



National Institute for Public Health
and the Environment
Ministry of Health, Welfare and Sport

***Clostridium perfringens* associated
food borne disease**

Final report

Report 330371005/2011

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This investigation has been performed by the order and for the account of the new Food and consumer Product Safety Authority, within the framework of Project V/330371/01/Cp.

Abstract

***Clostridium perfringens* associated food-borne disease: final report**

Release of the enterotoxin Cpe by *Clostridium perfringens* in the small intestine after the consumption of contaminated food may lead to diarrhoea. Potentially pathogenic bacteria seem to occur only in selected food commodities. Moreover, specific food preparation processes seem to be more involved in the onset of disease. These are the main conclusions from research carried out by RIVM by the order of the new Food and Consumer Product Safety Authority (nVWA).

Food contaminated with *C. perfringens* leads to approximately 160,000 disease cases annually in the Netherlands. The nVWA aims to obtain more insight into the most harmful combination food-commodity – preparation-process with respect to *C. perfringens*.

C. perfringens is frequently isolated from meat-containing food commodities such as soups and stews, and from herbs and spices. A minority of the isolated strains carry the gene encoding the enterotoxin Cpe and are thus potentially pathogenic. Strains isolated from meat-containing food commodities carry the Cpe gene more often than strains isolated from herbs and spices. Outbreak investigation reports show that inadequate cooling and/or inadequate reheating is often the main cause for the onset of disease. The most important food commodities involved in outbreaks are meat-containing dishes such as soups and stews. Cooling of food is a crucial but also a difficult step to control in the preparation cascade, especially in the private household. Reheating after cooling and before consumption is easier to control. Hence, more elaborate investigations into reheating have been carried out.

Key words:

Clostridium perfringens, diarrhoea, food preparation, prevention of disease

Rapport in het kort

Ziekte door voedsel besmet met *Clostridium perfringens*: eindrapport.

Mensen die voedsel eten dat de bacterie *Clostridium perfringens* bevat, kunnen daar diarree van krijgen. Deze bacterie komt vooral voor in producten die vlees bevatten, zoals soepen en stoofschotels, maar ook in kruiden en specerijen. Mensen worden voornamelijk ziek na het eten van vleesbevattende producten die onder verkeerde omstandigheden zijn gekoeld voor opslag. Dat kan ook gebeuren als dit soort voedsel onvoldoende is opgewarmd nadat het is gekoeld.

Dit zijn de belangrijkste conclusies uit onderzoek van het RIVM, dat in opdracht van de nieuwe Voedsel en Waren Autoriteit (nVWA) is uitgevoerd. Jaarlijks worden in Nederland ongeveer 160.000 mensen ziek nadat zij voedsel hebben geconsumeerd dat besmet is met de bacterie *Clostridium perfringens*. De nVWA wil meer inzicht krijgen welke combinaties van het voedseltype en het bereidingsproces tot ziekte kunnen leiden.

Als mensen bereid voedsel willen bewaren, wordt aanbevolen het zo snel mogelijk na de bereiding af te laten koelen. Om voedsel voldoende te verhitten wordt aanbevolen een kerntemperatuurmeter te gebruiken. Om zeker te weten dat alle levensvatbare bacteriën zijn gedood, kan als richtlijn worden gesteld dat de kern van voedsel minimaal gedurende 10 minuten 65 graden Celcius moet zijn. Het is lastiger te controleren of voedsel voldoende is afgekoeld.

Bij onvoldoende verhitten of afkoelen produceert de bacterie in de dunne darm het eiwit enterotoxine Cpe. Dit eiwit komt vrij als de bacterie daar tijdens het verteringsproces overgaat in een spore (een vorm die tegen veel externe factoren, zoals koude en zuren, bestand is). Het eiwit tast het epitheel aan, waardoor ziekteverschijnselen ontstaan.

Trefwoorden:

Clostridium perfringens, diarree, voedselbereiding, ziektepreventie

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Summary

Clostridium perfringens is a spore-forming anaerobic microorganism that may cause food borne disease by the action of enterotoxin Cpe. The bacterium grows in anaerobic conditions. The disease is characterised mainly by watery diarrhoea with an incubation time of 8 – 24 hours and a duration of the disease symptoms of 24 hours at most. The disease is self limiting. Although not known exactly, approximately 160,000 cases occur annually in the Netherlands.

In order to reduce the number of cases, the New Food and Consumer Product Safety Authority (nVWA) is interested in the combination of food commodities and preparation processes that pose the highest risk for developing *C. perfringens* associated disease. The outcomes of laboratory investigations and literature searches have been used to answer this question.

For these investigations we assumed that the most risky preparation process involves cooking food, with subsequent cooling for storage and reheating before consumption. In this process, vegetative cells are killed during cooking but spores survive the initial cooking process. These may germinate and grow during improper cooling and/or improper reheating. This assumption was confirmed by reports on outbreaks, where disease was most often caused by improper cooling and/or reheating after cooking.

Data on prevalence and concentration of *C. perfringens* in various food commodities were received from the nVWA. All food commodities are categorised in food commodity groups, for example *vegetables and vegetable products*, or *meat and meat products*. One such group is *prepared food*, which comprises food commodities consisting of ingredients from different commodity groups and which have experienced a preparation process already (e.g., soups and stews). It could be concluded from the nVWA data that the food commodity groups *spices and herbs* and *prepared food* pose the highest risk with respect to contamination with *C. perfringens*. The involvement of *prepared food* was confirmed by a survey of reported outbreaks, which made clear that often meat-containing dishes (included in the *prepared food* group) are involved, i.e., dishes with long preparation time and a cooling and a reheating step such as stews and soups.

Disease symptoms are caused by the enterotoxin Cpe. Approximately 8% of the investigated *C. perfringens* strains possessed the gene encoding the enterotoxin and are therefore potentially pathogenic. With this finding the estimated risk for diarrhoeal disease by *C. perfringens* is less than estimated on the basis of the prevalence of the micro organism. Importantly, investigation of nVWA isolates showed that strains isolated from *prepared food* commodities have a high prevalence of the Cpe encoding gene. Strains isolated from commodities from the *spices and herbs* group have a low prevalence of the gene.

Earlier research on *Bacillus cereus* learned that processes in the gastrointestinal tract may play an important role in the pathogenic process of food borne disease of this micro organism. Since *C. perfringens* is a Gram positive, spore forming, enterotoxin producing micro organism as well, investigations into simulated gastrointestinal conditions on the survival of both vegetative cells and spores were carried out. Spores survive the gastric conditions unharmed but germination and subsequent growth in simulated intestinal conditions could not be accomplished. From these results it was assumed that ingested spores take no part in the pathogenic process. Vegetative cells appeared to be very acid resistant, only at pH values ≤ 2 was rapid inactivation observed. At the ingestion of solid food *in vivo*, the

gastric pH rapidly rises to values near five. Therefore, we assumed that when present in solid food (including stews and pea soup), vegetative cells pass the stomach unharmed. Moreover, in simulated intestinal conditions *C. perfringens* was able to grow to levels comparable to those that are reached after the ingestion of the generally accepted number of cells necessary to invoke disease ($\geq 10^7$ colony forming units per gram of food). The cascade of cooking, cooling and reheating is considered a risky preparation process with respect to *C. perfringens* associated disease, as indicated earlier. Even though clear instructions are available for cooling food commodities, proper performance of this process was assumed to be hard to manage without proper equipment, especially in the private household. Therefore, we undertook investigations into the reheating process. Complete inactivation of vegetative cells could be accomplished without bringing the dish to boil. Importantly, proper reheating is easy to monitor with the use of a temperature probe.

The outcome of all investigations is that the most risky combination of the preparation process and food commodity is the preparation of meat-containing dishes with a long cooking time and a cooling and reheating step before consumption. Important dishes within this definition are stews and soups. Although frequently contaminated, commodities from the *spices and herbs* group pose a lesser risk because of the low concentrations of *C. perfringens* and the low prevalence of the Cpe-encoding gene in the strains. Decrease of the risk for disease can be accomplished by proper cooling but more easily by proper reheating. The latter is especially important for the private household.

1 General introduction

Clostridium perfringens type A is a spore forming, toxin producing bacterium first described by the American bacteriologist, Welch. The species is classified into five types (A – E) depending on the production of toxins. With respect to food borne disease, type A is important. The predominant disease symptom is watery diarrhoea.

C. perfringens type A can not only be found in soil, but also isolated from water, dust, sediments and raw and processed food commodities. Food commodities that are regularly associated with high prevalence of *C. perfringens* are commodities that contain meat and need long preparation times, cooling and reheating such as soups and stews. Spices and herbs are also known for their high prevalence of *C. perfringens*. Moreover, the micro organism is a common inhabitant of the human and animal gastrointestinal tract. Disease is caused by enterotoxin (Cpe) produced in the gut during the sporulation of vegetative cells (Adams and Moss 2004; Aguilera, Stagnitta et al. 2005). From here onward in this report, whenever *C. perfringens* is mentioned, type A is meant.

In the Netherlands, the number of cases of *C. perfringens* associated food borne disease is hard to estimate, since the symptoms last for a relatively short time, approximately 24 hours, restraining patients from seeking medical attention. The number of cases is estimated to be approximately 160,000 (range 65,000 – 290,000) cases annually. This number is based mainly on the outcome of the SENSOR study, a Dutch prospective population-based cohort study that was conducted from 1998 through 1999 in order to estimate the incidence of gastroenteritis and its pathogens (de Wit, Koopmans et al. 2001; Haagsma, Van Der Zanden et al. 2009).

Even though the exact number of cases is not known, the general policy is to reduce the number of food borne disease cases, including those caused by *C. perfringens*. As a result of that policy, the following research question was formulated by the Food and Consumer Product Safety Authority.

2 Research question

The overall research question was to identify combinations of food commodities and food preparation that may pose a risk for the development of *Clostridium perfringens* associated food borne disease. The main objective is to formulate measures leading to reduction of the number of disease cases caused by this micro organism.

3 Report set-up

The leading principle for our investigations was the following scheme:

Critical step	Assumed fate of <i>C. perfringens</i>
Cooking ↓	Food ingredients are contaminated with vegetative cells and spores; during cooking vegetative cells are killed, spores survive the process.
Cooling ↓	If cooling is adequate no germination and growth of spores takes place; if cooling is inadequate spores germinate and grow to high numbers of vegetative cells.
Reheating ↓	When time and temperature are adequate, food is safe for consumption; if time and/or temperature is inadequate the number of vegetative cells may increase to levels that may provoke diarrhoeal disease.
Gastric passage ↓	Spores survive gastric conditions without harm; the number of vegetative cells may be affected by the gastric conditions.
Intestinal passage	Spores may germinate, grow, and produce enterotoxin. Vegetative cells sporulate and produce enterotoxin.

This leading principle can be related to food commodities that need long cooking/simmering times, are cooled before storage and reheated before consumption, and contain meat. Examples of such food commodities are pea soup and stew-like food products.

In this report we describe the investigations deemed necessary to answer this overall research question and to get more insight in the processes as described above. These investigations comprise:

- an inventory of the prevalence of pathogenic *C. perfringens* strains in food commodities
- a literature-inventory into the background of outbreaks of *C. perfringens* induced diarrhoeal disease
- studies into preparation details of food commodities and simulation of reheating processes
- studies on the behaviour of *C. perfringens* in the gastrointestinal tract

Each of these subjects will either be described or referred to in appendices. The overall results will be described and discussed below in short. Based on the results and discussion, recommendations will be made for future investigation of food commodities for *C. perfringens*, for monitoring and outbreak research purposes. Recommendations will also be made for food

preparation, in order to reduce the number of cases of *C perfringens* associated diarrhoeal disease.

4 Results

4.1 Inventory of the presence of pathogenic *C. perfringens* in food commodities (see Appendix 1)

The inventory consisted of two different studies. Firstly, data on the presence and number of *C. perfringens* isolated from food commodities, received from the new Food and Consumer Product Safety Authority (nVWA), were reviewed and categorised. Secondly, presumptive *C. perfringens* strains from the culture collection of the (nVWA) were investigated for their potential pathogenicity. The investigations consisted of identification of strains as *C. perfringens* and determination of the presence of an enterotoxin-encoding gene (Cpe-gene).

For the first part of this study, all data received from the VWA were categorised into food commodity groups. The most important with respect to *C. perfringens* were *meat and meat products, prepared food commodities and spices and herbs*. The highest percentages of positive samples were found in food commodity groups *meat and meat products* and *spices and herbs*, 7.2 and 14.2% respectively. The majority of the positive samples from the *meat and meat products* group contained high numbers of *C. perfringens*, i.e., between 10^5 and 10^6 colony forming units (CFU) per gram of food. The majority of the positive samples from the *spices and herbs* group contained between 10^2 and 10^3 CFU per gram of food.

Products from the groups *meat and meat products* and *prepared food commodities* were related most often to *complaint* samples, i.e., samples received/collected after an outbreak or collected as a sequel to complaint about the quality of food. Only one sample from the *spices and herbs* group was categorised in the group of complaint samples.

For the second part of this study, 190 isolates were received from the nVWA, 135 of which were identified as *C. perfringens*, based on biochemical reactions and the detection by PCR of the phospholipase C gene. Twenty-five isolates were categorised as *complaint* samples and the other 110 isolates as *normal* samples, i.e., samples taken for monitoring within various projects.

The genome-borne Cpe gene is regarded as predominantly responsible for Cpe leading to diarrhoea (Collie and McClane 1998). From the 25 isolates from *complaint* samples, 10 contained a Cpe-gene (40%), 7 of which were characterised as genome-borne (28%). In the *normal* samples 11 isolates contained a Cpe-gene (10%), 6 of which were genome borne (5.5%).

4.2 Behaviour of *C. perfringens* in the gastrointestinal tract (see Appendix 2)

This study can be divided into two parts. Firstly, the survival of vegetative cells and spores in simulated gastrointestinal conditions. Secondly, the behaviour of different concentrations of vegetative cells in simulated intestinal conditions.

The first part of the study was intended to investigate the influence of passage through the gastrointestinal tract on the number of cells that may take part in the disease-provoking process. This study was laid down in a previous report (Wijnands, Van der Meij-Florijn et al. 2008).

The second part was carried out because although in general the disease causing dose is said to be $\geq 10^7$ CFU per gram of food, a review of outbreaks showed that outbreak-related food commodities usually contain lower amounts of *C. perfringens*. This part of the study will be published later (Wijnands, Van der Meij-Florijn et al. In preparation).

The first part of the study gave the following results:

- 1) Vegetative cells will pass the gastric barrier virtually unharmed. The investigations showed that when the pH of simulated gastric fluid was higher than two, vegetative cells remained viable. Upon lowering of the pH to two and less, viability decreased rapidly and vegetative cells were killed within minutes.
- 2) Spores pass the stomach unharmed regardless of the pH.
- 3) In simulated intestinal fluid, spores were unable to germinate and grow

The second part of the study gave the following result.

Two concentrations of *C. perfringens*, namely 10^5 and 10^7 CFU per gram of food, were compared in simulated conditions. A concentration of $\geq 10^7$ CFU per gram of food is generally regarded as the concentration necessary to provoke diarrhoeal disease (Adams and Moss 2004). In experiments with a high starting concentration, spores and enterotoxin were produced after approximately eight hours in simulated intestinal fluid, while at the lowest concentration spores and enterotoxin were produced after approximately 12 hours. In experiments with a low starting concentration the concentration first increased to levels detected in the experiments with the high starting concentration at the moment spore and enterotoxin production started. After having reached these levels, spore and enterotoxin production was initiated. The amounts of enterotoxin produced by each initial concentration were comparable. At both concentrations the spore and enterotoxin production was achieved within the generally accepted incubation time of 8 – 24 hours (Adams and Moss 2004). Based on these data, it is assumed that concentrations of *C. perfringens* in the food $< 10^7$ CFU per gram can induce diarrhoeal disease. It is also assumed that growth can take place in small intestinal conditions, resulting in higher concentrations of *C. perfringens* and the subsequent production of amounts of toxin comparable to the amounts produced by high starting concentrations in the food.

4.3 Background of outbreaks of *C. perfringens* induced diarrhoeal disease (see Appendix 3)

Many outbreak reports can be found in the scientific literature. A number of these reports have been screened for details with an emphasis on incriminated food commodity and preparation details. This exercise was carried out to obtain indications on the how and why of the described outbreak(s).

In general, all described outbreaks affected large groups of individuals and were the result of improper cooling and/or improper (re)heating. This means that cooling temperatures were too high for too long or that refrigerators were used that were not suitable for cooling the required amount of food. Such circumstances may lead to the germination of spores and subsequent growth. Sometimes, the equipment for reheating food was inadequate, resulting in reheating temperatures that promoted the growth of vegetative cells instead of killing them.

4.4 Simulation of reheating processes (see Appendix 4)

In order to better describe the conditions necessary for proper reheating of food, i.e., conditions suitable for killing all vegetative cells of *C. perfringens*, heating experiments with pea soup were carried out. Soup, inoculated with vegetative cells, was heated on a conventional electric heating plate and in a microwave.

Using an electric heating plate with continuous stirring no viable vegetative cells could be detected after 30 minutes at approximately 60 °C. When the temperature was raised to 65 °C the necessary time for killing all vegetative cells was reduced to several minutes.

Using a microwave at an output level of approximately 500 Watts and equipped with a rotating platter, no viable cells could be detected after approximately 10 minutes with regular stirring and after approximately 15 minutes without regular stirring.

5 General discussion and conclusion

C. perfringens is a spore forming micro organism that grows anaerobically and can cause food borne disease. Disease is induced by enterotoxin produced by the micro organism during sporulation in the small intestine. In general, high concentrations of *C. perfringens* in food ($\geq 10^7$ /g) are regarded as necessary for the onset of disease (Adams and Moss 2004).

For our research, the disease inducing mechanism was assumed to be: preparation of food such that spores survive, improper cooling of food leading to germination of spores and subsequent growth and improper reheating, leading to the ingestion of food with an increased load of vegetative cells.

Although the research question was to define combinations of preparation and food commodities that may pose a risk for the development of *Clostridium perfringens* associated food borne disease, research did not focus solely on food preparation processes. Investigations regarding the behaviour of *C. perfringens* after ingestion were also carried out. The rationale was that after ingestion, the food with the microbial cells is still subjected to specific gastrointestinal conditions, which may influence the viability of *C. perfringens*, sporulation and enterotoxin production. Such has previously been investigated with *Bacillus cereus* (Wijnands 2008).

All in all, the investigations consisted in four major subjects, namely prevalence of potentially pathogenic *C. perfringens*, influence of the gastrointestinal tract on the survival of *C. perfringens*, conditions leading to *C. perfringens* associated outbreaks, and conditions for properly reheating prepared food commodities.

The types of food commodities that have been identified as risky for the development of *Clostridium perfringens* associated food borne disease are foods that need long cooking time, that are cooled after preparation to be reheated again before consumption. This conclusion can be drawn from the, not exhaustive, description of outbreaks (see Appendix 3). Such food commodities comprise meat-containing dishes such as stews and several types of soup. From the inventory on the prevalence of *C. perfringens* (see Appendix 1), it can be concluded that the major food commodity groups in which the micro organism has been found include the prepared food commodities, the group in which the meat-containing dishes are categorised. Moreover, such commodities frequently appear to be investigated as *outbreak* samples, i.e., samples that are associated with suspected food borne disease. The long cooking time guarantees an anaerobic environment from which most other micro organisms are cleared, leading to a near mono-culture of *C. perfringens*. During an improper cooling phase, spores may germinate and grow and during an inadequate reheating stage, vegetative cells may multiply.

Importantly, it must not be overlooked that the enterotoxin Cpe is responsible for the onset of disease. We found that approximately 8% of all strains carry the gene encoding the enterotoxin (see Appendix 1). This is in agreement with other investigations into the prevalence of enterotoxigenic *C. perfringens* (Ridell, Björkroth et al. 1998; Miki, Miyamoto et al. 2008).

In previous research on *Bacillus cereus* as a cause of food borne disease it was found that the gastrointestinal passage plays an important role in the

pathogenicity of the micro organism (Wijnands 2008). Survival of *B. cereus* during the gastrointestinal passage influenced the number of viable cells that contribute to the onset of disease (Wijnands 2008; Wijnands, Pielaat et al. 2009). Therefore, the behaviour of *C. perfringens* in simulated gastrointestinal conditions was investigated as well (see Appendix 2). Vegetative cells appeared to be very acid resistant. Since the pH of the stomach rises rapidly at the ingestion of food (Takumi, De Jonge et al. 2000), the gastric barrier is virtually zero for *C. perfringens* cells. It is plausible that all ingested cells reach the small intestine and can as such contribute to a disease process. Moreover, in simulated small intestinal conditions we saw growth of *C. perfringens*. Assuming this to happen *in vivo* as well, it means that even concentrations lower than the generally accepted 10^7 CFU per gram of food may lead to disease. The finding that the concentration of *C. perfringens* in incriminated foods in outbreaks is usually lower than 10^7 cfu per gram of food supports the theory that concentrations $< 10^7$ cfu per gram of food may cause disease.

The cooling of food is an important part of the leading principle for the investigations described in this report. Many studies have been conducted investigating the effect of cooling on *C. perfringens* (Blankenship, Craven et al. 1988; Juneja and Marks 2002; Kalinowski, Tompkin et al. 2003; Taormina and Dorsa 2004; Le Marc, Plowman et al. 2008). A large study in the United Kingdom resulted in a model to determine the growth of *C. perfringens* during cooling processes (www.ifr.ac.uk/safety/growthpredictor). The described model is able to predict the degree of *C. perfringens* growth given a specific cooling regime. Predicting a cooling route given a maximum increase of *C. perfringens* per gram with this model is only possible by feeding the model numerous cooling regimes, reducing its practical feasibility.

The United States Department of Agriculture has published time/temperature guidelines for cooling heated products (Anonymous 1989). These guidelines, however, are not really applicable in the private household, due to the lack of refrigerators with forced circulation. Since quite a number of reported outbreaks are associated with the preparation of food in private households or in conditions resembling private household conditions, prevention of outbreaks through thorough cooling regimes was not our main research interest. We focused more on the final reheating process as the step in which prevention of disease can be accomplished more easily. The experiments that were carried out (see Appendix 4) were directed towards the prevention of survival and/or growth of *C. perfringens* in food during reheating, easy to perform in the private households as well.

Usually, the regular method for determining *C. perfringens* contamination of food commodities is determination of the total number of viable cells. No separate spore count is performed. Spores may play an important role, since they survive the initial cooking process and can germinate and grow during cooling and/or reheating. The higher the spore count, the higher the risk that such may take place. Therefore, we recommend including spore count determination in regular monitoring and in outbreak-related investigations. Moreover, in outbreak investigation we suggest including detection of the gene encoding the enterotoxin. Whenever a high number of *C. perfringens* is found in combination with the presence of the gene in the isolated strain in such investigations, it is more likely that the disease was caused by *C. perfringens* instead of another micro organism giving similar symptoms.

Recommendations

1. Spores play an important role in the possible onset of disease. They can survive cooking processes, ensuring that after preparation the food is still contaminated with viable cells. Therefore, when determining the number of viable *C. perfringens* cells in food commodities it is useful to determine not only the total number but also the number of spores after pasteurisation. The lower the spore count, the less risky the food.

2. Since the prevalence of potentially pathogenic strains is approximately 8%, it is useful to determine the presence of the Cpe gene in an isolated strain. Contamination with non-enterotoxin producing strains is indeed not proper, yet not harmful.

Especially in the investigation of (possible) outbreaks, determination of the presence of the Cpe gene is preferable in order to make sure the isolated strain was able to cause disease. If carried out with the duplex PCR described by Tansuphasiri (2001), not only the Cpe gene is detected, but also the identity of *C. perfringens* is confirmed by the detection of the phospholipase C gene.

3. Although not studied, it is useful to investigate the identity and presence of the Cpe gene of multiple colonies isolated from a sample. Often more than one type/clone can be isolated. This is, like item 2, useful in the investigation of (possible) outbreaks (Ridell, Björkroth et al. 1998).

4. The use of thermometers that measure the core temperature of food should be stimulated, especially where reheating of food is concerned. Often, during reheating the food is not brought to the boil again. Boiling temperature would ensure the killing of all vegetative cells of *C. perfringens*. But even at lower temperatures (65 °C), complete killing of vegetative cells can be accomplished in a short period of time.

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Appendix 1: The prevalence of potentially pathogenic *Clostridium perfringens* strains in food commodities

Introduction

Clostridium perfringens type A can cause short-lasting diarrhoeal symptoms when ingested with food through the action of enterotoxin Cpe, which is produced during sporulation in the small intestine. Therefore, the pathogenicity of *C. perfringens* strains is dependent on the presence of a gene encoding Cpe: if no gene is present, the strain will not be able to cause disease (Labbe 1989; Miwa, Nishina et al. 1998; Crouch and Golden 2005). However, two types of Cpe encoding genes have been described, a genome-borne gene and a plasmid-borne gene. In general, Cpe encoded by the genome-borne gene is able to cause food borne disease, Cpe deriving from the plasmid-borne gene is not responsible for food borne disease (Cornillot, Saint-Joanis et al. 1995; Collie and McClane 1998; Sparks, Carman et al. 2001; Brynestad and Granum 2002; Vaishnavi, Kaur et al. 2005). Therefore, in making an inventory on the prevalence of potentially pathogenic *C. perfringens* strains, not only the presence of strains in food commodities is of importance, the presence of a genome-borne gene is also relevant.

The research question formulated by the new Food and Consumer Product Safety Authority (nVWA) was to investigate what combinations of food commodity - preparation process are risky with respect to *C. perfringens* induced diarrhoeal disease. Therefore, the food source from which the micro organism can be isolated was also determined.

In these investigations two major questions were taken into account: 1) from what types of food commodities can *C. perfringens* be isolated and 2) what percentage of isolates carries a Cpe-encoding gene and is thus potentially pathogenic.

To answer the first question, an inventory from the nVWA was used in which numbers and counts of isolates from a large number of various food commodities were accumulated. These food commodities are categorised in various ways. The most important with respect to this report are: food commodity group and sampling reason. In the first category, food commodity group, comparable types of food are assembled, in the second, sampling reason, the background from the sample can be retrieved, for example, samples suspected of causing food poisoning or routine samples for monitoring the microbiological status of food. An overview of the reasons for sampling is shown in Table 1.1, where "Code" is the abbreviation for the sampling reason as explained under "Description". The column "Categorisation" in Table A1.1 indicates what sampling reasons have been combined to obtain a more comprehensive inventory of all samples. These new categories comprise 1) complaint samples, i.e., samples investigated in connection with complaints about food poisoning and 2) normal samples, i.e., samples investigated within projects or samples collected for regular monitoring. The 3rd category, non categorised, contains samples that do not fall within the scope of the other two categories, which are investigated for control of hygiene codes or for process control.

Table A1.1 nVWA sampling reasons

Code	Description	Categorisation
KL	Complaint samples, usually remains of suspected food samples	Complaint sample
NK	Sample collected in connection with complaint	"
NV	Sample collected after food poisoning, not necessarily complaint sample	"
VV	Food incident sample	"
MA	Normal sample, usually collected through shopping	Normal samples
NM	Normal sample, usually collected within the scope of special projects	"
PM	Project sample, collected within the scope of special projects	"
PB	Sample for process evaluation	Non categorised
RW	Sample for evaluation of hygiene codes	"

The inventory is not a representation of randomly taken samples. In general, samples were taken within the framework of special projects.

The second question was answered by investigating isolates from the nVWA for the presence of a Cpe gene and discrimination between the genome-borne and plasmid-borne gene.

Materials and Methods

A. Prevalence of *C. perfringens*

For the inventory of the presence of *C. perfringens* in a large variety of food commodities, a list with prevalence data from January 17, 2007 to August 7, 2009 from the Food and Consumer Product Safety Authority (VWA) was used. Not only the presence but also the number of colony forming units is described in this list. In total, 8495 samples were investigated for *C. perfringens* within various contexts. Per food commodity, the total number of investigated samples was mentioned; per positive sample more details were mentioned, such as origin and number of *C. perfringens*.

B. Prevalence of enterotoxigenic *C. perfringens*

For the inventory of the prevalence of *C. perfringens* strains carrying the genome-borne Cpe gene, approximately 190 putative *C. perfringens* isolates from the VWA-collection were investigated.

After receipt, the isolates were cultured in Luria Bertani Broth¹ (LBG) pH 7.0 and for purity control on Columbia agar with sheep blood (Oxoid, Basingstoke UK) under anaerobic conditions (Anoxomat, MART Microbiology, the Netherlands) at 37 °C. If pure, cells were scraped off the Columbia agar and transferred to a MicroBank vial (ProLab Diagnostics, USA) for storage at -70 °C. Nitrate-motility medium and lactose-gelatinase medium for confirmation of identity were inoculated from the LBG culture and incubated anaerobically at 37 °C overnight.

¹ LBG = 10 g Tryptone + 5 g yeast extract + 5 g sodium chloride + 4 g glucose per litre distilled water. Sterilise: 30 minutes 110 °C.

After reading the results of the biochemical tests and description of the growth on Columbia agar with sheep blood, fresh LBG medium was inoculated and incubated overnight anaerobically at 37 °C. 1 ml culture was used for isolation of genomic DNA with the WIZARD Genomic DNA purification kit (Promega, USA). Purified DNA was stored at -20 °C until further use.

Identity of the isolates was also investigated by PCR detection of a 280 base pair fragment part of the gene encoding phospholipase C(alpha toxin) using a previously described method (Tansuphasiri 2001).

The detection of a 420 base pair (bp) fragment of the Cpe gene was accomplished by a PCR method described by Tansuphasiri (2001).

The report by Tansuphasiri (2001) describes a duplex PCR, one for detecting the Cpe gene and the other for detecting the phospholipase C (Plc) gene. For our investigations we used the primers for detection of the Cpe gene and the primers for detecting the Plc gene separately, in combination with the corresponding PCR programme.

Discrimination between chromosomal and plasmid Cpe gene was accomplished using a previously described PCR method (Wen, Miyamoto et al. 2003). In this method a genome-borne Cpe gene is characterised by a 2000 bp fragment and a plasmid-borne Cpe gene by a 3000 bp fragment. The genome-borne gene is related to strains causing food-borne disease, the plasmid-borne gene is related to strains causing antibiotic associated diarrhoea (Collie and McClane 1998).

Results

A. Prevalence of *C. perfringens*

In Table A1.2 the number of investigated samples used to determine the prevalence of *C. perfringens* and the number and percentage of positive findings are listed per food commodity group number. Duplicate samples, i.e., samples taken at the same date at the same place for monitoring large batches of product, were not taken into account. The highest number of positive samples was seen in WSG 14 (*spices and herbs*).

Table A1.2 Prevalence of *C. perfringens* in various food commodities and overall for the investigated groups

Food comm. group nr	Food comm. group	Number investigated	Number Positive	Percentage positive
WSG 4	Nuts, seeds seed products	167	3	2
WSG 5	Milk and milk products	20	1	5
WSG 7	Meat and meat products	167	12	7
WSG 10	Prepared food commodities	4940	65	1
WSG 13	Special food commodities	170	2	1
WSG 14	Spices and herbs	2890	410	14
WSG 43	Hygiene code guide value	141	2	1
Total		8495	495	6

In Figure A1.1 a distribution is shown of the percentage of positive samples falling in log unit bins. The greatest interest is in groups 7 (*meat and meat products*), 10 (*prepared food*) and 14 (*spices and herbs*), since these are the groups with either the highest number of samples (14) or with the highest percentages of samples in the log 5 – 6 bin (7 and 10).

Notably, in food commodity groups *meat and meat products* and *prepared food* (groups 7 and 10 respectively), high concentrations of *C. perfringens* were frequently detected. The last food commodity group food contains items such as soups and meat-containing dishes.

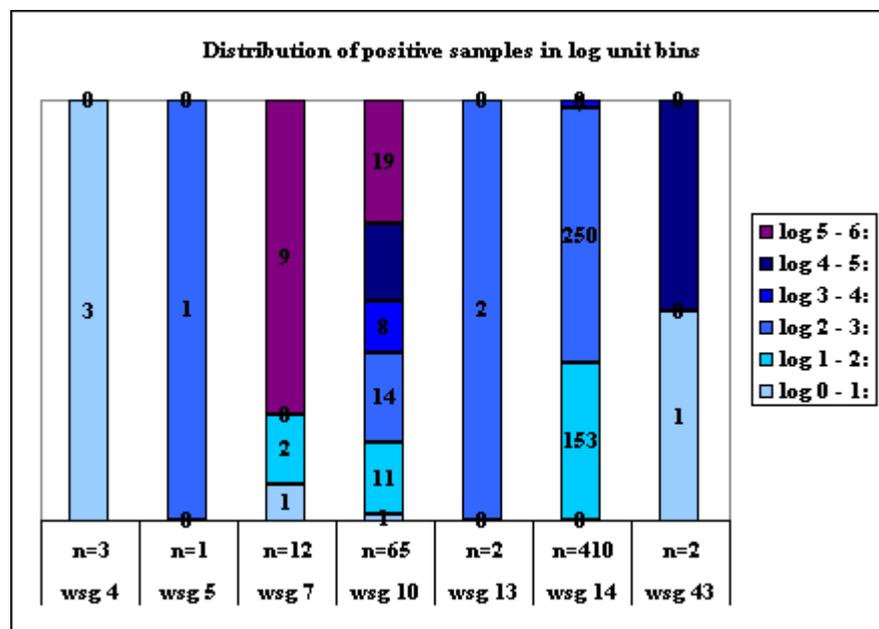


Figure A1.1 Distribution of positive samples per food commodity group in log unit bins. The number of samples per food commodity group and the food commodity group are indicated at the Y-axis. The different colours represent the various log unit bins as indicated in the legend.

All samples taken by the nVWA are investigated for some reason (see Table A1.2). As indicated in the Materials and Methods section, sampling reason categories have been combined for the samples investigated for this report, to simplify the distribution of the samples over three main groups: *complaint* samples, i.e., samples investigated in connection with outbreak or incident complaints, *normal* samples, i.e., samples investigated within the scope of special projects or for monitoring reasons and samples investigated for maintaining hygiene codes or processes (= *non categorised*).

For the three food commodity groups with the highest number of samples, these samples have been assigned to the *complaint* group, the *normal* group or the *non-categorised* group. As can be seen in Figure A1.2, in food commodity group 10, *prepared food*, the majority of samples fall in the *complaint* section. A quarter of the samples in the *meat and meat products* group also fall in the *complaint* section.

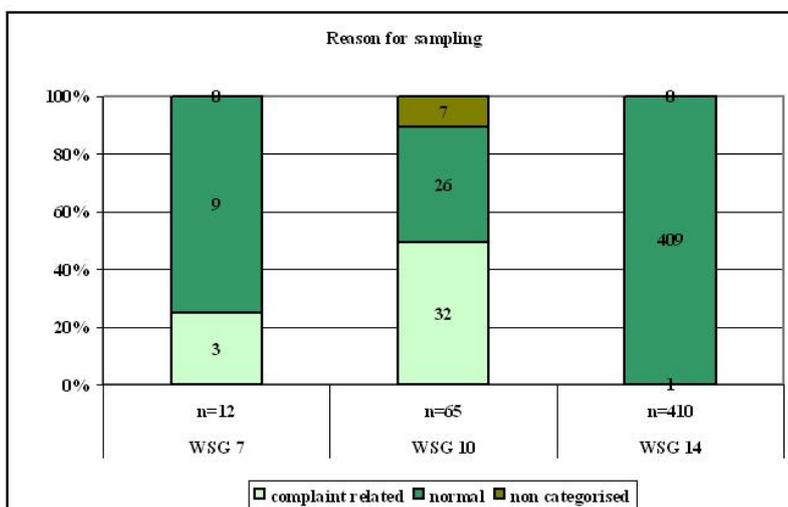


Figure A1.2 Distribution of reasons for sampling per food commodity group. Complaint samples comprise samples received after food outbreaks, samples collected as sequel to a complaint. Normal samples are routinely taken samples either at random or within the scope of special projects. Non categorised samples are samples taken for control of hygiene codes and process control samples.

B. Prevalence of enterotoxigenic *C. perfringens*

In total, 190 isolates were received from the nVWA: 135 isolates were identified as *C. perfringens* based on biochemical reactions, 52 isolates were classified as *Clostridium* spp., 3 isolates were not included in the investigations as the originating food commodity was not specified. The PCR identifying a 280 base pair (bp) fragment of the gene encoding phospholipase C (*plc*-PCR) matched with the strains identified as *C. perfringens* by biochemistry. None of the strains with aberrant biochemistry reacted in this *plc*-PCR. This last result is in agreement with the findings of Tansuphasiri (2001).

According to the earlier mentioned re-categorisation, 110 isolates originated from *normal* samples and 25 isolates from *complaint* samples (see Table A1.3).

The prevalence of Cpe genes has also been linked to sampling type, *complaint* or *normal*. The 420 bp fragment of the gene encoding enterotoxin Cpe was detected in 21 of the 135 isolates identified as *C. perfringens* (15.6%). The 420 bp fragment was not detected in the *Clostridium* spp. strains. In 10 of the 25 isolates marked as deriving from *complaint* samples, the Cpe encoding 420 bp fragment was detected (40%). In the 110 isolates from *normal* samples 11 contained the 420 bp Cpe-encoding PCR fragment (10%).

Table A1.3 Prevalence of Cpe grouped according to sampling reason

	# isolates	Cpe pos.	Cpe chrom.	Cpe plasmid
<i>C. perfringens</i> strains	135	21*	13	6
<i>Complaint</i>	25	10	7	3
<i>Normal</i>	110	11	6	3

* = in two cases of positive Cpe isolates, no discrimination could be made between the chromosomal or plasmid borne Cpe-gene.

The majority of samples (113 of 135, i.e., > 80%) fell into groups WSG 10 and WSG 14, *prepared food* and *spices and herbs*, respectively. In Table A1.4 the prevalence of Cpe-containing isolates discriminated by food commodity group is shown. In food commodity group *prepared food*, 11 of the 47 isolates contained the Cpe-encoding 420 bp PCR fragment (23%). Eight of these isolates were of the chromosomal type and three of the plasmid borne type. In the food commodity group *spices and herbs* 3 of 66 isolates (5%) contain a Cpe-encoding 420 bp PCR fragment. It was, however, not possible to link these three isolates to either the chromosomal- or the plasmid-borne type.

Table A1.4 Prevalence of Cpe grouped according to food commodity groups (WSG)

	# isolates	Cpe pos.	Cpe chrom.	Cpe plasmid
<i>C. perfringens</i>				
WSG 10 (prepared food)	47	11	8	3
WSG 14 (spices)	66	3	0	0

Discussion

The results regarding prevalence (part A) do not derive from a random survey, since the investigated samples fall into a small number of food commodity groups. However, these data give an insight into the contamination status of groups at risk for *C. perfringens* contamination. As shown in Figure 1, in food commodity groups 7 and 10 (*meat and meat products* and *prepared food*, respectively) some samples contain high numbers of *C. perfringens*, i.e., log unit bin 5 – 6. Most of these high counts were found in *complaint* samples, which comprise nearly 50% of the food commodity group *prepared food*. Such samples are composed of various food commodities but the individual components are normally not available for further investigation. It is therefore impossible to determine which of the components was contaminated and led to such high counts in these complaint samples.

One group, *spices and herbs*, appeared to have a high prevalence. In this group, the prevalence, with over 14%, was the highest of the investigated food commodity groups. In 98% of samples positive for *C. perfringens*, the concentrations did not exceed 1000 cfu/g. In the overall discussion, the impact of these findings will be discussed. From the *spices and herbs* only one sample belonged to the group of *complaint* samples, with a concentration in the 1 – 2 log unit bin. However, this sample was collected after a complaint had been filed.

The chance that the sample originated from the food that led to the complaint is very small.

The second part consisted of an inventory of the prevalence of the Cpe gene in isolates of *C. perfringens*, by determination of the presence of a Cpe gene with a PCR detecting a 420 bp fragment. Furthermore, a distinction was made between chromosomal- and plasmid-borne genes, even though this distinction is not absolute for potential pathogenicity of *C. perfringens* (Tanaka, Kimata et al. 2007).

The prevalence in the "prepared food" group may be biased by the fact that most *complaint* samples fall into this group. On the other hand, the absence of the chromosomal Cpe gene in isolates from the "spices and herbs" group indicates that these hardly play a role in food poisoning cases.

The overall prevalence of the chromosomal Cpe gene in our study is nearly 10% (13 isolates of 135). In comparison, Stagnitta et al. (2002) investigated 126 strains of *C. perfringens* isolated from meat products (hamburgers, sausages and minced meat samples) and found a prevalence of potentially pathogenic strains of approximately 7% (Stagnitta, Micalizzi et al. 2002).

Although strains carrying the chromosomal Cpe gene have been regarded as the major cause for *C. perfringens* food-borne disease, more evidence has recently become available on the contribution of strains carrying the plasmid-borne Cpe gene. Tanaka et al. (2007) showed that isolates carrying a plasmid-borne Cpe gene prevailed over the strains carrying a chromosomal Cpe gene (Tanaka, Kimata et al. 2007). An inventory on the type of Cpe gene in isolates from Finland and Germany resulted in a prevalence of 25% of strains carrying a plasmid-borne Cpe gene (Lahti, Heikinheimo et al. 2008). Therefore, it seems more obvious to determine the prevalence of a Cpe gene than to discriminate between the origin of the Cpe gene, chromosomal- or plasmid-borne.

Appendix 2: Gastrointestinal behaviour of *Clostridium perfringens*

Introduction

The pathogenic pathway of *Clostridium perfringens* associated food borne disease is as follows:

- Food contaminated with *C. perfringens* is consumed
- The contaminated food passes the stomach and enters the small intestine.
- In the small intestine *C. perfringens* sporulates and produces enterotoxin Cpe
- The Cpe influences the water – solute transport over the membrane of the epithelial cells, leading to increased release of water, thus inducing watery diarrhoea.

Since *C. perfringens* may occur as vegetative cell or spore, knowledge on the behaviour of both cell types is of value to understand the pathogenicity and possibilities to take measures to prevent disease.

Materials and methods

Details on the investigations with spores and vegetative cells in simulated gastrointestinal conditions are described elsewhere (Wijnands, Van der Meij-Florijn et al. 2008; Wijnands, Meij-Florijn et al. 2009).

Results and discussion

Investigations in simulated gastric conditions showed that spores are able to pass the stomach unharmed. For survival of vegetative cells, pH 2 appears to be a critical value: above that value, vegetative cells remain virtually unharmed but below that value vegetative cells are killed rapidly.

During the consumption of food, and especially solid food, the pH of the stomach reaches values of approximately 5 quite soon after ingestion ((Takumi, De Jonge et al. 2000; Wijnands, Pielaat et al. 2009). It is therefore plausible that vegetative cells present in contaminated food pass the stomach unharmed. These vegetative cells can sporulate and produce enterotoxin.

Spores also pass the stomach unharmed. Since sporulation is of vital importance for pathogenicity, spores should first have to germinate, possibly grow and sporulate again to contribute to pathogenicity. In simulated intestinal conditions we were unable to germinate spores and have them subsequently grow. Therefore, we concluded that spores do not contribute to the pathogenic process leading to diarrhoea.

In general it is assumed that a high concentration of *C. perfringens* in food, i.e., $\geq 10^7$ cfu/g, is necessary to lead to disease. Since the incubation time of the disease ranges from 8 – 24 hours, we investigated the possibility for concentrations $< 10^7$ cfu/g to lead to diarrhoeal symptoms. Based upon investigations carried out in simulated conditions, we concluded that even at concentrations of approximately 10^5 cfu/g food, the micro organism was able to grow to numbers high enough to produce amounts of enterotoxin similar to the amount produced by a starting concentration of 10^7 cfu/g (in preparation).

Conclusion

Although vegetative cells play an important role in the onset of disease by *C. perfringens*, spores are not to be overlooked. Spores may contaminate food as well and will survive not only cooking processes, but may germinate and grow out during cooling and reheating processes.

Vegetative cells of *C. perfringens* appear to be resistant to pH > 2, implicating that in the presence of solid food they may pass the gastric barrier easily. Moreover, vegetative cells can grow in simulated intestinal conditions. In general, $\geq 10^7$ cfu per gram of food or higher are assumed to be necessary to invoke disease. As a consequence of the ability to grow in the small intestine, the contamination level in food does not necessarily have to be $\geq 10^7$ cfu per gram of food, but can even be as low as 10^5 cfu/gram of food.

Appendix 3: Background and cause of *Clostridium perfringens* food borne disease.

Introduction

An important research question within this project is to assess what food preparation processes lead to germination of *C. perfringens* spores. This must eventually lead to the denomination of product/process combinations with a heightened risk for public health.

Outbreak reports usually contain data on type of food and details on the preparation of the food that led to food borne disease. A (random) selection of such reports, obtained after searching Scopus and PubMed with the keywords "*Clostridium perfringens*" and "outbreak", was studied and described in more detail here in order to try and assess the cause of the reported outbreak and with that, to assess what preparation process(es) plays an important role in the germination and subsequent survival of *C. perfringens* spores. This information will be used to carry out laboratory experiments on the most critical preparation process(es).

Outbreak survey

Outbreak 1

Reference: (Holtby, Tebbutt et al. 2008)

Affected population: > 92 guests at a buffet lunch at a multicultural event

Main symptoms: fever, abdominal pain, diarrhoea, nausea, vomiting approx. eight hrs after attending buffet

Implicated food: chicken curry, chick pea curry, boiled rice

Culture data: chicken curry 9.6×10^5 *C. perfringens*/g

Food preparation: 9 kilos of diced chicken cooked for 30 – 40 minutes; cooked chicken was added to vegetables and cooked on hob for 40 – 60 minutes; left with lid on to cool at ambient temperature for 10 hrs; reheating at event site (3 pans over 2 gas rings). Anecdotal description: food served lukewarm.

Comment on probable cause: poor temperature and time control; insufficient refrigerator capacity for safely cooling this large quantity

Outbreak 2

Reference: (Parikh, Jay et al. 1997)

Affected population: Inmates and staff of a juvenile detention facility

Main symptoms: diarrhoea, abdominal pain, nausea, vomiting 5 – 19 hrs after consumption

Implicated food: cooked turkey and gravy

Culture data: none

Food preparation: frozen turkey breasts were thawed and cooked until visually done; cooked turkey meat was piled up in large stock pot, covered with foil and placed in a walk-in refrigerator; the next day, the turkey was sliced, reheated and served; the juice of the turkey meat was used to make gravy, which was also cooled overnight in a large pot. Anecdotal: some of the turkey was still warm when removed from the walk-in refrigerator.

Comment on probable cause: inadequate cooking of large pieces of meat; slow and insufficient cooling and subsequently, insufficient reheating.

Outbreak 3

Reference: (Lederman, Crum et al. 2004)

Affected population: laboratory personnel attending a Mardi Gras celebration, 30/59 developed symptoms (USA)

Main symptoms: diarrhoea, abdominal pain, flatulence, nausea,

Implicated food: gumbo

Culture data: none

Food preparation: home prepared, no exact preparation description given; served over a 1.5 hour period; leftovers distributed to the rest of the day and night shifts; leftovers eaten as such or reheated in microwave

Comment on probable cause: improper serving and cooling temperatures; food left at ambient temperature for extended periods.

Outbreak 4

Reference: (Hsieh, Archer et al. 2009)

Affected population: inmates of a Wisconsin county jail, 100/550 (USA)

Main symptoms: diarrhoea, nausea, vomiting

Implicated food: casserole with macaroni noodles, ground beef, ground turkey, frozen mixed vegetables and gravy

Culture data: gravy at least 4.3×10^4 *C. perfringens*/g

Food preparation: meals are prepared and portioned in a central kitchen; served to the inmates; macaroni and beef were cooked the day before serving; no proper recording and documenting of food and cooling temperatures; some respondents commented on the unusual taste of the casserole

Comment on probable cause: casserole was made with food items that were prepared and stored improperly.

Outbreak 5

Reference: (Young, Smith et al. 2008)

Affected population: 7/25 attendants of a celebration in a local restaurant (Australia)

Main symptoms: not specified gastrointestinal symptoms

Implicated food: pre-prepared meat dish (spore counts in)

Culture data: meat dish 2.5×10^7 *C. perfringens*/g; faecal samples 2.1×10^5 – 8.7×10^6 spores/g

Food preparation: not specified

Comment on probable cause: large quantities of cooked food were left to cool at ambient temperature for more than 12 hours.

Outbreak 6

Reference: (Simmons and Manning 1998)

Affected population: 64/139 attendants of a hui (social meeting) (New Zealand)

Main symptoms: diarrhoea, stomach pain, nausea

Implicated food: roast pork

Culture data: none

Food preparation: loins of pork were rolled and stuffed with bread-based stuffing; cooked in an oven, 30 min at 200 °C and 4.5 hrs at 180 °C

Comment on probable cause: meat was not inspected (home-killed pig); meat was transported non-refrigerated; no temperature control during cooking; cooked meat left to cool for 1.5 hrs at room temperature before serving; poor hygiene with kitchen utensils and no separation of cutting boards for raw and cooked foods.

Outbreak 7

Reference: (Mol, Vincentie et al. 1988)

Affected population: 100/350 inhabitants of a retirement home

Main symptoms: diarrhoea, mild symptoms

Implicated food: stuffed breast of veal

Culture data: veal 5.0×10^4 *C. perfringens*/g

Food preparation: prepared by supplying butcher; after receipt on Friday, cooked in an oven during 2 hrs; first cooling at room temperature for 1.5 hrs and subsequently placed in refrigerator; early Sunday morning sliced, portioned and replaced in refrigerator until approx 11:45; served on warm plates with gravy, potatoes and beans

Comment on probable cause: preparation process uncontrolled; cooling at ambient temperature before refrigeration.

Outbreak 8

Reference: (Anonymous 1994)

Affected population:

1. Customers of a delicatessen, 156 illnesses
2. Attendants of a St Patrick's Day dinner, 86/115 ill

Main symptoms:

1. Abdominal cramps, vomiting
2. Diarrhoea, abdominal cramps, vomiting

Implicated food:

1. Corned beef (prepared by the delicatessen)
2. Corned beef

Culture data:

1. Corned beef $> 10^5$ *C. perfringens*/g
2. Corned beef $> 10^5$ *C. perfringens*/g

Food preparation

1. March 12: start of cooking of 1400 pounds of raw, salt-cured product; portions were boiled for 3 hrs, allowed to cool at room temperature and refrigerated; March 16 and 17 removal from refrigerator; held at 49 °C; sliced and served; March 17 corned beef sandwiches were also prepared for catering, kept at room temperature
2. Frozen, commercially prepared, brined corned beef 13 pieces of 10 pounds each; cooked in ovens in 4 batches; first three batches cooled in home refrigerator, last batch taken directly to event; 90 minutes before serving, the meat was sliced and placed under heat lamps.

Comment on probable cause

1. Insufficient cooling; temperature at which dish was kept warm too low.

2. Improper and insufficient cooling; reheating temperature before consumption too low.

Outbreak 9

Reference: (Roach and Sienko 1992)

Affected population: conference attendants, 32/42 ill

Main symptoms: diarrhoea, abdominal cramps

Implicated food: minestrone soup

Culture data: none

Food preparation: 1-day old chicken stock, fresh vegetables, canned tomatoes, commercial chicken base, cooked navy beans, cooked split peas (25 gallons in total); soup was simmered for 3.5 hrs and served to 400 persons; five gallons left-over was cooled in a plastic container with ice around it and then refrigerated; Two days later, the soup was boiled for 5 minutes and simmered for 15 minutes before portioning; portions placed in hot holding cart and served two hours later

Comment on probable cause: insufficient rapid cooling of stock and soup; reheating and holding temperature insufficient for prevention of multiplication or killing of vegetative cells.

Outbreak 10

Reference: (Pollock and Whitty 1991)

Affected population: patients in hospital long-stay department, 58/647 ill, 2 dead

Main symptoms: diarrhoea

Implicated food: minced meat (soft diet)

Culture data: none

Food preparation: minced meat was cooked two days prior to consumption, cooled and reheated before serving

Comment on probable cause: serving was done per ward, some in larger heated containers, some individually packaged; minced meat in heated trolleys never reached an adequate temperature; also reports on inadequate chilling and storage, refrigerators above recommended temperatures.

Outbreak 11

Reference: (Hewitt, Begg et al. 1986)

Affected population:

1. guests at a festival supper, 50 ill
2. Masonic dinner, 56/219 ill
3. function meal, 60/850 ill
4. dinner/dance, 402 attendants
5. group of 455 hotel guests

Main symptoms:

1. severe diarrhoea
2. diarrhoea
3. diarrhoea, abdominal cramps
4. watery diarrhoea, stomach cramps, nausea
5. difficult to obtain information due to origin of attendants from different parts of the country

Implicated food:

1. salmon mayonnaise
2. cooked salmon
3. cooked salmon
4. most probably salmon
5. most probably salmon

Culture data:

1. 10^8 *C. perfringens*/g
2. $6 \times 10^4 - 4 \times 10^5$ *C. perfringens*/g
3. $10^2 - 10^3$ *C. perfringens*/g
4. no data
5. no data

Food preparation

1. large quantity boiled and left overnight to cool in own juice (refrigerator was used); after portioning, freshly prepared mayonnaise was added
2. whole salmon (approx. 8 pounds each) defrosted, seasoned, wrapped in tin foil, placed in baking tin lined with foil; water added to cover the wrapped fish, cooked for 1.5 hrs over gas rings; wrapped fish left to cool overnight in the pan with water at ambient temperature; at noon the following day unwrapping, skinning and portioning; plates stood 2-4 hrs at room temperature before start of dinner
3. salmon defrosted on day 1; fish cooked on day two; fish portioned and served on day three
4. salmon fillets placed in bouillon, boiled for 15 – 30 minutes; fish left to cool at ambient temperature for 2 – 3 hrs before placing in walk-in cold room overnight; following day fish broken into pieces and mixed with lettuce, capers and mayonnaise
5. as 11.4

Comment on probable cause

1. insufficient cooling
2. insufficient cooling
3. not clear, insufficient cooling(?)
4. most probably insufficient cooling
5. as 11.4

Overall comment on outbreak 11: all incidents happened in the same hotel; no food remnants were available for investigation in incidents 4 and 5.

Outbreak 12

Reference: (Deseo and Engeli 1984)

Affected population: inhabitants (90) and staff (10) of a retirement home, 70/100 ill

Main symptoms: diarrhoea, nausea, abdominal cramps

Implicated food: veal tongue

Culture data: none

Food preparation: 15.6 kg tongue cooked with vegetables in 25 litres of water for approx. 3 hrs; tongues were peeled and covered with the still hot (70 – 80 °C) broth; the pan was left on the kitchen table overnight at ambient temperature, next day a fat layer had formed on top of the broth;

the next day early, the tongues were portioned; before serving the tongues were reheated but not cooked any more, and kept warm.

Comment on probable cause: insufficient reheating did not kill vegetative cells.

Outbreak 13

Reference: (Helstad, Mandel et al. 1967)

Affected population: students, 366/2954 ill

Main symptoms: diarrhoea, abdominal cramps

Implicated food: gravy for roast beef

Food preparation: 27 gallons of left-over gravy cooled in 9-gallon portions in plastic containers in a refrigerator overnight; the next day mixed with 7 gallons of freshly prepared gravy; mixture brought to boil and served

Comment on probable cause: cooling of too large quantities → insufficient cooling; insufficient heating the next day of the mixture of left-over and fresh gravy

Outbreak 14

Reference: (Eriksen, Zenner et al. 2010)

Affected population: guests from 2 weddings, 57/150 and 36/400 ill (known through interviews)

Main symptoms: diarrhoea, abdominal pain, nausea

Implicated food: lamb dish and chicken dish prepared by the same food handler, the caterer

Food preparation: not specified in detail, both dishes prepared by the same caterer and transported to the two venues. Cooling after preparation performed with a blast chiller but inappropriately, transport from caterer to venues in non-refrigerated vans.

Comment on probable cause: poor temperature control of the food during preparation, cooling, transport and reheating. Insufficient personal hygiene by the catering staff, causing contamination of the prepared dish.

Conclusions

In general the described outbreaks were caused by one or a combination of the following factors:

1. too large quantities are cooled at room temperature
2. cooling took place in unsuitable refrigerators
3. reheating is carried out at too low temperatures
4. keeping dishes warm is carried out at too low temperatures
5. insufficient hygiene and/or knowledge of hygiene by food handler(s)

Some of the described outbreaks are due to the circumstance that the 'caterers' overestimate their ability to cook for large groups of people. Sometimes one could say that an individual of 'good will' has been preparing the meal that caused the food poisoning, instead of a licensed caterer. In these cases, the chance of improper time/temperature combinations is quite large

Even though cooling regulations state that large quantities should be portioned and cooled in a suitable refrigerator, quite a few of the described outbreaks can be attributed to a failure to do so. Such failures lead to the following scenario. Spores are not killed by regular cooking temperatures; thus, when cooling is carried out too slowly, spores may germinate and grow out to high number of vegetative cells. This need not necessarily be a problem if the food is properly reheated before consumption. Proper reheating ensures killing all vegetative cells. If, however, reheating is not carried out properly, at too low a temperature for too short a time, the vegetative cells will not be killed and may enter the gastrointestinal tract, leading to food borne disease.

Control of temperature and time are therefore crucial for preventing germination and growth of *C. perfringens* during cooling and subsequent (re)heating.

Appendix 4: Survival of vegetative cells of *Clostridium perfringens* in pea soup during various heating conditions.

Introduction

The ability of *Clostridium perfringens* to appear in both vegetative form and as a spore poses an extra problem for food safety, since spores are able to survive adverse environmental conditions, such as heating and elevated salt concentrations.

In general, heating processes during cooking are considered to assure that all vegetative bacterial cells are killed. Spores, however, can withstand heating processes, causing only partial decontamination of the food. If these spores subsequently germinate and grow out to larger numbers, the heating process did not ensure food safety. This process is described in the following life cycle of *C. perfringens* during these cooking processes:

1. One or more of the ingredients is contaminated with *C. perfringens*, vegetative cells and spores. During cooking, the vegetative cells die off, while the spores survive (Wijnands, Meij-Florijn et al. 2009). Also during cooking, most competitive flora is removed and oxygen is expelled from the food, thus creating an anaerobic environment.
2. During improper cooling, the temperature drops slowly, and *C. perfringens* spores are triggered to germinate and grow when the temperature comes below 50 °C.
3. Reheating and keeping warm are done at temperatures supporting the growth of *C. perfringens* instead of killing the organisms. In some reports, 74 °C is advised as the reheating temperature or warm-keeping temperature. During reheating, people should take care that the temperature at the centre reaches the advised temperature.

From this description it can be deduced that two processes are of vital importance to ensure that food, initially contaminated with *C. perfringens*, can safely be consumed after preparation, namely proper cooling after cooking and proper (re)heating.

Proper cooling consists in portioning the prepared food into small quantities and rapid cooling of these, especially in the region from 50 °C and down. This prevents spores, which survive the cooking process, from germinating, and if germinated, growing to large quantities of vegetative cells. Guidelines for proper cooling are also formulated by the United States Department of Agriculture (Anonymous 1989).

A method to determine the quality of a cooling process is given by the "Perfringens Growth Predictor", as set up by The Institute for Food Research in Norwich (UK). This application predicts the growth of *Clostridium perfringens* after feeding the programme with the data of a cooling course (time and temperature) (www.ifr.ac.uk/safety/growthpredictor). By looking more closely at this programme, two things become evident: 1) the outcome of the programme is a trend (increase, decrease or no change) of numbers of *C. perfringens* after an input of time and temperature data, and 2) the programme is not suitable for rapidly determining a time/temperature combination feeding a maximum change in *C. perfringens* numbers.

Thus, in general proper cooling of heated food commodities before storage is recommended to ensure the quality and safety of food. However, proper

cooling facilities are mandatory in professional kitchens, but household refrigerators are not suited for this.

Proper reheating also is a time/temperature controlled process. Sufficient heat for a sufficient time period ensures killing of vegetative cells, thus reducing the risk of food-borne disease. Time and temperature depend on a number of factors, such as type of food commodity, amount of food and type of contamination.

The Food Safety and Inspection Service of the United States Department of Agriculture provides a list with recommended internal temperatures of food to ensure safe consumption (Fact Sheet Internal Cooking Temperatures, FSIS,

http://www.fsis.usda.gov/Fact_Sheets/Cooking_For_Groups_Index/index.asp

p) In contrast, the Dutch "Voedingscentrum" only recommends to heat food properly but fails to show internal temperatures (<http://www.voedingscentrum.nl/nl/eten-veiligheid/hygiene/koelen-en-verhitten.aspx>).

To obtain better insight into the heat stability of vegetative cells of *C. perfringens* present in a food commodity, we investigated the time and temperature necessary to kill vegetative cells in pea soup. Heating was performed in a microwave oven and on a temperature-controlled electrical heating plate, both with and without stirring. The temperature was recorded regularly in combination with the time elapsed since the start of the experiment.

Materials

- Canned pea soup, homogenised. For each experiment the contents of one can was used, i.e., 1 litre.
- Cooking pot, suitable for approximately 1 litre of soup
- Glass bowl
- Heating plate with temperature controller
- Microwave oven
- Thermometer, digital or analogue
- *C. perfringens*, strain Cp 8, food isolate obtained from the Food and Consumer Product Safety Authority, Eindhoven, the Netherlands
- Trypton Soy agar plates
- Peptone saline
- Anaerobic pots
- Anoxomat (Mart Microbiology, Lichtenvoorde, the Netherlands)
- Timer

Methods

In general, vegetative cells of an overnight culture of *Clostridium perfringens* were added to homogenised pea soup, the soup was heated and the temperature was measured and samples were taken at regular intervals. When a microwave was used, the temperature was measured in the centre of the bowl and at the sides of the bowl; samples were also taken from the centre and the sides of the bowl.

Four different heating and measuring methods were used:

1. heating in a cooking pot on a temperature-controlled heating plate with overhead stirring, culture was added before heating the soup and sampling was started when heating was started, temperatures were set at 55, 60 and 65 °C respectively,

2. heating in a cooking pot on a temperature-controlled heating plate with overhead stirring, adding the culture and starting the sampling at the time the desired temperature was reached
3. heating in a glass bowl in a microwave oven with rotating platter; with regular manual stirring, sampling was started when the microwave oven was switched on, samples were taken only from the centre of the bowl. The microwave was used at approximately 500 Watts output.
4. heating in a glass bowl in a microwave oven with turning platter; without regular manual stirring, sampling started when the microwave oven was switched on, samples were taken from the centre and from the side of the bowl. The microwave oven was used at approximately 500 Watts output.

The preset heating temperatures in method 1 were 55, 60, and 65 °C. The desired temperatures in method 2 were 50, 55, 60 and 65 °C.

Results and discussion

In Figure A4.1 typical results are shown of method 1. The actual final temperatures were not exactly the set temperatures but all within 2 degrees of the set temperatures. From this figure can be read that in all experiments the temperature reaches the set value after approximately 10 minutes. At 55 °C the number of *C. perfringens* cells slowly drops, but does not reach zero by far after 30 minutes at the set temperature (= time = 40 in the graph). At 60 °C the number of *C. perfringens* reaches zero after 30 minutes at the set temperature and at 65 °C no *C. perfringens* could be detected the moment the set temperature was reached.

In Figure A4.2 typical results of the experiments according to method 2 are shown.

At 50 °C no loss of *C. perfringens* is detected within the duration of the experiment. At 55 °C the detectable number of *C. perfringens* dropped approximately 1 log-unit from the original concentration, i.e., from ca. 5×10^6 /ml to 5×10^5 /ml.

At 60 °C the reduction was a little more than 3 log units, but did not drop to zero within the duration of the experiment.

At 65 °C the concentration *C. perfringens* dropped to zero cfu/ml, although the results are not very uniform. To reach zero cfu/ml, approximately 30 minutes were necessary.

In Figure A4.3 the average results of three heating experiments in a microwave with stirring (method 3) are shown. At regular intervals the microwave was opened to draw a sample and measure the temperature of the soup after stirring. As soon as the temperature reaches approximately 60 °C (approx. 6 minutes after the start of the experiment) *C. perfringens* counts drop rapidly.

In Figure A4.4 the average results of three heating experiments in a microwave without stirring (method 4) are shown. At regular intervals the microwave was opened to draw a sample and measure the temperature in the centre and at the edge of the bowl. In comparison with the method with stirring, the soup heated more slowly and thus before no *C. perfringens* cells could be detected takes longer (12 to 15 minutes, dependent on the place of measurement)

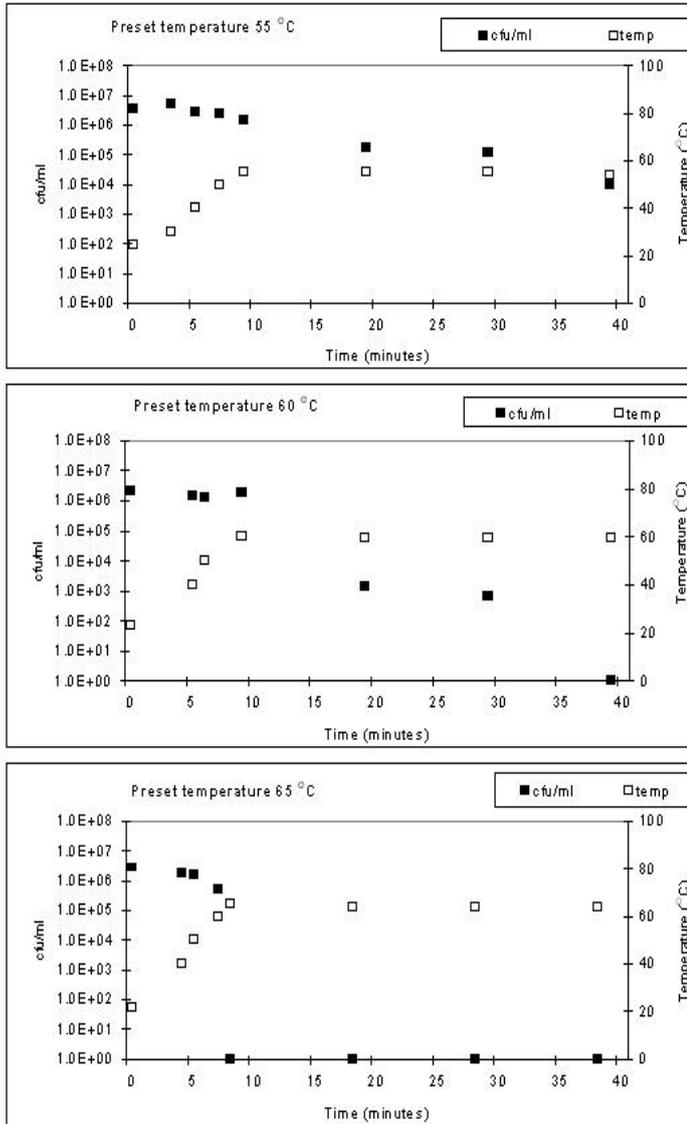


Figure A4.1 Survival of *C. perfringens* Cp 8 in pea soup during heating to preset temperatures 55, 60 and 65 °C on a temperature-controlled heating plate. Cells were added before heating was started.

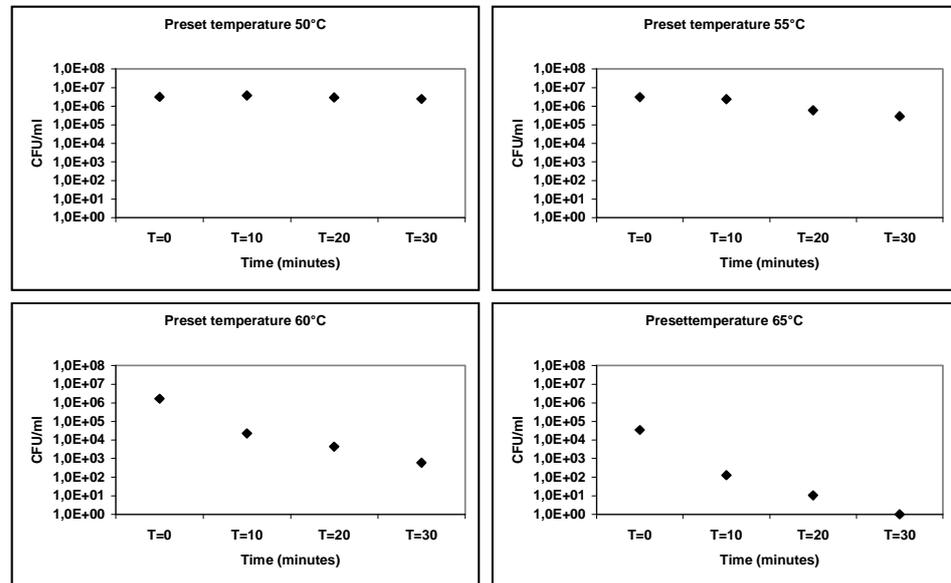


Figure A4.2 Survival of *C. perfringens* Cp 8 in pea soup during heating to preset temperatures 50, 55, 60 and 65 °C on a temperature-controlled heating plate. Cells were added at the moment the preset temperature was reached.

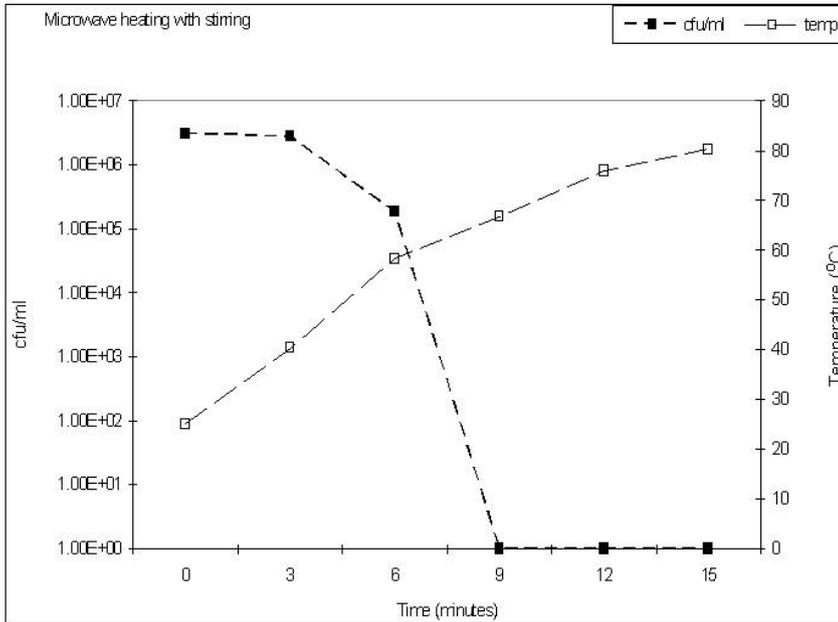


Figure A4.3 Survival of *C. perfringens* in and temperature of pea soup during heating in microwave oven with regular stirring

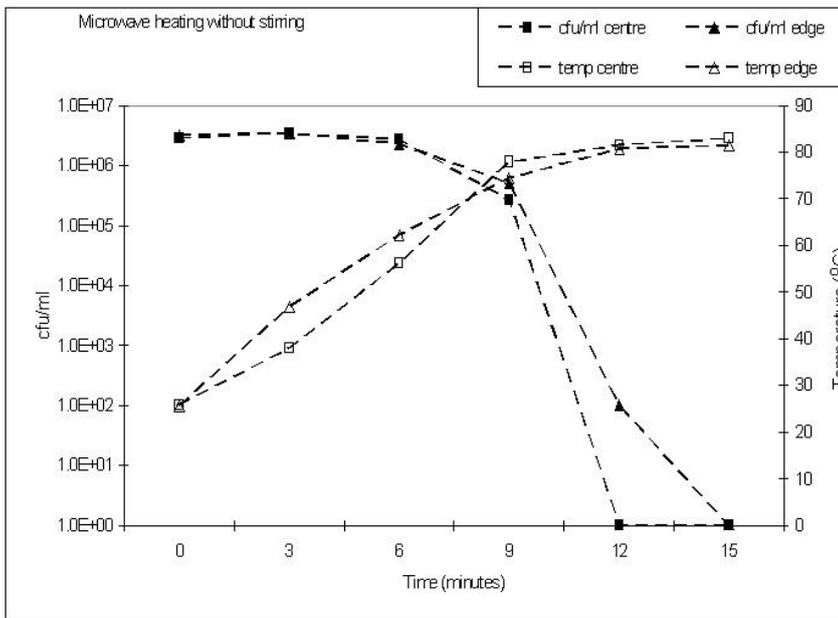


Figure A4.4 Survival of *C. perfringens* in and temperature of pea soup during heating in microwave oven without regular stirring Measurement of temperature of the soup in two places, centre and edge of the bowl.

Discussion and conclusions

The experiments described in this Appendix start with the following assumption: food is prepared, cooled and stored and reheated again for consumption. According to the descriptions of food outbreaks as laid down in Appendix 3, most outbreaks result from improper cooling after preparation and/or improper reheating before consumption. We also assumed that proper cooling is a process that is hard to control, especially in a private household. Therefore, we investigated the conditions needed for proper reheating of food before consumption, instead of focusing on the cooling process.

The most efficient and realistic method in terms of killing the vegetative cells of *C. perfringens* and control of temperature was number 1, in which the bacteria was already in the food before heating was started. Within 10 minutes, no viable bacterial cells were detected.

From the results of method 2, it can be seen that an increase in the set temperature from 60 to 65 °C leads to the very rapid killing of bacterial cells, within a minute after reaching 65 °C, no viable cells were detected. When the food is heated to 60 °C, it takes approximately 30 minutes at that temperature to kill all bacterial cells. Such a sudden change in survival is also seen when determining the heat stability of spores and their stability in an acid environment (Wijnands, Van der Meij-Florijn et al. 2008; Wijnands, Meij-Florijn et al. 2009). The reasons for such a drastic drop in survival are not known, nor did we try to investigate this phenomenon.

A highly popular way of reheating food is by microwave oven. The type of microwave oven we used was equipped with a revolving platter, which ensures a more even heat distribution through the food. Microwave ovens without a revolving platter give a less even heat distribution, which may lead to cold spots in the food. The more or less even heat distribution can also be derived from the temperature readings, as shown in Figure 4.4, where hardly any difference in the temperature of the food was detected between the measurements in the centre and at the edge of the bowl.

In the experiments with the microwave oven, the use of a permanent device for determining the food temperature during heating was impossible, due to the metal in the temperature probe. We tried to circumvent this handicap by stopping the microwave as briefly as possible for temperature measurement and sampling. At the output level we used, which was not the maximum possible level of the microwave, no *C. perfringens* could be detected after 9 minutes. Without stirring, the time needed to reduce the *C. perfringens* count to zero cfu/ml increased to at least 12 minutes (in the centre the count was reduced to zero cfu/ml, while at the edge, the count was reduced to zero cfu/ml after 15 minutes).

C. perfringens cells can be efficiently killed during the (re-)heating of food. A prerequisite is that the temperature is high enough for a sufficiently long time. We found that when the temperature is raised above 60 °C, *C. perfringens* vegetative cells are rapidly killed (within ten minutes on a hot plate). A microwave oven can also be used to eradicate all *C. perfringens* vegetative cells from the pea soup we used. At the output level we used, no survival was seen within 10 minutes with regular stirring, and within 15 minutes without regular stirring. Even though we did not test this, raising the output level would lead to more rapid killing of *C. perfringens* cells.

The use of a thermometer to monitor the internal temperature of the food is strongly recommended. If not used, one might over-estimate the actual temperature and thus not be sure all cells have been killed.

