *(19)* found that HBL is identical to the toxin of Thompson et al. *(157)* by performing Western blots and immunodiffusion assays with antiserum to HBL components and antiserum that Thompson et al. *(157)* produced to their enterotoxin.

The three components of HBL, B, L_1, and L_2, have been purified from *B. cereus* strain F837/76 and have molecular masses of 37.8, 38.5, and 43.2 kDa, respectively, and pI values of approximately 5.3 *(21)*. Nucleotide and deduced amino acid sequences have been reported for all components in strain F837/76 (GenBank accession nos. L20441 and U63928) *(73, 133)* and ATCC 14579 (GenBank accession no. AJ237785) *(117)*. The genes *hblC* (L_2), *hblD* (L_1), and *hblA* (B) are arranged in tandem in an operon *(117, 133)* with the promoter located upstream of *hblC*. Alignment of the deduced amino acid sequences of the three proteins revealed significant similarities *(13, 14, 23)*. The proteins are 20 to 24% identical to each other. Structural analysis of the HBL proteins indicates that all three components consist almost entirely of alpha-helix. Components B and L_1 contain predicted transmembrane segments of 17 and 60 amino acid residues, respectively, in the same position, whereas L_2 does not contain predicted transmembrane segments. These observed similarities suggest that the HBL components resulted from the duplication of a common gene *(13, 14)*.

The DNA sequences of the *hbl* genes of strains F837/76 and ATCC 14579 are 97% identical *(73, 117)*. The major difference is at the C-terminal of L_1; 22 additional amino acids are present in ATCC 14579 that are absent in F837/76. Immediately downstream of *hblA* is *hblB*, which has a predicted amino acid sequence that is about 69% identical to that of the mature B component. There are 91 amino acids at the C-terminal of the protein encoded by *hblB* that are absent from the B component encoded by *hblA*. This difference could be the result of a C-terminal fusion with an open reading frame during the duplication process *(117)*. The protein encoded by *hblB* has not been isolated, but the presence of a signal peptide is predicted from the nucleotide sequence, suggesting that the protein could be exported from the cell.

All three components in HBL are required for biological activity. HBL produces a unique discontinuous hemolysis pattern on blood agar *(15, 21, 22)*. Hemolysis begins several millimeters from the edge of a colony or a well containing HBL, forming a ring-shaped clearing zone (discontinuous). With time, the zone moves inward toward the source. Hemolytic potency varies depending on the species of mammalian blood tested, with guinea pig > swine > calf > sheep > goat > rabbit > human > horse. Beecher and Wong *(22)* found that the discontinuous hemolysis phe-

nomenon is mediated by the B and L_1 components. Sheep erythrocytes do not lyse when incubated with the B component alone. Rather, the erythrocytes become sensitized or primed and are rapidly lysed with the addition of L_1 and L_2. Excess concentrations of B, however, inhibit the activity of L_1 on the lysis of B-primed erythrocytes, and excess L_1 inhibits the priming activity of B. The L_2 component is required for lysis but does not interfere with the action of B or L_1. Therefore, hemolysis of erythrocytes in the blood agar plate assay occurs at the point in the diffusion gradient (away from the well) where appropriate concentrations of both B and L_1 exist.

In addition to its hemolytic activity, HBL is dermonecrotic, increases vascular permeability in rabbit skin *(21)*, and is cytotoxic to Chinese hamster ovary cells *(12)* and retinal tissue both in vitro and in vivo *(18)*. It causes fluid accumulation in the rabbit ileal loop assay, and necrosis of villi, submucosal edema, interstitial lymphocytic infiltration, and variable amounts of blood were also observed in loops that were positive for fluid accumulation *(19)*. The potency in the rabbit ileal loop assay (5 μg per component) is in a range similar to that of cholera toxin (≤1 μg), and the tissue necrosis is similar to that observed by Turnbull et al. *(163)* when working with two chromatographic fractions that exhibited lethal activity.

HBL forms pores in eukaryotic cell membranes, with each of the components binding the membrane independently and reversibly *(22)*. One hypothesis was that once bound, the components oligomerize and form transmembrane pores consisting of at least one of each component. The transmembrane segments in B and L_1 may serve as mediators of oligomerization *(14, 23)*. Membrane receptors have not been identified.

Heterogeneity of HBL. A high degree of molecular heterogeneity exists in HBL from different strains. In a study of 127 *B. cereus* isolates by Western blot analysis, four sizes of B (38, 42, 44, and 46 kDa), two L_1 (38 and 41 kDa), and three L_2 (43, 45, and 49 kDa) were identified *(136)*. Individual strains produced various combinations of single or multiple bands of each component. In addition, some strains produced only one or two of the three HBL components. A total of 13 different band patterns were observed with various forms of B, L_1, and L_2.

Beecher and Wong *(24)* reported the isolation of two distinct sets of HBL proteins from a single *B. cereus* strain, MGCB 145. One set of proteins, 87 to 100% identical in their respective N-terminal amino acid sequences to those of the prototype *B. cereus* strain F837/76, was designated HBL. The second set, designated HBL_a, was 62 to 65% identical to the prototype and consisted of B_a, L_{1a}, and L_{2a}. These antigens reacted to polyclonal antibodies raised against HBL purified from strain F837/76. The authors suggested that high sequence similarity between the two sets of homologs indicates that genes may have been either duplicated or transferred horizontally.

HBL and HBL_a from strain MGBC145 induced hemolysis in erythrocyte suspensions and increased vascular permeability *(24)*. As with HBL, all three HBL_a components were required for activity, and in most activity assays the components of HBL and HBL_a were interchangeable. No notable differences in activity between individual L_1 and L_{1a} or L_2 and L_{2a} components were observed, but B and B_a were significantly different in the hemolysis assay. On blood agar plates, B_a with L_{1a} and L_{2a} or with L_1 and L_2 produced a continuous rather than the discontinuous pattern typical of HBL. In the erythrocyte suspension assay, excess B inhibited the activity of L_1 in lysing erythrocytes that had been primed by exposure to B but not those primed by exposure to B_a. Excess B_a enhanced lysis of B_a-primed cells. The exact mechanism is unknown, but B and B_a must interact differently on the target cell membrane.

Two sets of HBL proteins were also isolated from a second *B. cereus* strain, S1C *(135)*. The N-terminal amino acid sequences of the first set of B, L_1, and L_2 had 90 to 100% identity with their respective HBL counterparts from strain F837/76, whereas the second set shared 50 to 71% identity. The second set of proteins had 45 to 86% identity with HBL_a from strain MGBC 145.

Nhe. After screening over 300 *B. cereus* strains using PCR, Western blot, and cell cytotoxicity tests, Granum and coworkers *(61, 101)* concluded that there was at least one enterotoxin complex in addition to HBL involved in *B. cereus* enterotoxigenicity. *B. cereus* strain 0075-95, responsible for a Norwegian diarrheal syndrome food poisoning outbreak, was used to produce and characterize Nhe *(101)*. Three proteins (39, 45, and 105 kDa) reacted to polyclonal antiserum that was reported to detect *B. cereus* enterotoxin. The 39- and 45-kDa proteins were isolated. Some similarity was observed between the N-terminal amino acid sequence determined for the 39-kDa protein and that of the HBL L_1 component. The 45-kDa protein, NheA, was the same as the main antigen in the *Bacillus* Diarrhoeal Enterotoxin Visual Immunoassay kit from Tecra, previously reported by Beecher and Wong *(20)*. The 105-kDa protein was identified as a protease that is not part of the Nhe complex *(102)* but is similar to ColH from *Clostridium histolyticum* and to the collagenase encoded by *colA* from *C. perfringens*, possessing both gelatinolytic and collagenolytic activity.

Granum et al. *(65)* cloned and sequenced *nhe* from *B. cereus* strain 1230-88 (EMBL accession no. Y19005). The *nhe* operon contains three open reading frames that correspond to the genes *nheA*, *nheB*, and *nheC*. The deduced sizes of the encoded proteins are 41 kDa (NheA), 39.8 kDa (NheB), and 36.5 kDa (NheC), with predicted pI values of 5.13, 5.61, and 5.28, respectively. NheA and NheB have been isolated, but NheC has not. The functions of these proteins have not been determined.

The Nhe proteins demonstrate homology with each other and with HBL components. NheA is 19% identical to NheB and NheC, and NheB is 44% identical to NheC. The identities observed between NheA and L_2, NheB and L_1, and NheC and B are 24, 37, and 25%, respectively *(14, 65)*. Similarities were also observed in predicted transmembrane helices for the proteins. Like L_1 and B, NheB and NheC have two and one predicted transmembrane helices, respectively, located in the same position, whereas NheA,

like L_2, has none. These similarities suggest that HBL and Nhe are homologs in a family of tripartite toxins consisting of HBL, HBL_a, and Nhe *(14)*.

Location of *hbl* and *nhe*. Numerous studies on genome mapping of *B. cereus* and *B. thuringiensis* have revealed that the chromosome sizes vary greatly, ranging from 2.4 to 6.3 Mb, and extrachromosomal bands are frequently observed *(33, 37, 91)*. The genetic organization of one region of the chromosome appears to be constant, whereas that of the other is variable in terms of presence and location of genes. The constant region contains housekeeping and ribosomal genes *(33–36)*, whereas genes in the variable region are often plasmid encoded. One of the strains studied by Carlson and Kolstø *(37)*, F837/76, considered the prototype strain for production of HBL *(12, 16, 21)*, has the smallest chromosome (2.4 Mb) of the 10 strains analyzed; however, it carries a large amount of extrachromosomal DNA (2.6 Mb), which exists as large plasmids that are each reported to be greater than 40 kb and stably maintained. The small chromosome of F837/76 contained many of the genes in the constant part of the larger chromosomes observed in other strains, whereas genes in the variable region were absent or present in the extrachromosomal elements. The *hbl* operon is located in the variable region, and the *nhe* operon is in the constant portion of the *B. cereus* and *B. thuringiensis* chromosome *(34, 35)*.

Distribution of HBL and Nhe. Production of both HBL and Nhe is quite widespread; 34 to 84% of *B. cereus* isolates produce HBL *(20, 45, 67, 104, 128, 132)*. Some isolates secrete only one or two of the HBL components *(136, 156)*, whereas others possess the HBL genes but the proteins are not expressed *(128, 136, 156)*. The ability of *B. cereus* to produce Nhe is even more common, with estimates of its production in 92 to 100% of isolates *(31, 44, 132)*. HBL and Nhe production is also prevalent among *B. thuringiensis (41, 128, 131)*, and HBL can be produced by *B. mycoides (128)*. The *B. anthracis* genome contains the genes for Nhe but not those for HBL *(130)*. Whether Nhe is expressed is not known.

Regulation of *hbl* and *nhe*. PlcR is a 34-kDa protein that was first described in *B. thuringiensis* as a *trans* activator for the phosphatidylinositol-specific phospholipase C gene *plcA*. PlcR regulated its own transcription at the onset of stationary phase *(97)*. Agaisse et al. *(1)* provided evidence that PlcR is a pleitropic regulator that controls the expression of at least 15 genes, many of which encode virulence factors in *B. cereus* and *B. thuringiensis*. These genes encode for degradative enzymes, cell-surface proteins, and toxins including Nhe and HBL. A highly conserved palindromic region ($TATGNAN_4TNCATA$), known as the PlcR box, is the specific recognition target for PlcR activation and is located at various positions upstream of the transcription start site of the target gene *(1, 117)*. The genes regulated by PlcR are widely dispersed and do not constitute a pathogenicity island. Many of the proteins encoded by these genes are regulated by PlcR in *B. cereus*, as determined by two-dimensional gel electrophoresis *(56)*.

Agaisse et al. *(1)* found that *plcR* is present in *B. anthracis* and *B. cereus* but not *B. subtilis*. The polypeptide produced by the *B. cereus* gene is similar to that of *B. thuringiensis*, but the *B. anthracis* polypeptide is truncated because of a nonsense mutation in the *plcR* gene and presumably is not functional. The genome sequences of *B. cereus* strains 14579 *(85)* and 10987 *(129)* contain 55 and 57 putative PlcR-binding motifs, respectively, which potentially could regulate over 100 genes in each isolate. In *B. anthracis* Ames, there are 56 putative PlcR-binding motifs in the chromosome and two on the virulence plasmid pXO2 *(130)*.

Gominet et al. *(57)* found that the *B. thuringiensis* oligopeptide permease system was required for *plcR* expression, and Slamti and Lereclus *(146)* found that a small peptide, PapR, was secreted from the cell and reimported via the oligopeptide permease system. Once inside the cell, PapR is processed into a pentapeptide and activates the PlcR regulon by promoting binding of PlcR to its DNA target.

Results of two recent studies suggest that components of the flagellar apparatus may be involved in the secretion of virulence-associated proteins, including HBL. Senesi et al. *(138)* found that a *fliY* mutant of *B. cereus* lost the ability to swarm and secrete L_2 and that L_2 was only produced in the swarm-cell state. Ghelardi et al. *(53)* observed that *flhA* was required for export of flagellin, HBL, and phosphatidyl-preferring phospholipase C in *B. thuringiensis*.

SUMMARY

B. cereus is widespread in nature and frequently contaminates a wide variety of food products. The incidence of both the diarrheal and emetic syndromes caused by *B. cereus* probably has been underestimated because the illnesses are usually self-limiting with mild symptoms. Despite the recognition of *B. cereus* as a foodborne pathogen over 50 years ago, its virulence mechanisms are still not fully elucidated. Cereulide has been identified as the causative agent in the emetic syndrome, and HBL is associated with diarrheal food poisoning. Nhe, a homolog of HBL, probably possesses biological activities similar to those of HBL and could be a factor in the diarrheal syndrome; however, this hypothesis has not been tested. In addition to causing food poisoning, HBL can play a role in nongastrointestinal infections caused by *B. cereus*. The in vivo roles of many of the putative and potential virulence factors produced by *B. cereus*, such as hemolysins, phospholipases, and proteases, have not been defined. Based on their biological activities, these factors are likely to be involved in *B. cereus* infections and illnesses. Many of these factors are also expressed by *B. thuringiensis* and *B. anthracis*, which are close relatives of *B. cereus*. Continued research is needed to elucidate the virulence mechanisms of *B. cereus* and the regulation and significance of virulence factor expression in infections and in nature.

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Food Safety Knowledge of Consumers and the Microbiological and Temperature Status of Their Refrigerators

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ABSTRACT

The objectives of this study were to examine domestic food safety knowledge levels of consumers, establish the levels and incidence of bacterial contamination and operational temperatures in domestic refrigerators, and identify areas in which consumer food safety education is necessary in Ireland. A food safety knowledge questionnaire applied to a representative sample of households ($n = 1{,}020$) throughout the island of Ireland found the gaps in consumer food safety knowledge. Analysis of swab samples ($n = 900$) recovered from the domestic refrigerators in these households showed average total viable counts of 7.1 log CFU/cm^2 and average total coliform counts of 4.0 log CFU/cm^2. Analysis of swab samples also detected the incidence of *Staphylococcus aureus* (41%), *Escherichia coli* (6%), *Salmonella enterica* (7%), *Listeria monocytogenes* (6%), and *Yersinia enterocolitica* (2%). *Campylobacter jejuni* and *E. coli* O157:H7 were not detected in domestic refrigerators. The temperature profiles of a subset of the sampled refrigerators (100) were monitored for 72 h, and 59% were found to operate, on average, at temperatures above the recommended 5°C. Knowledge and temperature survey results varied considerably, but consumers who scored better in terms of basic food safety knowledge had reduced levels of bacterial contamination in their refrigerators and reported a reduced incidence of food-associated illnesses. This study confirms the effect of basic food hygiene knowledge on hygienic practice and identifies specific areas for emphasis in the development and delivery of effective food safety risk communication messages to consumers.

Foodborne illness is a threat to the health and well-being of consumers, involving major economic losses to individuals in terms of lost working days and substantial clinical and other costs to the health and welfare sectors. Although many consumers tend to associate foodborne illness with eating outside the home, research suggests that many food poisoning cases are associated with domestic food preparation *(19).* Many of these cases are associated with the most common faults in domestic food hygiene practice, such as inappropriate storage, inadequate cooking, and cross-contamination *(16).* The importance of domestic food preparation practices in foodborne illness is supported by epidemiological data. Outbreak investigations in Scotland suggest that most food poisoning occurs in private houses and is associated with mishandling of food *(3).* A sentinel study in The Netherlands by Hoogenboom-Verdegaal and Postema *(21)* indicated that 80% of *Salmonella* and *Campylobacter* infections were associated with domestic food preparation. Similarly, the domestic kitchen has been implicated as a major source in the high incidence of family-associated foodborne disease in Germany *(26)* and France *(20).*

Currently, much of the government and regulatory authority effort to reduce the dangers posed by foodborne pathogens has focused on the application of more effective food safety management systems, such as hazard analysis and critical control point (HACCP) within the food production, processing, and retail elements of the food chain. Such efforts are certainly worthwhile, but it is also clear that poor knowledge and practice among domestic food handlers can often negate much of the effort made in improving and maintaining food safety at earlier stages of the food chain *(5, 23).*

Progress in resolving or reducing the risks involved in failures in consumer food safety practice is dependent on correctly identifying the hygiene errors that consumers make in their own kitchens and on developing education or information strategies that get the corrective messages and practices to consumers, or to relevant subgroups of consumers *(6).* In line with the above emphasis on food production, processing, and retail, relatively few investigations have examined consumer food safety knowledge and practices. The objective of this study was to identify gaps in food safety knowledge among consumers and the hygiene errors that most frequently occur in domestic kitchens so that education programs can be tailored to address these issues.

MATERIALS AND METHODS

Knowledge survey. A food safety questionnaire was designed involving 33 questions covering food safety issues (such as refrigerated storage of food, cross-contamination, defrosting and reheating, cooking, domestic food poisoning, food hygiene education, and microbial knowledge) and 5 questions covering sociodemographic issues. A pilot study of the questionnaire took place in 10 houses in a suburb of Dublin, Ireland. No revisions were made to the questionnaire following this pilot as no problems

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were evident in either administration, timing, or consumer understanding of the questionnaire. Administration of the interviews took the form of face-to-face interviews in the respondents' homes, immediately after their agreement to take part in the study. The questionnaire took approximately 10 min to complete. All responses to the questionnaire were unprompted.

Representative sampling areas ($n = 102$) were identified across the island of Ireland by the Marketing Research Bureau of Ireland (MRBI), in line with the overall population patterns on the island of Ireland. Quota controls were established in terms of the number of people in respondent households, occupations of the chief wage earners, and socioeconomic status of the main wage earner. In terms of the socioeconomic status of the chief wage earner, each person was classified into one of seven social grades on the basis of their occupation. Social class A includes upper middle-class occupations such as senior professionals, chartered accountants, architects who are partners in the business, dentists, and senior executives. Social class B includes middle-class occupations ranging from recently qualified professionals and army lieutenants to head masters in small schools. Class C1 are other white-collar workers, including junior civil servants and secretaries. Class C2 are skilled workers, such as electricians. Class D are unskilled workers. Class E includes casual workers and pensioners dependant on state pensions with no other income and those on social security. Class F are farmers.

Households were selected at random. The random route procedure involved going to the starting address, completing the first interview there, then calling at every fifth house on the left-hand side of the road. On reaching the end of that road, a left turn was taken and every fifth house was called at; on reaching the end of that road, a right turn was taken and every fifth house was called at. Ten or more households in each location were visited by this random route procedure until the quota and target number (10) had been achieved in each area. No notification was given before the visit. The questionnaires were administered during daylight hours.

In total, information was recovered from 1,020 households. All the respondents were ''mainly'' responsible for food preparation and cooking in their household. After completion of the questionnaire, permission was sought to take swab samples for subsequent microbiological analysis. Permission was also sought from 100 householders to measure their refrigerator temperature for a 72-h period.

Microbiological survey. Cellulose sponge swabs (10 by 10 by 1 cm, Sydney Heath and Son, Stoke-on-Trent, Staffordshire, UK), previously tested to ensure they did not contain inhibitory substances for bacteria, were prepared in sterile bags, moistened with 5 ml of maximum recovery diluent (Oxoid, Unipath Ltd., Basingstoke, UK) and sterilized by autoclaving. Each swab was used to sample the inner sides and floor (~2,076 cm^2) of the refrigerator by the inverted bag technique *(27)*, transported to the laboratory in a cool box at approximately 4°C, as described by Cagney et al. *(10)*, and microbiologically examined within 48 h.

Swabs were aseptically transferred into a sterile stomacher bag (Stomacher 400, Seward Medical, London, UK) and homogenized for 2 min with 250 ml of buffered peptone water (BPW CM509, Oxoid) with a Colworth Stomacher (model BA 6021, A.J. Seward and Company Ltd., London, UK). Serial dilutions of the resultant bacterial suspensions (in maximum recovery diluent) were plated onto plate count agar (tryptone glucose yeast agar, Oxoid) to obtain total viable counts (TVCs). These plates were incubated at 30 ± 1°C for 48 ± 3 h *(17)* or onto Chromocult coliform agar (Chromocult, Merck, Darmstadt, Germany) to obtain total coliform counts (TCCs). These plates were incubated at 37 ± 1°C for 24 ± 1 h *(14)*.

Presumptive *Escherichia coli* (dark blue/violet colonies on the Chromocult coliform agar) was confirmed by plating onto Levine's eosin methylene blue agar (Oxoid) and phenol red sorbitol agar, and completion of the range of biochemical tests described by Finney et al. *(14)*. Colonies displaying a green metallic sheen on Levine's eosin methylene blue agar and no fluorescence on UV-illuminated phenol red sorbitol agar with 4-methylumbelliferyl-B-D-glucuronide (Oxoid) were analyzed further as described by Cagney et al. *(10)*.

Salmonella spp. were isolated and confirmed as described by Pearce *(31)*. *Campylobacter* spp. were isolated and confirmed as described by Cloak et al. *(11)*. *Yersinia enterocolitica* was isolated and confirmed as described by Logue et al. *(28)*. *Listeria monocytogenes* was isolated and confirmed as described by McClain and Lee *(29)*. *Staphylococcus aureus* was isolated by plating onto Baird Parker medium (Baird Parker agar base with egg yolk tellurite emulsion, Oxoid). The plates were incubated at 37°C for 48 h. From each plate, five typical colonies of *S. aureus* were tested by Gram stain and tested for the production of coagulase, catalase, and DNase; the fermentation of mannitol, and oxidation. Primary identification involved subculturing of typical *S. aureus* colonies onto DNase plates (Oxoid) and blood agar plates (Columbia base agar and 5% lysed horse blood, Oxoid) and incubating at 37°C for 24 h. Colonies positive for all the aforementioned tests were maintained on tryptic soy agar (TSA; Oxoid) and confirmed by testing of the clumping factor (Staphylase Test Kit, DR595, Oxoid).

Temperature survey. Testo 175 temperature data loggers (Testo Ltd., Alton, Hampshire, UK), adjusted to record the internal refrigerator temperature every 10 min over a 72-h period, were placed on the middle shelves of 100 of the domestic refrigerators swabbed during the study.

Data analysis. The questionnaire responses and microbiological results were analyzed with SPSS (version 10.1) software. Pearson's chi-square tests were performed to examine the relationships between responses and microbial status of the refrigerator.

RESULTS

Demographics. The demographic details of the survey respondents are given in Table 1.

Food handling, refrigeration, and storage. The survey questions and results are summarized in Table 2. The first question asked was, ''Thinking about the last time you went shopping for raw meat, how much time lapsed between placing it in your shopping basket and storing it in your fridge or freezer?'' Respondents reported the time lapse from retail to domestic refrigerator as less than 30 min (58%), 30 to 90 min (35%), 90 min to 3 h (6%), or more than 3 h (1%). The next question was, ''At what temperature do you think that your refrigerator should be?'' Only one-fifth of respondents (22.4%) were aware of the correct refrigeration temperature of 0 to 5°C. Respondents were asked whether they had a thermometer in their refrigerator. Just less than a quarter (23.2%) of surveyed refrigerators reportedly contained a thermometer. Respondents in lower income households (C2DE) were significantly ($P < 0.001$) less likely to know the correct refrigeration temper-

TABLE 1. *Demographic details of the food safety knowledge respondents*

	Demographic of respondents	Response (%)
Location	Urban	75
	Rural	25
Gender	Male	23
	Female	77
No. of people in household	1–3	63
	4–5	28
	5+	9
Socioeconomic group	ABC1	39
	C2DE	51
	F1:F2	9
	Refused to answer	1
Employment status	Full time	24
	Part time	20
	Unemployed	6
	Student	4
	Retired	19
	Full-time housewife	26
	Refused to answer	1
Age	>18	1
	19–24	10
	25–34	20
	35–44	19
	45–54	17
	55–64	15
	65+	19

ature. Younger respondents (<25 years old) were significantly ($P < 0.005$) more likely to know the correct refrigeration temperature than older respondents (>25 years old). With regard to storage of raw meat in the refrigerator, approximately half of consumers reported storing raw meat correctly (on the bottom shelf or in the bottom drawer) in their refrigerators.

Respondents were asked, ''How do you clean your refrigerator?'' Respondents indicated a variety of agents used for cleaning their refrigerator, including washing-up liquid (39.4%), sanitizer or detergent (31.7%), baking soda (16.3%), vinegar (10.4%), salt (0.1%), and water (2.1%). They used these cleaning agents hot (10.8%), warm (71.3%), or cold (7%), applied with a dishcloth (54.7%), a clean cloth (21.1%), a paper towel (12%), a disposable dish cloth (9.4%), or a sponge (2.8%).

Cross-contamination. Responses in relation to practices to prevent cross-contamination on knives included washing the knife with detergent and hot water (72.8%), wiping the knife with a cloth (13.3%), rinsing the knife with cold water (7.5%), using the knife as-is (i.e., without cleaning, 2.8%), using separate knives for raw meat and cooked foods (2.2%), and washing the knife with detergent, hot water, and bleach (1.3%). Responses in relation to practices to prevent cross-contamination on cutting boards included washing the board with detergent and hot water (72%), wiping the board with a cloth (13.1%), rinsing the board with cold water (6.1%), reusing the board without cleaning (3.5%), using a separate cutting board (3%), and washing the board with detergent, hot water, and bleach (3%).

Of 56.5% of the respondents who had a pet, 302 (52.4%) allowed the pet into the kitchen.

Respondents were asked, ''On what occasions do you think it is important to wash your hands?'' The occasions mentioned were before meals (69.9%), after handling raw meat (64.6%), and after using the toilet (49.6%). Only 7% reported that hand washing was important after touching a pet. Respondents described a variety of hand washing (see Table 2). The methods mentioned included ordinary soap and warm or hot water (63.7%), antibacterial soap and warm or hot water (21.7%), washing-up liquid and hot water (4.1%), rinsing with warm or hot water (without soap, 6.6%), rinsing with cold water (3.0%), and wiping with a tea towel, dish cloth, or disposable cloth (J-cloth, 0.9%).

Defrosting, storing, and reheating. Respondents reported defrosting frozen meat at room temperature (56.2%), in the refrigerator (23.4%), or in a microwave oven (13.1%). Respondents stored meat leftovers in the refrigerator (57.6%), in the oven (6.0%), at room temperature on a table or counter (5.2%), in the freezer (2.9%), and in an unrefrigerated cupboard (1%). Almost one third (27.8%) of respondents cited that this question regarding storage of leftovers was not applicable to them. In terms of reheating meat, respondents reported heating leftovers until they were hot (42%), warm (10.1%), or cold (20.1%), and this question was not applicable to 27.8% of respondents.

Cooking. When respondents were asked how they cook roast beef, steak, and beef burgers, they reported that meats were cooked until well done (roast beef, 83.7%; steak, 75.6%; beef burgers, 82.7%), medium (roast beef, 9.7%; steak, 15.8%; beef burgers, 2.5%), or rare (roast beef, 3.2%; steak, 5.3%; beef burgers, 0.1%). No respondents reported eating raw roast beef or beef burgers, but 0.3% reported eating raw steak. Some respondents reported that the question was not applicable (roast beef, 3.3%; steak, 3%; beef burgers, 14.7%).

Respondents used a variety of tests to check that red meat was sufficiently cooked, including visible inspection (39.7%), until the juices ran clear (28%), when brown inside (12.5%), when cooked for a specified period (7.5%), or until the meat fell from the bone (5%). For poultry, respondents checked that such meat was sufficiently cooked by visible inspection (32.5%), until the juices ran clear (30.6%), until brown outside (13.6%), after cooking for a specified period (8.4%), until meat fell from the bone (8.5%), or by taste (2.8%).

Domestic food poisoning. When asked to estimate the percentage of food poisoning associated with domestic food preparation, 38.9% of respondents suggested up to 20% of cases, 18.7% of respondents suggested between 21 and 40% of cases, 23.4% of respondents suggested between 41 and 60% of cases, 13.9% of respondents suggested between 61 and 80% of cases, and 1.7% of respondents suggested between 81 and 100% of cases. The remainder, (3.3%) did not express an opinion. Those consumers who believed that

TABLE 2. *A summary of the main survey questions and their respective answers*

Question	Answer
Thinking of the last time you went shopping for raw meat, how much time lapsed before it was stored in the refrigerator or freezer?	<30 min (58%) 30–90 min (35%) 90–180 min (6%) >180 min (1%)
At what temperature do you think your refrigerator should be operating?	<0°C (4%) 1–5°C (22%) 6–10°C (6%) >10°C (1%) Unsure (67%)
Do you have a thermometer in your refrigerator?	Yes (23%) No (77%)
Do you have a thermometer in your freezer?	Yes (12%) No (64%) Not applicable (14%)
When you refrigerate raw meat, where do you store it?	Top shelf (13%) Middle shelf (10%) Bottom shelf or drawer (53%)
What cleaning agent do you use to clean your refrigerator?	Washing-up liquid (39%) Sanitizer (18%) Baking soda (16%) Detergent (13%) Vinegar (10%) Water (2%) Salt (0.1%)
What temperature is the cleaning agent you use to clean your refrigerator?	Hot (11%) Warm (60%) Luke warm (11%) Cold (7%)
What cleaning cloths/other do you use to clean your refrigerator?	Disposable cloth (J-cloth) (9%) Paper towel (12%) Clean cloth (21%) Dish cloth (55%) Sponge (3%)
When you cut raw meat and need to use the knife again, what do you do?	Reuse the knife as it is (3%) Rinse with cold water (8%) Wipe with a cloth (13%) Wash with detergent and hot water (73%) Wash with detergent, hot water, and bleach (1%) Use a different knife (2%)
When you cut raw meat and need to use the cutting board again, what do you do?	Reuse the cutting board as it is (4%) Rinse with cold water (6%) Wipe with a damp cloth (13%) Wash with detergent and hot water (72%) Wash with detergent, hot water, and bleach (3%) Use a different board (3%)
Do you allow a pet into your kitchen?	Yes (30%) No (27%) Not applicable (43%)
On what occasions do you think it is important to wash your hands?	Before preparing meals (70%) After using the toilet (50%) After handling raw meat (65%) After feeding or touching pets or other animals (7%) After gardening (1%) When I come home from work (1%) After changing a baby's nappy (2%)
How do you wash your hands?	Ordinary soap and warm or hot water (64%) Antibacterial soap and warm or hot water (22%) Washing-up liquid and hot water (4%) Warm or hot water only (7%) Cold water only (3%) Wipe with a tea towel, dish cloth, or J-cloth (1%)

TABLE 2. *Continued*

Question	Answer
How do you defrost frozen meat?	At room temperature (56%) In the refrigerator (23%) In the microwave (13%) Cook frozen (1%) In boiling water (1%) Not applicable (6%)
Where do you store leftovers?	In the refrigerator (58%) In the freezer (3%) On the countertop or table (5%) In the oven (6%) In a press or cupboard (1%) Other (please specify) (0%) Not applicable (28%)
Considering the last time you ate leftovers, how would you describe their temperature?	Cold (20%) Warm (10%) Hot (42%) Not applicable (28%)
How well cooked do you like roast beef?	Raw (0%) Rare (3%) Medium (10%) Well done (84%) Not applicable (3%)
How well cooked do you like steak?	Raw (0%) Rare (5%) Medium (16%) Well done (76%) Not applicable (3%)
How well cooked do you like beef burgers?	Raw (0%) Rare (0%) Medium (3%) Well done (83%) Not applicable (15%)
How do you check that red meat is sufficiently cooked?	When the juice runs clear (28%) When it tastes cooked (4%) When it looks cooked (visible inspection) (40%) When the meat falls away from the bone (5%) When the meat is brown on the inside (13%) When the meat has the correct thermometer reading (2%) When the meat has been cooked for the stated time (8%) Other (please specify) (0) Not applicable (1%)
How do you check that poultry is sufficiently cooked?	When the juice runs clear (31%) When it tastes cooked (3%) When it looks cooked (visible inspection) (33%) When the meat falls away from the bone (9%) When the meat has a brown outer coating (14%) When the meat has the correct thermometer reading (2%) When the meat has been cooked for the stated time (8%) Other (please specify) (1%) Not applicable (1%)
What percentage of food poisoning do you think is associated with the home?	Up to 20% (39%) 21–40% (19%) 41–60% (23%) 61–80% (14%) 81–100% (2%)
Have you or any member of your family suffered from food poisoning in the last 12 months?	Yes (19%) No (81%)

TABLE 2. *Continued*

Question	Answer
If you or your family experienced food poisoning in the last 12 months, where do you think you got it?	Restaurant (32%) Home (27%) Take-away (20%) Mobile take-away (13%) Barbeque (2%) Crèche (1%)
Where did you learn about food safety?	Parent, guardian, or grandparent (52%) Friend (5%) Cookery class (4%) School (28%) Third-level course (7%) On-the-job training (8%) Magazine or newspaper (17%) Experience (26%) Television (21%) Food safety agency (3%) Internet (1%) Food safety brochure (6%) Medical professional (1%) Radio (2%) Brochure (5.7%) Other (2%)

less than 20% of food poisoning occurred in the home were significantly ($P < 0.05$) less likely to wash their hands correctly.

When questioned about direct experience of food poisoning within the last 12 months, approximately four fifths (80.5%) of respondents claimed the question was not applicable because neither they nor a family member had suffered from food poisoning during that time. Of those who had experience of food poisoning within the last 12 months, respondents suspected restaurants (31.8%), the home (26.7%), take-away (20%), mobile take-away (food van) (12.8%), barbecues (2.0%), and crèches (day care/nursery) (0.5%). Of those who had experience with food poisoning within the last 12 months, 6.2% did not express an opinion as to the potential source of the illness.

Food hygiene education. Respondents reported learning correct food safety practices from their parents or grandparents (52.1%), school (28.4%), general experience (25.6%), television (21%), newspapers or magazines (16.6%), work (8%), third-level education (6.5%), brochures (5.7%), friends (4.9%), cooking classes (4.3%), food safety agencies (2.9%), radio (1.8%), medical doctors (1.1%), and the internet (0.5%).

Microbial knowledge. The levels of awareness of food poisoning agents is summarized in Table 3. Respondents reported having heard of *Salmonella* (92.9%), *E. coli* O157 (77%), and *L. monocytogenes* (45.2%). A range of other pathogens, such as *Campylobacter*, *Bacillus cereus*, *S. aureus*, *Clostridium perfringens*, *Clostridium botulinum*, *Y. enterocolitica*, and viruses, were reported by <20% in each case. Respondents were asked the open-ended question, ''Do you associate any food with . . .'' each pathogen mentioned. In general, respondents had limited success in associating pathogens with particular foods. The most fre-

TABLE 3. *Knowledge of different food poisoning agents and associated foods*

Bacteria	Rate of respondent recognition (%)	Correct association with relevant food (% of those who had heard of the agent)		
Salmonella	92.9	Poultry: 23.1%	Pork: 4.7%	Eggs: 44.0%
L. monocytogenes	45.2	Beef: 9.1%	Soft cheese: 28.1%	Vegetables: 2.9%
Shigella	5.6	Meat: 7.1%	Water: 8.9%	Salads: 1.8%
E. coli O157	77.0	Beef: 38.7%	Raw milk: 2.1%	Burgers: 15.9%
Campylobacter	10.2	Poultry: 19.6%	Pork: 1.0%	Eggs: 3.9%
B. cereus	10.1	Rice: 9.9%	Cream/milk: 5.0%	Soup: 0.0%
S. aureus	2.5	Milk: 2.5%	Eggs: 0.6%	People: 21.7%
C. perfringens	5.2	Meat: 9.6%	Spices: 0.0%	
C. botulinum	16.7	Canned foods: 14.4%	Meat: 12.6%	
Y. enterocolitica	1.5	Pork: 33.3%		
Viruses	18.3	Shellfish: 6.6%		

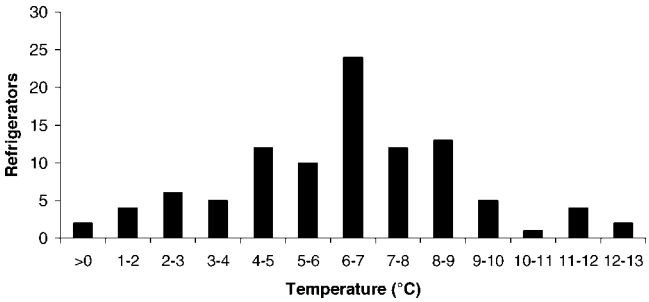

FIGURE 1. *Mean temperature frequency distribution for domestic refrigerators in Ireland.*

quently reported association was between *Salmonella* and eggs (44%). Some of the other associations reported included *E. coli* O157 and beef (38.7%) and *L. monocytogenes* and soft cheese (28.1%). Interestingly, respondents with more correct microbial knowledge were significantly ($P < 0.05$) more likely to correctly store raw meat and to correctly clean knives and cutting boards.

Refrigerator temperatures. In the survey of 100 refrigerators, the observed mean temperatures varied between −1.7 and 11.8°C, and 71 had average temperatures above 5°C. The maximum temperature was 20.7°C, and the minimum temperature was −7.9°C (Fig. 1).

Microbiological survey. TVC varied between not detected and 9.56 log CFU/cm^2. TCC varied between not detected and 6.43 log CFU/cm^2. The average TVC and TCC were 7.1 and 4.0 log CFU/cm^2, respectively (see Table 4). A number of the target pathogens were detected in the sampled refrigerators (i.e., *S. aureus* [41%], *Salmonella* spp. [7%], *E. coli* [6%], *L. monocytogenes* [6%], and *Y. enterocolitica* [2%]). *Campylobacter* spp. and *E. coli* O157:H7 were not detected. Fifty-two percent of refrigerators contained at least one of the aforementioned pathogens.

Statistical analysis of consumer knowledge and microbial survey data suggested the following.

(i) Urban consumers had significantly ($P < 0.05$) higher TVCs and general incidence of pathogens in their refrigerators than rural consumers (Table 5).

(ii) Respondents younger than 25 years of age are more likely to have one or more pathogens present in their refrigerators.

(iii) Respondents from the socioeconomic group ABC1 had a significantly ($P < 0.05$) higher general incidence of pathogens than the C2DE group.

(iv) Respondents from the socioeconomic group ABC1 had significantly ($P < 0.05$) higher TVCs than the C2DE group.

(v) Respondents with a third-level qualification had significantly ($P < 0.05$) higher TVCs in their refrigerators than respondents without a third-level qualification.

(vi) Respondents who reported correct hand washing procedures (89.5%) had significantly ($P < 0.05$) lower TVCs, TCCs, and incidences of pathogens than respondents who washed their hands incorrectly (10.5%).

(vii) Respondents who had heard of *Salmonella, Campylobacter, E. coli,* or *L. monocytogenes* were more likely to wash their hands correctly ($P < 0.05$).

(viii) Respondents who reported correct knife and cutting board cleaning procedures were more likely to know the correct refrigeration temperatures ($P < 0.05$) than respondents who reported an incorrect cleaning procedure for these utensils. The respondents who reported this correct cleaning procedure were more likely to cook meat and poultry correctly ($P < 0.05$). These respondents also had significantly ($P < 0.05$) lower TVCs and incidences of pathogens in their refrigerators than the respondents who reported an incorrect procedure for washing the knife and cutting board. Interestingly, respondents who reported that they or a member of their family had suffered food poisoning in the previous 12 months had significantly ($P < 0.05$) higher TVCs and incidences of pathogens in their refrigerators.

TABLE 4. *Quantitative and qualitative microbial contamination in Irish domestic refrigerators*

Bacterial count (log CFU/cm^2)	
Mean total viable count (TVC)	7.1
Mean total coliform count (TCC)	4.0
% incidence (mean)	
Campylobacter	0
E. coli O157	0
Y. enterocolitica	2
L. monocytogenes	6
E. coli	6
Salmonella	7
S. aureus	41

TABLE 5. *A summary of the significant associations between demographics, food safety knowledge, and microbiological survey data*

Urban consumers had significantly higher TVCs and general incidence of pathogens in their refrigerators
Respondents younger than 25 years of age were more likely to have pathogens present in their refrigerators
Socioeconomic group ABC1 had a significantly higher TVC and incidence of pathogens in their refrigerators compared with C2DE group
A knowledge of correct hand washing procedures resulted in significantly reduced refrigerator TVC, TCC, and pathogen incidence
Respondents who had a basic awareness of pathogens like *Salmonella, Campylobacter, E. coli,* or *L. monocytogenes* tended to wash their hands correctly
Respondents who knew the correct knife and cutting board hygienic procedures to prevent cross-contamination were more likely to know the correct refrigeration temperatures and safe meat and poultry cooking practices
Those with a lack of knowledge of how to prevent cross-contamination via knives and cutting boards had significantly higher refrigerator TVC and incidence of pathogens
Respondents who reported that they or a family member had suffered food poisoning in the previous 12 months had significantly higher refrigerator TVC and incidence of pathogens

DISCUSSION

Some potential for temperature abuse exists because 7% of respondents allow more than 90 min to lapse between shopping and chilled or frozen storage. Any practice in which food is held over time in temperatures conductive to bacterial growth is a potential risk in terms of food safety because it allows more rapid growth of spoilage microorganisms and the growth of food pathogens, if they are present *(21).* One method of helping to ensure that perishable food remains microbiologically safe after purchase is to place it in a refrigerator or freezer as soon as possible.

In this study, 22.4% of consumers were aware of the correct refrigerator temperature. This can be compared with results from surveys in the United Kingdom, the United States, and Australia, where 10%, 54%, and 32.3% of consumers knew the correct refrigeration temperature *(4, 23, 32).* More than three quarters of respondents in this survey (76.8%) reported that they had no refrigerator thermometer; thus, the refrigeration temperature could not be monitored. In this respect, knowledge of the correct refrigeration temperature is of no benefit to respondents who have no means of monitoring it. The observed refrigerator temperature means varied between −1.7 and 11.8°C, with an average temperature of 5.4°C. Of the refrigerators surveyed, 59% had an average temperature higher than 5°C, and 6% had an average temperature higher than 10°C. A similar study in Northern Ireland reported mean temperature ranged from 0.8 to 12.6°C, with 71.3% over 5°C and 6% over 10°C *(15).* If food is held over time in temperatures conductive to bacterial growth, there is a potential risk in terms of food safety because it allows more rapid growth of spoilage microorganisms and the growth of food pathogens, if they are present *(23).* This is important at the domestic refrigeration stage, which can be the last line of defense in terms of controlling bacterial proliferation.

In general, the majority of respondents in this all-Ireland study did correctly identify occasions when hand washing is important in terms of good domestic food preparation practice, such as before meals (69.9%). However, this result is less satisfactory than in other countries. For example, Australian consumers scored higher (81.6%) on hand washing, reporting hand washing before meals. More significantly, the fact that more than 30% of those preparing food in Irish homes do not wash their hands at such times continues to present considerably increased risks to themselves and their families.

Respondents to this survey scored quite well (i.e., 64.6%) in terms of the need to wash hands after handling raw meat, which is better than in other similar studies. For example, only 53% of Australian consumers scored well in this area. One of the most worrying observations in this study was that less than half (49.6%) of respondents reported the importance of washing hands after using the toilet. The critical importance of fecal-to-oral transfer is well established in the epidemiology of pathogens, and such responses indicate the need for significantly greater education in this area. It has long been established that pets carry a range of human pathogens and can facilitate cross-contamination. Thus, the very low percentage of respondents (7%) who recognized the need to wash hands after contact with pets is of concern, especially because this percentage is so much lower than reported in other studies. In comparison, almost all survey respondents (99%) in New Zealand did consider hand washing important after touching a pet *(8).* This suggests that Irish consumers require significantly more information and education in relation to the role of domestic pets as significant sources of human pathogens *(9).* However, Ireland is not alone in this regard, because poor knowledge and practice in relation to pets as sources of human pathogens have been reported in other countries (e.g., Australia *(22)*). This suggestion is confirmed by the observation that more than half (52.4%) of respondents who had pets allowed their pet into the kitchen.

A considerable percentage of respondents did not use effective means of properly cleaning cutting boards (23%) and knives (24%) after cutting raw meat, leaving themselves and their families subject to significant risk of consuming cross-contaminated foods. Such cross-contamination in domestic kitchens is suggested to be responsible for 14% of all cases of food poisoning in the United Kingdom *(33).* Such undesirable results have also been reported in Australia, where similar percentages of consumers failed to clean utensils correctly after use with raw meat and vegetables *(23).* Such observations confirm the more general finding of a Ministry of Agriculture, Fisheries, and Food survey, which found that 70% of British consumers were unaware of the risks, potential, and nature of cross-contamination *(2).* Because it is currently impossible to prevent undesirable pathogens from entering the domestic kitchen on raw meat, vegetables, etc., considerable efforts could be required to improve consumer understanding and action in this area.

Irish consumers had a limited knowledge of food-associated pathogens. Although most respondents had heard of *Salmonella* (92.9%) and *E. coli* O157 (77%), less than half (45.2%) had heard of *L. monocytogenes,* and the other significant foodborne pathogens (*Campylobacter, B. cereus, S. aureus, C. perfringens, C. botulinum, Y. enterocolitica,* and viruses) were less well known (<20% awareness; Table 3). Overall, there was considerable ignorance regarding the association between pathogens and specific high-risk foods. This is in agreement with Scott *(35),* who reported that many British consumers were unaware of the link between foods, especially raw foods, and bacterial pathogens. Even the best scores of correct association were less than 50%. Thus, only 44% of respondents were able to associate *Salmonella* with eggs, 39% *E. coli* O157:H7 with beef, 33% *Yersinia* with pork, and 28% *Listeria* with soft cheeses. These exceptions probably relate to recent large or high-profile outbreaks involving those pathogens. In most other cases, correct associations were less frequent (i.e., <20%), with many scoring less than 10%.

The relatively strong recognition of *Salmonella* and its association with eggs is in agreement with Jay et al. *(23),* who reported *Salmonella* as the best known foodborne pathogen in a study of Australian consumers, although the degree of association was much higher in that study (96%).

Other, higher degrees of association between *Salmonella* and eggs have been reported; for example, Altekruse et al. *(1)* observed that 80.2% of U.S. respondents associated *Salmonella* with eggs. However, such patterns do not extend to other pathogens; for example, in this study, only 14% of respondents associated *C. botulinum* with canned foods, which is much lower than in the Australia (62%, *(23)*) or the U.S. (74.8%, *(1)*). Higher recognition in Australia and the United States might reflect the greater popularity of home canning of low-acid foods in these countries.

This study found that socioeconomic status and level of education were inversely associated with the incidence of pathogens and overall microbial numbers (TVCs) in domestic refrigerators. ABC1 consumers with a third-level qualification had higher incidences of pathogens, and higher TVCs in their refrigerators. Those with a third-level education or higher paid job might lack adequate food skills because of a lack of food handling experience *(38),* or they might follow different lifestyles, such as proportionally greater consumption of convenience foods *(25),* or have a lower aversion to risk-taking behaviors *(4, 24).*

Interestingly, consumers under 25 years of age were also more likely to have pathogens in their refrigerator. Griffith et al. *(18)* and Williamson et al. *(39)* reported a link between age and food hygiene, with older people more likely to properly store and cook food. Younger consumers often lack basic food handling and preparation skills because of changes in lifestyle and the increased consumption of convenience foods *(25).* For whatever reason, it is clear that younger consumers face higher risks of domestic foodborne illness. Thus, the findings of this study support Williamson et al. *(39)* in recommending a greater focus on food safety educational efforts for consumers under 35 years of age.

The hygiene status of refrigerators is indicated by TVC and TCC. The average TVC was 7.1 log CFU/cm^2 and varied between not detected and 9.56 log CFU/cm^2. Gorman et al. *(17)* similarly reported a TVC of 7 log CFU/cm^2 in domestic kitchens. The average TCC was 4.0 log CFU/cm^2 and varied between not detected and 6.43 log CFU/cm^2. In other studies, it was found that the kitchen environment was more heavily contaminated with coliforms than the bathroom *(30, 34).* This study shows that there is a wide range of undesirable bacteria and pathogens in domestic refrigerators. *S. aureus* was the most common pathogen found (41%), followed by *Salmonella* (7%), *L. monocytogenes* (6%), and *Y. enterocolitica* (2%). Other studies have also detected bacterial pathogens in the domestic kitchen environment, including *E. coli (37), Listeria* spp. *(7), L. monocytogenes (12, 36),* and *S. aureus (13).* The pathogens found in this study of domestic refrigerators could contaminate food directly or indirectly and pose an important risk to consumers in terms of food poisoning. Thus food safety knowledge with a focus on kitchen hygiene and prevention of cross-contamination is necessary if the scale and effect of domestic foodborne illness is to be reduced.

Although Irish consumers generally had limited knowledge of high-risk foods, those respondents who had better microbial knowledge were more likely to practice hygienic food storage and preparation and had lower TVCs and incidence of pathogens in their refrigerators. Furthermore, those households with ''cleaner'' refrigerators (i.e., lower TVCs) reported lower incidences of food poisoning within the last 12 months. This is in line with Woodburn and Raab *(40),* who reported that the ability to correctly identify foods associated with a specific pathogen was an important motivator for safe food preparation in U.S. homes. Overall, these findings are in agreement with Scott *(35)* and suggest that effective and appropriate food safety education can deliver significant reductions in the burden of foodborne illness among consumers.

The results of the food safety knowledge, microbial, and temperature surveys give cause for concern about consumer food storage and food preparation practices. Food safety agencies, such as the Food Safety Promotion Board on the island of Ireland should tailor the food hygiene message to educate consumers about bacterial pathogens and foods associated with them in order to motivate changes in cleaning, chilling, cross-contamination, and cooking in the domestic kitchen. This message should be targeted at younger audiences (<35 years), and parents should be reminded of their responsibility for teaching their children the fundamentals of food hygiene.

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