AN INVESTIGATION OF GRAM-POSITIVE PATHOGENS IN POWDERED FOODS

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ABSTRACT

The purpose of the project was to obtain and test a range powdered food products that are marketed for consumption by individuals that may be immunocompromised. Hence seventeen infant formula milks (0-6months), twelve over-the-counter, elderly, build-up products and nineteen sports powdered protein shakes were examined. These products were tested for the presence of four different Gram-positive pathogens: Two spore formers; *Bacillus cereus* and *Clostridium perfringens* and two non-spore formers; *Listeria monocytogenes* and *Staphylococcus aureus.*

Products were tested according to the ISO standardised methods of testing for described for each of the organisms and samples plated on different diagnostic agars. The presumptively positive organisms that formed characteristic colonies were then further identified. This identification was confirmed in two ways; biochemically, using a wide range of tests including API, and molecularly using PCR-based assays. The results from the project showed that from the 48 products tested; 16 contained *B. cereus,* nine *S. aureus,* three *L. monocytogenes* and one with *C. perfringens.*

To further investigate whether the non-spore forming organisms that survived in these products were more resilient than expected, a heat inactivation experiment was carried out. A simulated high-temperature-short-time (HTST) pasteurisation was set up and the results gained suggested that the *S. aureus* isolate was able to survive pasteurisation but not subsequent cold and heat shock. In contrast the *L. monocytogenes* was sub-lethally damaged by the heat treatment but then recovered during cold storage. Thus postulations about how or where these organisms entered food could be made.

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ABBREVIATIONS

ISO	International Standard Organisation
HTST	High Temperature Short Time (Pasteurisation)
INET	East Midlands Development Agency Food Innovations Network
IFM	Infant Formula Milk
SD	Sports Drinks
EBU	Elderly Build Up
RTE	Ready-to-Eat
HPA	Health Protection Agency
CIP	Cleaning-in-Place
GMP	Good Manufacturing Practices
CCPs	Critical Control Points
ICMSF	International Commission on Microbiological Specifications of Food
BPW	Buffered Peptone Water
PCA	Plate Count Agar
MYP	Mannitol Egg Yolk Polymyxin B
BP	Baird Parker
TSC	Tryptose Sulphite Cyclosine
BHI	Brain Heart Infusion
TSYE	Tryptose Soy-Yeast Extract
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
CFU	Colony Forming Units
ARDRA	Amplified Ribosomal DNA Restriction Analysis
ELISA	Enzyme Linked Immunosorbant Assay
ATCC	American Type Culture Collection
NCTC	National Collection of Type Cultures
ANOVA	Analysis of Variance

g	gram
mg	milligram
µg	microgram
l	litre
ml	millilitre
µl	microlitre
M	Molar
mM	millimolar
bp	Base pairs
Kbp	Kilo base pairs

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1. POWDERED FOODS

The drying of food products has been used for a very long time as a food preservation method and, as the name suggests, generally involves removing the moisture content of the food. The first drying techniques involved using the sun and dry air to desiccate foods. For modern manufacturers, in addition to extending the life of the product as it is thought that organisms may not be able the survive in such a low moisture contents (Ratti *et al.,* 2001), drying has many additional benefits, such as reducing the overall weight and space the product would normally take up and therefore reducing the transport costs.

The focus of this project is to determine whether dried powdered food products that are used regularly by those who may be immunocompromised are safe enough to consume. This is because there are organisms that are known to be able to survive the drying processes involved in making these products, as well as being able to survive in the harsh dry environment accompanied by powdered food. For example *Enterobacter sakazakii* has been found to be a frequent contaminant in powdered infant formula. This organism causes Neonatal necrotizing enterocolitis, characterized by intestinal necrosis and pneumatosis intestinalis and is the most common gastrointestinal emergency in the newborn (Acker *et al.,* 2000). This is a very serious problem especially for neonates, whose immune system is not fully developed. With this is mind, many other individuals may be under threat if they are immunocompromised. If this organism can survive in infant formula, then there is a potential for other organisms to survive in a wide range of powdered products aimed at potentially immunocompromised individuals.

Three products formed the focus of the work; infant formula milk for neonates aged between nought to six months, protein based drinks used by individuals to support high performance sport and weight gain products aimed at the elderly. All the people that use these powdered products have the potential to be in

some way immunocompromised. Newborn's have very little immunity, which is exacerbated if they are not being breast fed. This is because there is no passive immunity being transferred to the new born baby. Therefore babies being fed infant formula from birth may be at risk of illnesses from organisms that are in their food. Individuals undertaking sports whilst taking protein based drinks may also be at higher risk. It is suggested that exercise can be employed as a model of temporary immunosuppression that occurs after severe physical stress (Bente Klarlund and Laurie Hoffman-Goetz, 2000). This suggests that the level exercise one undertakes may be linked to the immune system becoming depressed. Elderly individuals are also seen to be potentially at risk. This is because as they get older, their immune system begins to decline, leaving them more vulnerable to pathogens that would not normally be a problem for healthy adults.

Four Gram-positive bacteria that are known to be able to survive in harsh environments will be under investigation: Two spore formers; *Bacillus cereus* and *Clostridium perfringens* and two non-spore formers; *Listeria monocytogenes* and *Staphylococcus aureus.*

1.2. LITERATURE REVIEW

This work was funded by the East Midlands Development Agency Food Innovations Network (iNet) and part of the work required was a survey of the published literature describing the contamination of powdered foods.

1.2.1. *Bacillus cereus*

Bacillus cereus is a spore-forming, Gram-positive, rod-shaped, motile organism. It is responsible for only a small amount of food borne illnesses (2-5%; (Kotiranta *et al.,* 2000). However due the relatively short duration of symptoms, notification is generally low (HPA, 2010). This organism is normally associated with two forms of human food poisoning; either diarrhoea and

abdominal distress, which is caused by three pore forming cytotoxins haemolysin BL (Hbl), nonhaemolytic enterotoxin (Nhe) and cytotoxin K or nausea and vomiting, which is caused by cereulide (Arnesen *et al.,* 2008). Both the emetic and diarrhoeal syndrome can occur in healthy individuals however contaminated, hospital-prepared food dietary supplements may be a potential risk to immunocompromised patients (Rowan and Anderson, 1998). The diarrhoeal type normally associated with a longer incubation time compared to the emetic type which has a relatively short incubation time (Kotiranta *et al.,* 2000). The emetic form is almost invariably associated with contaminated cooked rice (Gilbert and Taylor, 1976).

1.2.1.1. Bacillus cereus in powdered foods

Bacillus cereus has particular importance in powdered foods as it can form spores which can survive various drying and heat-treatments used in the food industry such as pasteurisation (Andersson *et al.,* 1995). The spores have also been seen to adhere to stainless steel and to resist a cleaning in place (CIP) procedures in and around food factories (Tauveron *et al.,* 2006). This has obvious implications as the equipment could infect the food and therefore cause high numbers of the spores to be found in the food products.

Bacillus cereus has been found in many different dried food products. Dried milk products and infant food are known to be frequently contaminated with *Bacillus cereus* (Becker *et al.,* 1994). This can give rise to both the diarrhoeal or emetic syndrome. As described above, the spores can survive the pasteurisation and storage in a desiccated environment over an extended period time. When the powdered product has been rehydrated the spores may then return to their vegetative state and begin the release of their toxins. If there is a high enough number of these cells, or if enough toxin has been made, then the syndromes

described above may be caused, especially in someone who has a compromised immune system.

1.2.1.2. Bacillus cereus spores

Generally dried milk products will be contaminated with *B. cereus* via raw milk that contains the organism frequently in low numbers (Jayne-Williams and Franklin, 1960). The initial heat treatment step applied in the production of dried milk is very important for the activation and germination of *B. cereus* spores, as their many different layers can protect the organism in extreme environments (Figure 1.1). Where raw milk does not generally support the germination of spores, a high temperature short time pasteurisation treatment renders the milk as a good germination medium (Becker *et al.,* 1994). This then gives the organism a good hold in the food product as a spore which can survive further treatments down the production line, such as desiccation. As mentioned in section 1.2.1.2, spores can be found on equipment used in processing the food which can either contaminate a product with a low microbial load or add to the spore numbers already in the final food product.

There are therefore two main routes identified in which *B. cereus* spores can be linked in contaminating a powdered milk based product. Firstly it is likely that the contamination of milk powders with this spore forming organism is due to the detachment of microorganisms from biofilms that have developed on stainless steel surfaces which can resist CIP procedures (Stadhouders *et al.,* 1982). Secondly from spores that formed in milk pre-pasteurisation and can therefore survive the heating process and persist in the dried milk.

Figure 1.1: The *B. cereus* spore

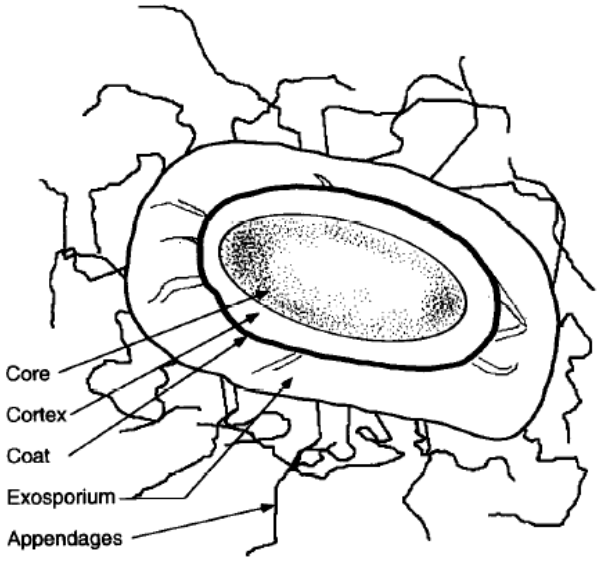


Diagram showing the different layers that a *B. cereus* cell has when it is in spore form (Husmark, 1993).

1.2.1.3. Bacillus cereus in other dried food products

The occurrence of *B. cereus* in other dried food products other than dried milk and infant dairy products has also been noted. In a survey of 39 dried food samples which represented 12 different pulses and cereals, 56% were found to be contaminated with *B. cereus* (Blakey and Priest, 1980). However the most significant findings involving dried pulses was that during normal cooking procedures and storage at room temperature, the *B. cereus* numbers residing on red lentils and kidney beans increased to a level at which enterotoxin production could become significant (Blakey and Priest, 1980). As the organism is naturally found on these products the initial numbers are not unexpected. However the fact that after processing during normal cooking times and conditions - and significantly the drying process - the bacterium could survive and make significant levels of enterotoxin represents a health concern.

Another food group that has been studied is dried herbs and spices. The main impact being on public health risks is when using these spices and herbs as an addition to ready-to-eat foods. This is especially true for those that undergo little further processing after being added to these foods. Studies have shown that overall 3.0 % of herbs and spices contained high counts of *B. cereus* (Sagoo *et al.,* 2009). Mckee (1995) describes how traditional drying methods can involve placing the herbs on the ground and letting the sun dry them, which can expose these foods to a wide range of microorganisms and many spices are imported from countries that still use such traditional preservation methods. For instance issues have been raised concerning spices imported from India. Four unprocessed Indian spices sampled at the point of export were shown to be highly contaminated with bacteria. *B. cereus* was detected in more samples of these spices than any other spore-forming aerobe (Seenappa and Kempton, 2008). However knowing that this is a risk, the key aspect to reducing the count of potential pathogens in the herbs and spices should be good hygienic practice

in factories, including identifying necessary CCPs and maintaining good CIP regimes.

Powdered dietary supplements have also been scrutinised as they have been linked to problems in immunocompromised individuals, in hospitals, especially where supplemented food is served to people that may have undergone surgery or who are HIV positive. For instance researchers in an HIV ward have found that the reconstitution and build-up of powder in pasteurised semi-skimmed milk (PSSM) followed by storage resulted in growth of *B. cereus* and synthesis of diarrhoeal enterotoxin (Rowan and Anderson, 1998).

1.2.2. *Clostridium perfringens*

Clostridium perfringens is a Gram-positive, rod-shaped, spore-forming bacterium. *C. perfringens* is ubiquitous and therefore found in a variety of places including; food, water and air. Its survival under extreme conditions is largely a factor of differentiation from metabolically active, vegetative cells to highly resistant (100 °C for 60 min), dormant spores (Novak and Juneja, 2002). Their highly heat resistant spores pose major problems within the food industry as this organism can survive cooking temperatures. This organism is responsible for a large majority of food poisoning cases in the UK (HPA, 2010). This bacterium can release five different types of toxins, the most common involved in food poisoning for humans is type A. *C. perfringens* enterotoxin (CPE) has been shown to be the virulence factor responsible for causing the symptoms of *C. perfringens* type A food poisoning. This bacterium is also responsible for the rare but severe food borne necrotic enteritis (Brynestead and Granum, 2002).

1.2.2.1. Clostridium perfringens' stress response

As a spore former, *C. perfringens* can survive in a variety of different stressful environments. It is also an anaerobic organism that has the ability to survive in

its vegetative form in an aerobic atmosphere. *C. perfringens* does not readily form spores, however when it does the ability of *C. perfringens* type A to produce an enterotoxin active in human food poisoning has been shown to be directly related to the ability of the organism to sporulate (Duncan *et al.,* 1972).

The main specific problem with *C. perfringens* is that wild type strains isolated from the environment are almost always enterotoxin negative (Skjelkvile et al., 1979). In contrast to has been postulated that industrial treatment of heating and cooling of the pathogenic strains can contribute to selecting for enterotoxin-production (Sarker *et al.,*2000). Food poisoning caused by C. *perfringens* is therefore almost always seen to be the responsibility of the food manufacturer, as environmental strains, at least initially, are not likely to cause food poisoning (Andersson *et al*., 1995).

1.2.2.2. Clostridium perfringens powdered milk based food

This organism has not been widely associated with powdered foods, especially milk-based products. The main food group that has been linked with food poisoning from *C. perfringens* are meat products that have been cooked but not stored at an adequate temperature to prevent growth or toxin production.

1.2.2.3. Clostridium perfringens in other dried or powdered foods

Natural or alternative health foods have recently become more popular with the public, especially in the western world, where natural remedies are thought to be healthier than modern medicines. However problems have occurred as these products have been dried and processed without stringent hygienic practices to prevent contamination of potential pathogens. *C. perfringens* is recognized as being incriminated in food poisoning cases related to these dried food products (Kunene *et al*., 1999). From studies carried out by Martin *et al.* (2001) 19.2% of dried medicinal plants had levels *C. perfringens* levels above

10^3 spores/g. Corn silk, flowers of linden tree and leaves of orange tree were the main culprits from this study contaminated with the highest levels of *C. perfringens* spores.

As described for *B. cereus, C. perfringens* spores can be found in a variety of different herbs and spices quite readily. As per International Commission on Microbiological Safety of Foods (ICMSF) specifications, the total aerobic mesophilic bacteria (TAMB) count found in coriander spices showed that 51% of the samples were in the unacceptable level, and 59% of these products were found to be contaminated with *C. perfringens* (Banerjee and Sarkar, 2003). The possibility also exists that pathogen growth is higher when spices are used in foods that are not subjected to complete thermal treatment (Aguilera *et al.,* 2005). This may be because during the heat treatments used may trigger sporulation of the organism, leading to toxin production. Therefore the best way to try and prevent the spread of *Clostridium* in foods is in the manufacturing stage. By preventing contamination of powdered herbs and spices with the use of good hygiene practices during growing, harvesting and processing from farm to fork, and effective decontamination, as well as good food handling practices the risk may be reduced (Sagoo *et al.,* 2009).

1.2.3. *Listeria monocytogenes*

This organism is a Gram-positive, facultative-anaerobic, rod-shaped bacterium. It does not form spores and is mainly motile when at room temperature. Since 1983 major food borne listeriosis outbreaks have been reported in Europe and North America mainly due to ready-to-eat foods (Tham *et al.,* 2000 and Okutani *et al.,* 2004). *L. monocytogenes* can cause many different ailments in humans and can come from a wide range of different food products. Clinical manifestations range from febrile gastroenteritis to more severe invasive forms including meningitis, encephalitis, abortions, and prenatal

infections (Dussurget, 2008). Its non-invasive form can cause gastroenteritis in anyone; symptoms include vomiting, nausea, stomach cramps and diarrhea. The invasive form from *L. monocytogenes* can cause a potentially fatal disease that mainly affects people that are immunocompromised. In these people it can infect the nervous system, first causing 'flu like' symptoms, developing into meningitis which may cause convulsions or affect other motor functions (Farber and Peterkin, 1991).

In pregnant women it can also cross the placental barrier (Lecuit, 2005); the organism can induce fevers and flu like symptoms in the mother as well as premature labour or cause stillbirths. New born babies can contract listeriosis from their mothers in the womb, which can cause sepsis, meningitis and spontaneous abortion.

A major concern with *L. monocytogenes* is that it has the ability to grow at temperatures as low and 3 ^{o}C, which has obvious implications for transporting or storing food and low temperatures (Harwig *et al.,* 1991). Another problem is that *L. monocytogenes* can survive quite high temperatures (around 50 ^{o}C, Doyle *et al.* 1987) for short periods of time. This is problematic, especially if ready to eat foods are contaminated and they are not held at high enough temperatures during production to kill the bacteria.

1.2.3.1 Listeria monocytogenes in powdered food

The organism's ability to survive in such a wide range of temperatures is a recognised problem within the food industry. A solution to this problem was thought to be drying. It was thought that by drying the food, there would be less chance of the organism surviving in the harsh desiccated conditions. However research has found that *Listeria* is able to survive the drying process and has been found in powdered food products (Lammerding and Doyle, 1990). This is particularly interesting as *Listeria* is a non-spore forming organism. And its

ability to survive as a vegetative cell poses quite a serious problem within the food industry.

Further studies with *L. monocytogenes* involving powdered egg products have also been carried out. It is more likely that products that have undergone commercial handling and processing are more likely to harbour the organism (Leasor and Foegeding, 1989). Dried powdered whole egg and dried egg yolk was used in the study to determine how long *L. monocytogenes* could survive in the powdered products in different storage temperatures (5 ^{o}C and 20 ^{o}C). The results indicated that *L. monocytogenes* can survive throughout the normal shelf life of powdered products, better at 5 ^{o}C but still with significant viable numbers remaining at 20 ^{o}C after six months storage (Brackett and Beuchat, 1991).

1.2.3.2. Listeria monocytogenes stress response

This organism has three main well characterised stress responses. These relate to extreme environmental changes such as; heat, pH and osmotic variations. *L. monocytogenes* are usually resistant to small changes in a particular environmental parameter, but in the presence of more prominent changes the induction of complex stress responses occur that are generally directed to survival instead of growth (Faleiro et al., 2003). This response enables the bacterium to alter its gene expression (Liu, 2008). This leads to alterations to the proteins being synthesised allowing the organism to survive harsh osmotic stressed environments for a long time.

As well as being mainly linked with survival some of the stress responses can also increase the virulence of the organism. This increase in pathogenicity, which is induced following exposure to a variety of stress responses, may also increase the bacterial resistance to the host defences (Gahan et al., 2001). These responses, as well as enabling the organism to infect their host, effectively also allow them to survive in the harsh conditions during the processing of food. The

ability of *L. monocytogenes* to survive desiccated stressed environments is extremely relevant during the process of drying foods.

1.2.4. *Staphylococcus aureus*

This organism is a Gram-positive, coccus-shaped bacteria. *S. aureus* is commonly carried asymptomatically on human skin, typically around the nose and nails. Occasionally the organism enters the bloodstream to cause invasive disease (Fan *et al.,* 2009). It is also a significant cause of a wide range of infectious diseases in humans. *S. aureus* often causes life-threatening deep seated infections like bacteraemia, endocarditis and pneumonia (Kanafani and Fowler, 2006). Food poisoning occurs when food containing preformed enterotoxins made by the organism are ingested. The disease is characterized by symptoms including nausea, vomiting, abdominal cramps, and diarrhoea lasting from 24 to 48 h and the complete recovery usually occurs within a few days (Pinto *et al.,* 2005).

S. aureus can grow between 7 ^{o}C and 45 ^{o}C. Its optimal temperature range is around 35-39 ^{o}C. Generally the organism grows best at a neutral pH; however it can grow between a range of pH 4-9. It can grow in both aerobic and anaerobic conditions (better in the presence of oxygen) and can survive in highly desiccated environments.

There are up to 21 different enterotoxins that this organism can produce. These may all be able to cause a wide range of illnesses in humans. Food poisoning is attributed to around 11 of these toxins. However toxins; SEs SEA to SEE and only SEH, SEG and SEI (McLauchlin *et al.,* 2000, Omoe *et al.,* 2002 and Bukowski *et al.,* 2010) have been proven to induce gastroenteric syndrome. However the most prevalent toxin associated with food poisoning is SEA (Klotz *et al.,* 2003). *S. aureus* is readily killed by cooking; however its toxins are very heat resistant for example the Decimal reduction time of enterotoxin B at 149 ^{o}C

is 100 min (NZFSA, 2001). This is extremely problematic, as cooking times may not extend this long, suggesting that viable toxin may survive and cause food poisoning. The toxins are also highly stable and resist most proteolytic enzymes, such as pepsin or trypsin, and thus keep their activity in the digestive tract after ingestion (Loir *et al.,* 2003).

1.2.4.1. Staphylococcus aureus in powdered foods

S. aureus has been noted to be able to survive for a long time in powdered food products. However in products such as dried infant formula milk, the contamination normally occurs from non-sterile spoons or from human contact with the milk. In a study of infant powdered milk the highest *S. aureus* counts occurred on the day of opening the formula and it survived for 12 days within the powdered milk without its numbers diminishing (Umoh et al., 1985). Umoh *et al* (1985) also stated that there was no enterotoxins production in the milk samples. However it is known that staphylococci multiply to a very high level and produce detectable amount of enterotoxins within 3 h in heat treated milk (Gosh and Laxminaraya, 1973). Therefore milk that may have been rehydrated and left in a warm place, could start to accumulate high levels of enterotoxins and therefore cause an illness to the infants drinking the milk.

1.2.4.2. Stress response

For an organism to survive the harsh conditions of the drying process and storage, many stress responses would be needed, as well as responses for when the organism enters the harsh environments in the human digestive system. Evolution has led to the development of *S. aureus* to become a highly adaptable organism, as even subtle changes in environmental conditions affect its physiology (Chan and Foster, 1998). *S. aureus* has the ability to be highly desiccation resistant. This important attribute is paramount in the organism's

survival in dried or powdered food products. Little is known about the mechanism of desiccation tolerance; however, there does seem to be a correlation between pigmentation of the organism and resistance to desiccation (Lacey and Lord, 1981).

S. aureus also have some resistance to acid environments. This organism is rapidly killed by acid at pH 2, but can develop resistance to the 'killing' pH if first adapted by exposure to a higher, non-lethal pH (Clements and Foster, 1999). This can be potentially hazardous, as food after rehydration may be only slightly acidic, which would then infer a greater resistance to stomachs acids and therefore allow the organism to survive the harsh pH in the stomach and cause food poisoning lower in the gastrointestinal tract.

1.3. AIM

From this survey of the literature, it can be seen that all these Gram-positive bacteria are of potential concern in dried products that are consumed by immunocompromised individuals. One aim of this project was to contribute to an educational programme targeted at training workers in the food industry. Specifically relevant information describing these organism would be collated to produce a web-based CPD package. The aim of the practical part of the project was to obtain a range of baby infant formula, powdered sports drinks and elderly build up powders and to test these using international standard protocols to see if they contain any of these four Gram-positive organisms. Tests on the non-spore forming organisms (*S. aureus* and *L. monocytogenes*) isolated would also be carried out to determine their heat tolerance to see if those isolates recovered from powdered food have any characteristics that account for their survival.

1.4. EDUCTAIONAL SERIES

CosmosBiomedical is a company that runs a continuing professional development (CPD) scheme that offers microbiologists the opportunity to learn about different aspects of microbiology (Bacteriology, virology, mycology and parasitology). On this company's website there is information about a range of different organisms. This study involved creating web pages on the organisms under investigation in this project. The four bacteria under investigation in this study was *Bacillus cereus, Clostridium perfringens, Staphylococcus aureus* and *Listeria monocytogenes.* Thus there were four sections of web-based information created for this company on these bacteria (see Figure 1.2 for an example of the web text and Appendices 1.1-1.4, for the full information collated). CPD question cards (Fig. 1.3) would then be developed by the company based on this information. The CPD scheme works by microbiologists who register with the course studying the web based material and then answering the questions on the cards that are sent out to them. Their work is then returned to the company for assessment and on the basis of this CPD points are awarded.

Microscopic images of the organisms have also been produced that allow detailed observation of colony and cell morphology that can be used on the CPD cards and on the web text.

At the beginning of this project the company only supported material for training Biomedical scientists, mostly those working in medical diagnostic laboratories. As a result of the work presented here the scheme has been extended to workers in food microbiology laboratories and a formal launch is planned by the company at the Society for Applied Microbiology meeting in July 2010.

Figure 1.2: Illustration of the material procedure for CPD website

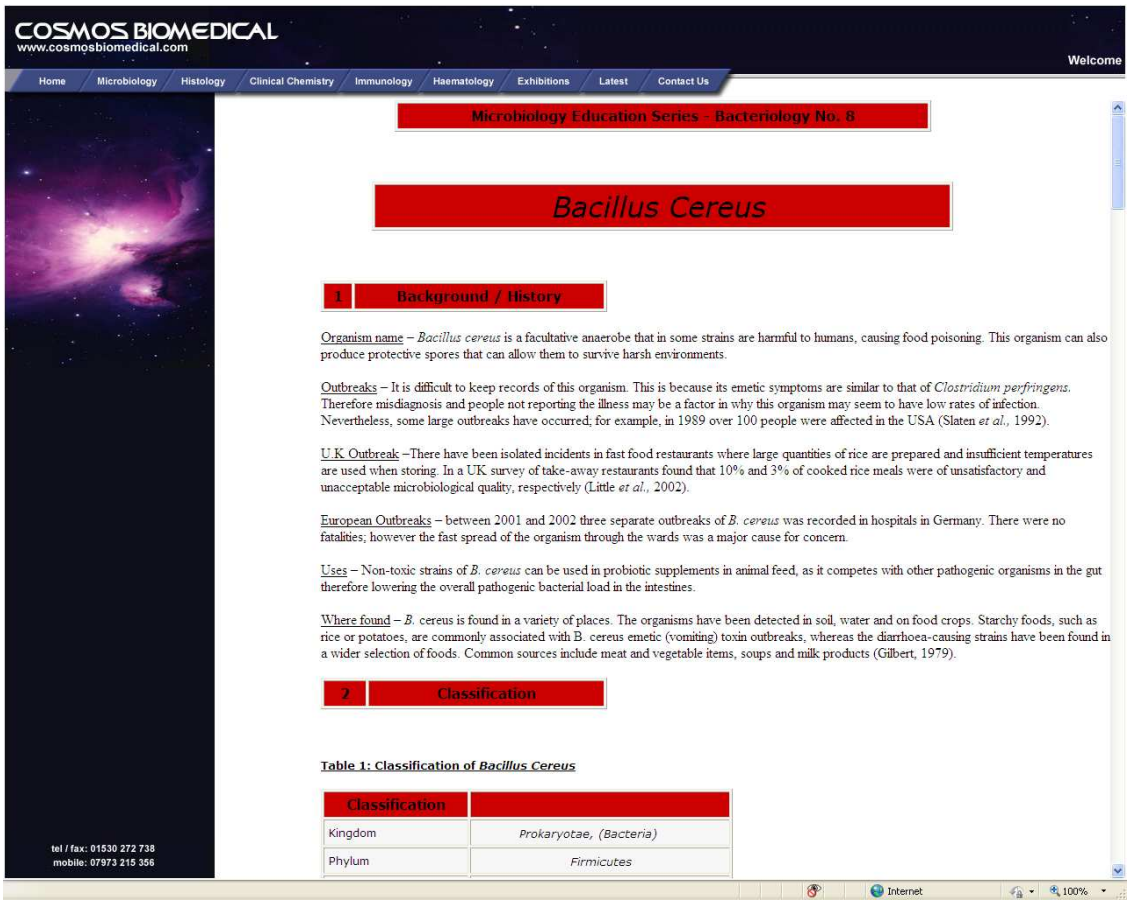
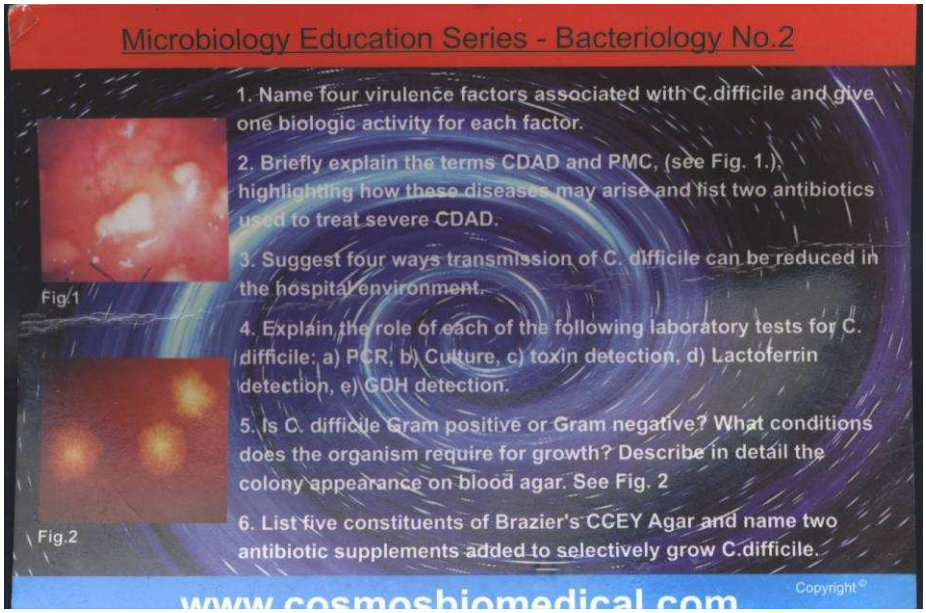


Figure 1.3: Example of CPD card produced from web text



CHAPTER 2

MATERIALS, METHODS AND STANDARD PROCEDURES

2.1. GENERAL MEDIA AND REAGENTS

2.1.1. Plate Count Agar (PCA)

This was achieved by reconstituting 17.5 g into 1l of RO water. This is then sterilised for 15 min at 121 ^{o}C. Around 20 ml of the PCA was then poured into separate Petri dishes in a sterile environment.

2.1.2. Buffered Peptone Water (BPW)

Twenty grams of the dehydrated medium was dissolved into 1l of RO water. This was then autoclaved for 15 min at 121 ^{o}C to sterilise. After sterilisation 225 ml of the buffered peptone water was poured into separate sterile bottles for use in future experiments

2.1.3. Brain Heart Infusion (BHI, Oxoid) Agar

Brain Heart Infusion (BHI, Oxoid) agar was made by adding 37 g of the dehydrated medium and 5 g of agar to 1l of RO water. This was mixed and then autoclaved at 121 ^{o}C for 15 min to sterilise. The BHI agar was then mixed and poured into sterile Petri dishes under aseptic conditions.

2.1.4. Blood Agar Plates

Blood agar (Oxoid) was made up by suspending 40 g of the dehydrated medium into 1l of RO water. This was then autoclaved for 15 min at 121 ^{o}C to sterilise the agar. After the agar cooled to hand hot (approx. 45 °C), defribrinated sheep blood was then aseptically added to 5% (v/v). The agar was then poured into sterile Petri dishes very thinly, so that haemolysis can be seen easily.

2.1.5. Tryptone Soy Yeast Extract (TSYE) Agar

This was made up by reconstituting 30 g of Tryptone Soy Broth, 6 g of yeast extract and 10 g of agar with 1l of RO water. This was then mixed thoroughly and autoclaved at 121 ^{o}C for 15 min to sterilise it. Each sterilised agar was then poured into sterile Petri dishes under aseptic conditions and left overnight to dry.

2.2. MEDIA AND PRODCEDURES FOR ISOLATION OF SPECIFIC ORGANISMS

2.2.1. *Bacillus cereus*

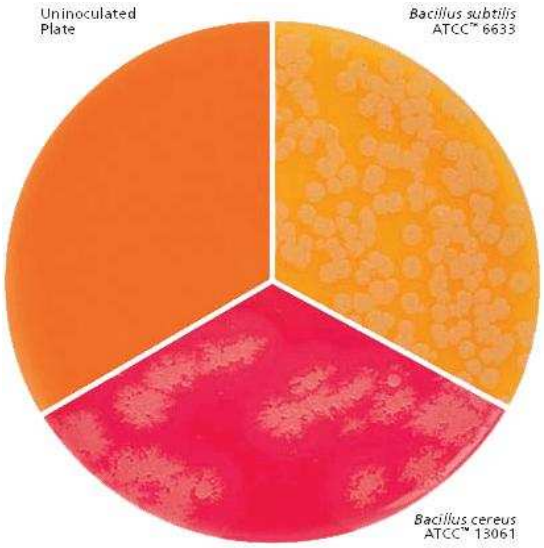
2.2.1.1. Mannitol Egg Yolk Polymyxin agar

For the isolation of *Bacillus cereus,* mannitol egg yolk polymyxin (MYP, Oxoid) agar was used for the isolation of *Bacillus cereus*. This was made by dissolving 21.5 g of media into 450ml of reverse osmosis (RO) water. This was then sterilised by autoclaving at 121 ^{o}C for 15 min. 50 ml of egg yolk emulsion and one vial of reconstituted polymyxin B supplement was aseptically added to the sterilised medium. Polymyxin B supplement was reconstituted by adding 2 ml of sterile RO water into the vial. The agar was then cooled and roughly 20 ml was poured into petri dishes in a laminar flow hood.

2.2.1.2. Sampling for B. cereus

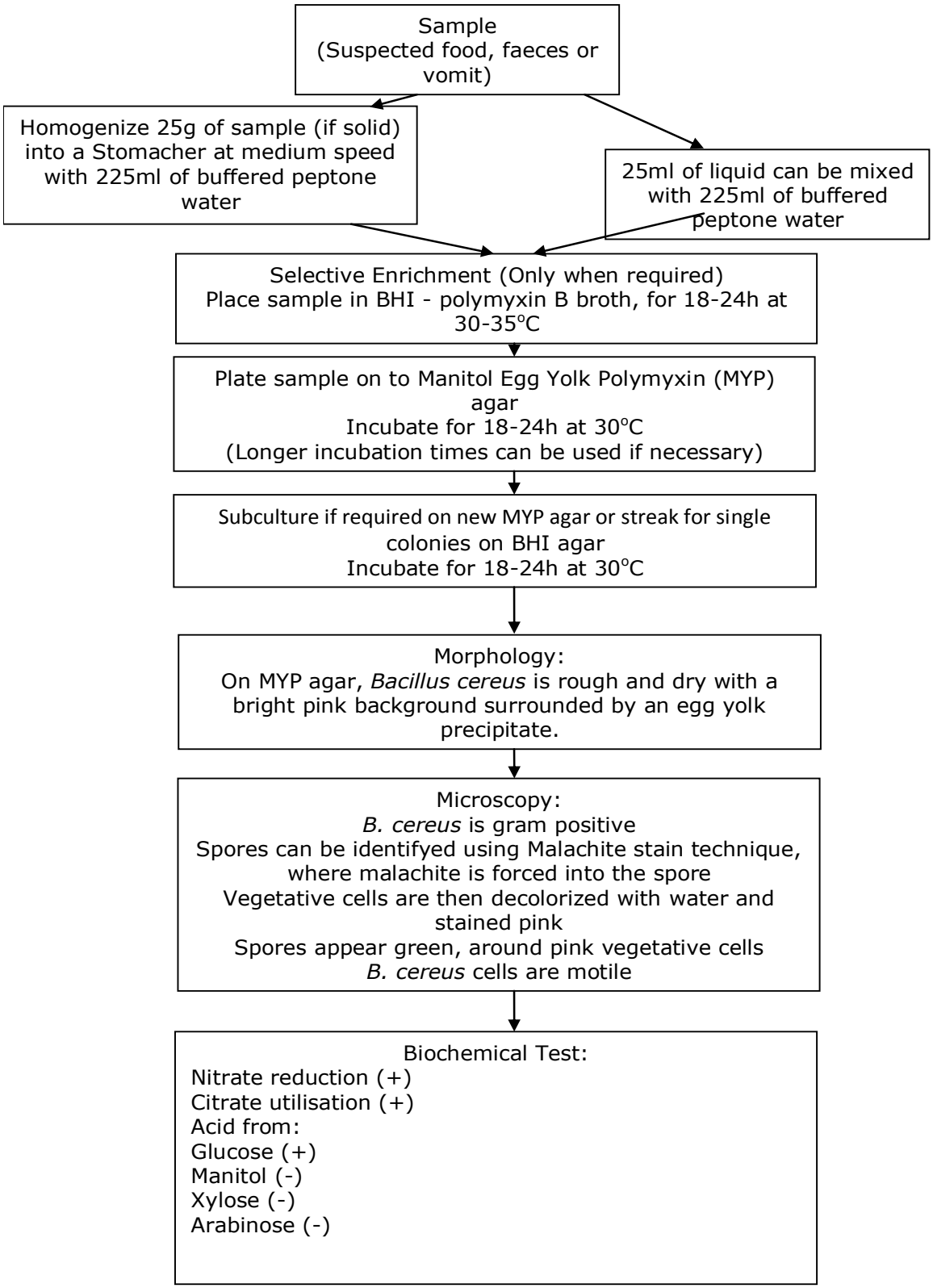
Using the international standard method ISO: 7932 (Figure 2.2), 0.1 ml samples in triplicate, were spread on to MYP agar. These were incubated aerobically for 24 h at 30 ^{o}C. Results were recorded only as positive or negative for presumptive colony growth. On MYP agar a positive result was the presence of bright pink, rough and dry colonies, with a zone of egg yolk precipitate (see Figure 2.1).

Figure 2.1: Growth characteristics of *Bacillus* spp. on MYP agar



The diagram shows the possible growth results of different *Bacillus* spp. when isolated on MYP agar (BBCorp, 2005). *B. cereus* colonies give a characteristic pink-red colonies, because mannitol is not fermented, with zones of precipitate around the colonies due to the production of lecithinase. In contrast *B. subtilis* produces yellow colonies with no precipitation. It should be noted that the media used in this ISO method is not 100% selective and other *Bacillus* spp. are often recovered (Downes *et al.* 2001).

Figure 2.2: Scheme for the isolation of *Bacillus cereus*



Generalised scheme for isolation of *B. cereus* showing recommended confirmatory tests to be performed following isolation on MYP agar. Taken from Shinagawa (1990).

2.2.2. *Clostridium perfringens* Identification

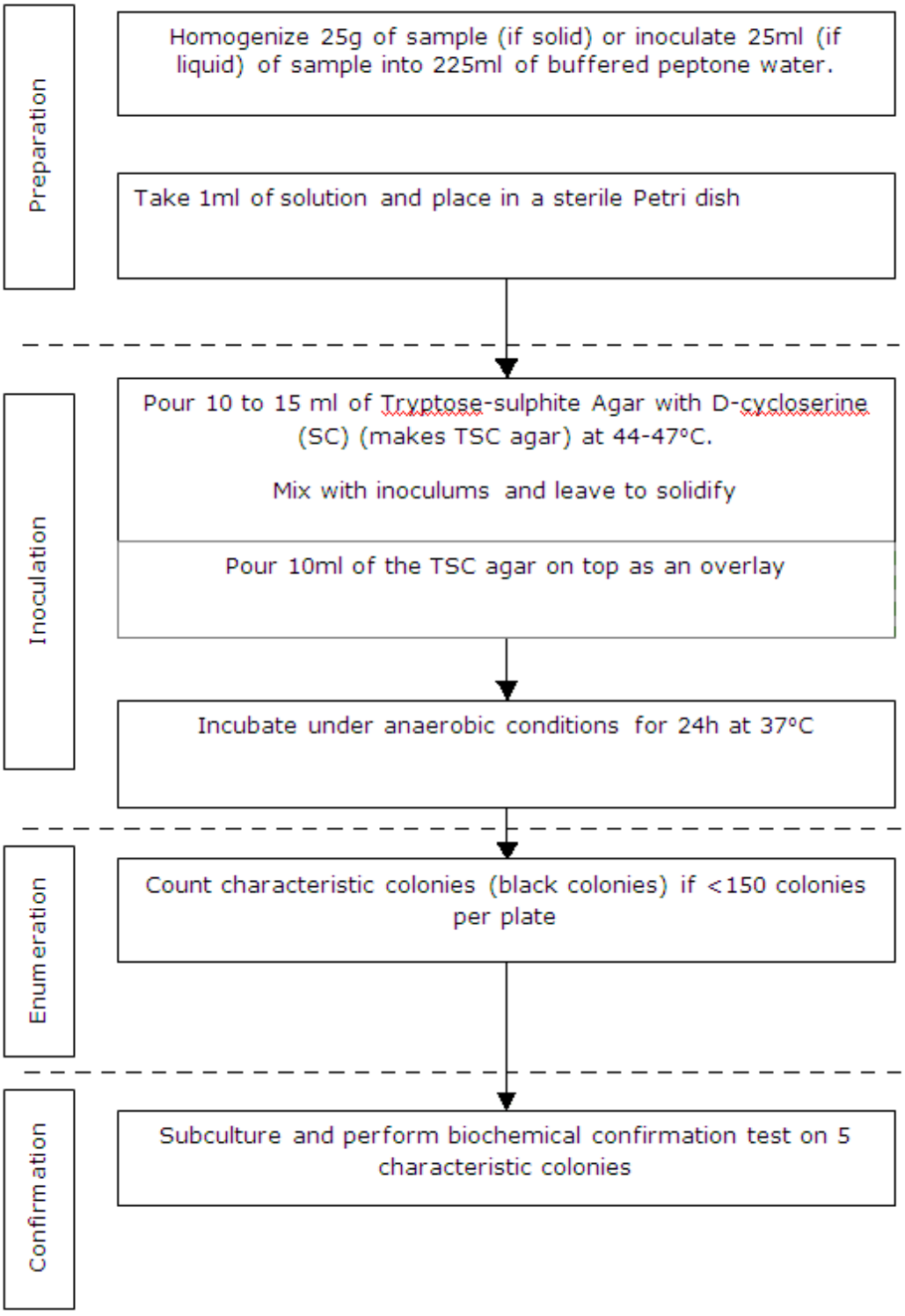
2.2.2.1. Tryptose Sulphite Cycloserine agar

Tryptose sulphite cycloserine agar (TSC, Oxoid) was used for the isolation of *Clostridium perfringens*. This was made by dissolving 23 g of the dehydrated media into 500 ml of RO water. This was the autoclaved for 15 min at 121 ^{o}C. One vial of TSC supplement was then reconstituted by adding 2 ml of sterile RO water into the vial. The reconstituted TSC supplement was then added with 25 ml of egg yolk emulsion aseptically. 15 ml of the prepared agar was then poured into petri dishes in a laminar flow hood.

2.2.2.2. Clostridium perfringens identification

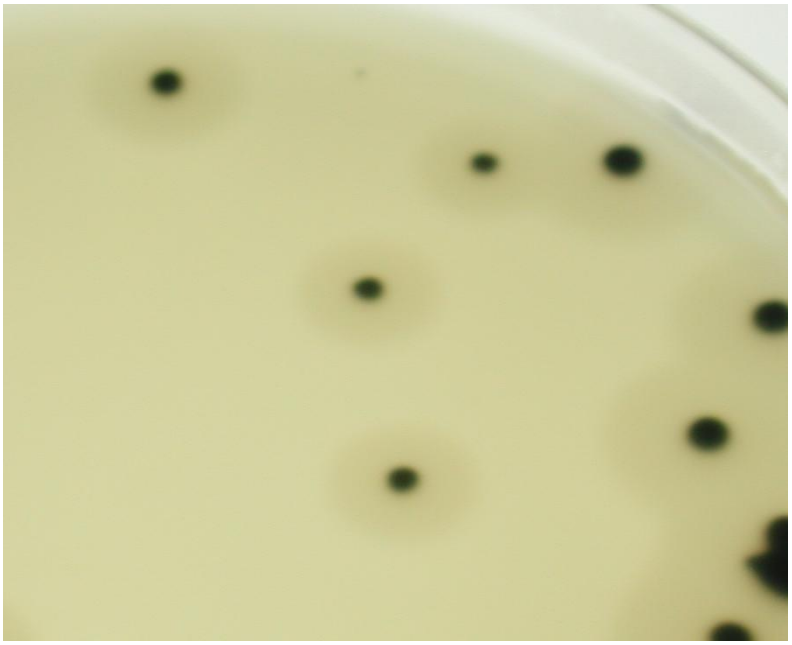
Samples were screened for the presence of *Clostridium perfringens* using the standard phenotypic ISO 7937 method (Figure 2.3), which uses tryptose sulphite cycloserine (TSC) agar (Section 2.2.2.1). Samples (1 ml) of the reconstituted powdered foods (Section 2.3.2) were pipetted into a sterile petri dish. Cooled TSC agar (10-15 ml; Section 2.2.2.1) was then mixed with the 1 ml sample and this was left to cool and set. A sample (10 ml) of the same medium was then layered on top of the first layer of agar and food sample. When the agar had set, the plates were incubated under anaerobic conditions for 24 h at 37 ^{o}C. The organism should form round black colonies on the agar, which can be accompanied with a zone of clearing of the egg yolk emulsion (see Figure 2.4). However the presence of this zone of clearing is not definitive for *C. Perfringens* if characteristic black colonies (with or without zone of clearing) were seen these were taken to be a presumptive positive isolate of *C. perfringens.* Presumptive positive colonies were further characterised using confirmatory tests. Definitive identification is normally based on the following tests: sulphite reduction and lactose fermentation in lactose sulfite broth, gelatine liquefaction, and nitrate reduction and Gram stain (Araujo *et al.,* 2004).

Figure 2.3: Scheme for isolation of *Clostridium perfringens*



Generalised scheme for isolation of *C. perfringens* (Biomerieux, 2004). For details of other tests performed see Sections 2.2.2.3.

Figure 2.4: Growth characterisitcs of *C. perfringens* on TSC agar



This image shows the colony morphology of presumptive *C. perfringens* on TSC agar, with the zone of clearing using a camera. Presumptive *C. perfringens* may also from black colonies without the zone of clearing.

2.2.2.3. API 20A test (Biomerieux)

Colonies formed from TSC agar were streaked for pure culture onto BHI agar. This was then incubated overnight anaerobically at 37 ^{o}C. Using a sterile swab, all of the growth from the BHI plates was collected and inoculated into the API 20A medium. The swab was rotated against the side of the walls, being careful to not create bubbles. The final turbidity of the medium was around McFarland standard No. 3. The API 20A incubation box was incubated by pouring 5 ml of sterile RO water into the honeycombed wells in the tray. The API 20A strips were removed from the packaging and placed in their incubation trays. Using a sterile pipette, each strip was inoculated with separate suspected *C. perfringens* isolates. Each tube on the strip was filled being careful to not create bubbles. However the 'GEL' tube was filled as well as its cupule. The 'IND' tube was filled and its cupule was topped up with mineral oil. The API 20A strip was placed in its incubation box and the lid was placed on top. Each strip was incubated anaerobically at 37 ^{o}C for 24 h, with an extra 24 h if required. The result was processed on APIWeb (Biomerieux) to determine what the organism was.

2.2.3. *Listeria monocytogenes* Identification

2.2.3.1. Listeria monocytogenes enrichment broths and agars

Fraser broth was made by adding 500 ml of RO water to 28.7 g of dehydrated media. This was then sterilised by autoclaving for 15 min at 121^{o}C. One vial of Fraser supplement was then reconstituted by adding 4 ml of RO water and ethanol in a 1:1 ratio and aseptically adding it to autoclaved Fraser broth.

Half Fraser supplement was then made by adding 12.9 g of media into 225 ml of RO water. This was then autoclaved for 15 min at 121 ^{o}C. 4 ml of sterile water and ethanol in a 1:1 ratio was then added to Half Fraser supplement. This was then mixed in with the sterilised Half Fraser broth.

PALCAM agar (Oxoid) was made dissolving 34.5 g of the dehydrated medium into 500 ml of RO water. This was then autoclaved for 15 min at 121 ^{o}C to sterilise it. The supplement for PALCAM was then rehydrated with 2 ml of sterile RO water and then was aseptically added to the sterile PALCAM agar. 20 ml of the agar was then poured aseptically into Petri dishes.

2.2.3.2. Sampling for L. monocytogenes

The 25 g of the powdered food were dissolved in 225 ml Half Fraser broth (Section 2.3.2) and incubated for 24 h at 30^{o} C as a pre-enrichment step. A sample (0.1 ml) of this was then aseptically added to 10 ml of Fraser Broth. This was then incubated for 48 h at 37 ^{o}C to act as the selective enrichment step. Finally 0.1 ml of this culture was spread onto the surface of the PALCAM agar and incubated under microaerophilic conditions for 24 h at 37 ^{o}C. Characteristic black or dark green colonies surrounded by a black 'halo' (Figure 2.5) were considered to be presumptive *L. monocytogenes* colonies.

Figure 2.5: *L. monocytogenes* colonies on PALCAM agar.

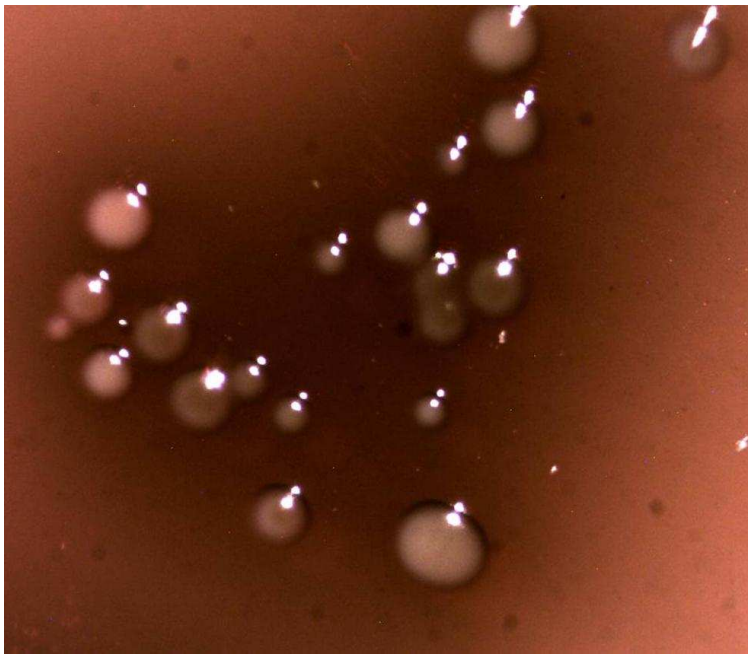
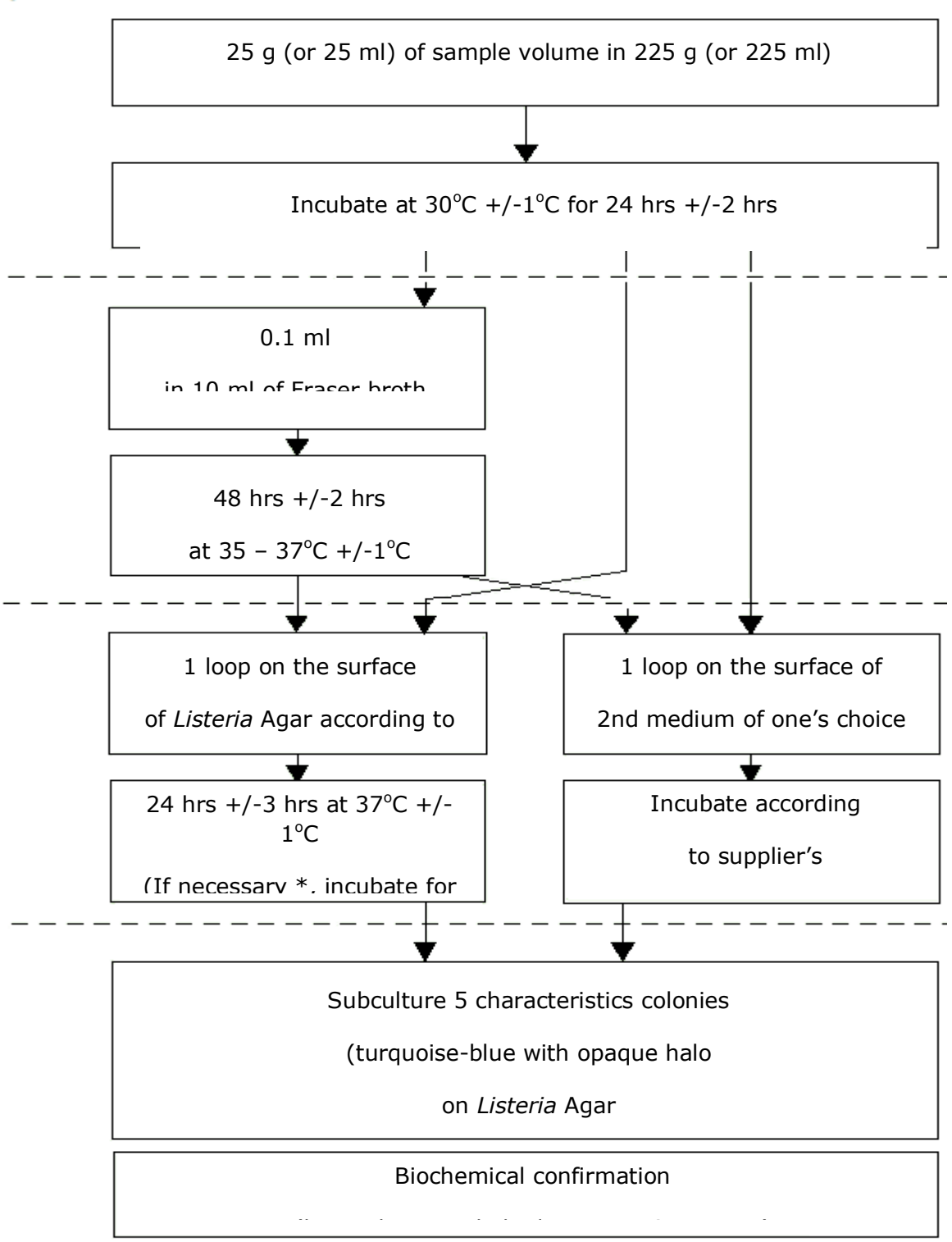


Image showing *L. monocytogenes* colonies on PALCAM surrounded by a black halo, using a digital eye piece attached to a microscope.

Figure 2.6: Scheme for isolation of *Listeria monocytogenes*



Generalised scheme for isolation of *L. monocytogenes* (Biomerieux, 2004). For details of other tests performed see sections 3.2.2.5.

2.2.3.3. API Listeria (Biomerieux)

Pure cultures were prepared on BHI agar plates from each isolate to be tested. These were incubated overnight in microaerophilic conditions at 37 ^{o}C. The incubation containers for the API Listeria strips were prepared by pouring 3 ml of sterile RO water into the honeycombed wells. Each strip was then removed from the packaging and placed in the incubation box. The API Listeria suspension medium was prepared by inoculating it with colonies grown from the overnight culture on BHI agar to a turbidity of McFarland standard No.1. Around 50 µl of the prepared medium was then pipetted into all of the cupules except for the 'DIM' cupule, where 100 µl was added. The strip was then enclosed in the incubation box and put placed in the incubator for 24 h at 37 ^{o}C under aerobic conditions. To read the strip after incubation, a drop ZYM B reagent is added to the 'DIM' test. The reactions were all read within three minutes of coming out of the incubator and interpreted using APIWeb (Biomerieux).

2.2.4 *Staphylococcus aureus* Identification

2.2.4.1 Baird Parker agar

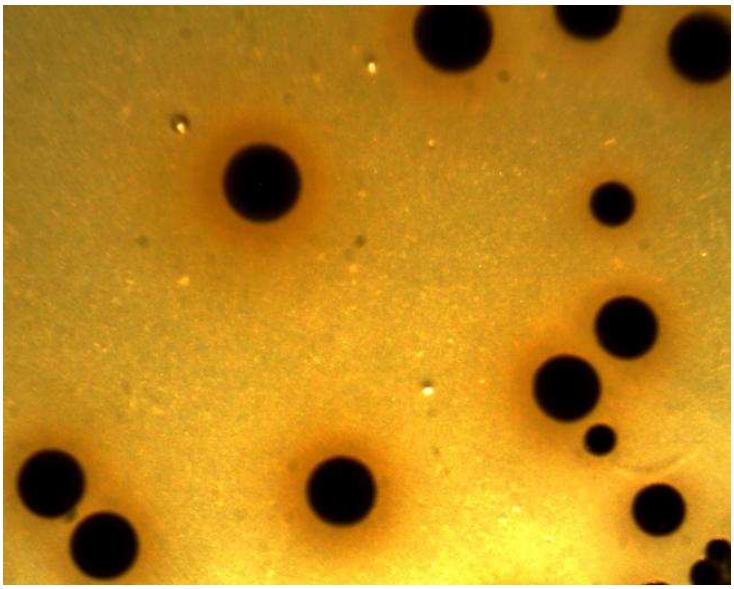
Baird Parker (BP, Oxoid) agar was used for the isolation of *Staphylococcus aureus*. This agar was made by dissolving 31.5 g of dehydrated medium into 500 ml of RO water. This was then autoclaved for 15 min at 121 ^{o}C. 50 ml of egg yolk tellurite emulsion was then aseptically added to the medium. About 20 ml of the agar was then poured into Petri dishes in a laminar flow hood.

2.2.4.2. Sampling for Staphylococcus aureus

This organism was tested for using the international standard method ISO: 6888 (Figure 2.8). Samples (0.1 ml) of the reconstituted powdered food (section 2.3.2) were spread onto the surface of BP agar (Section 2.2.4.1) plates and left to dry. The plates were inverted and then incubated at 37 ^{o}C for 24 h. If

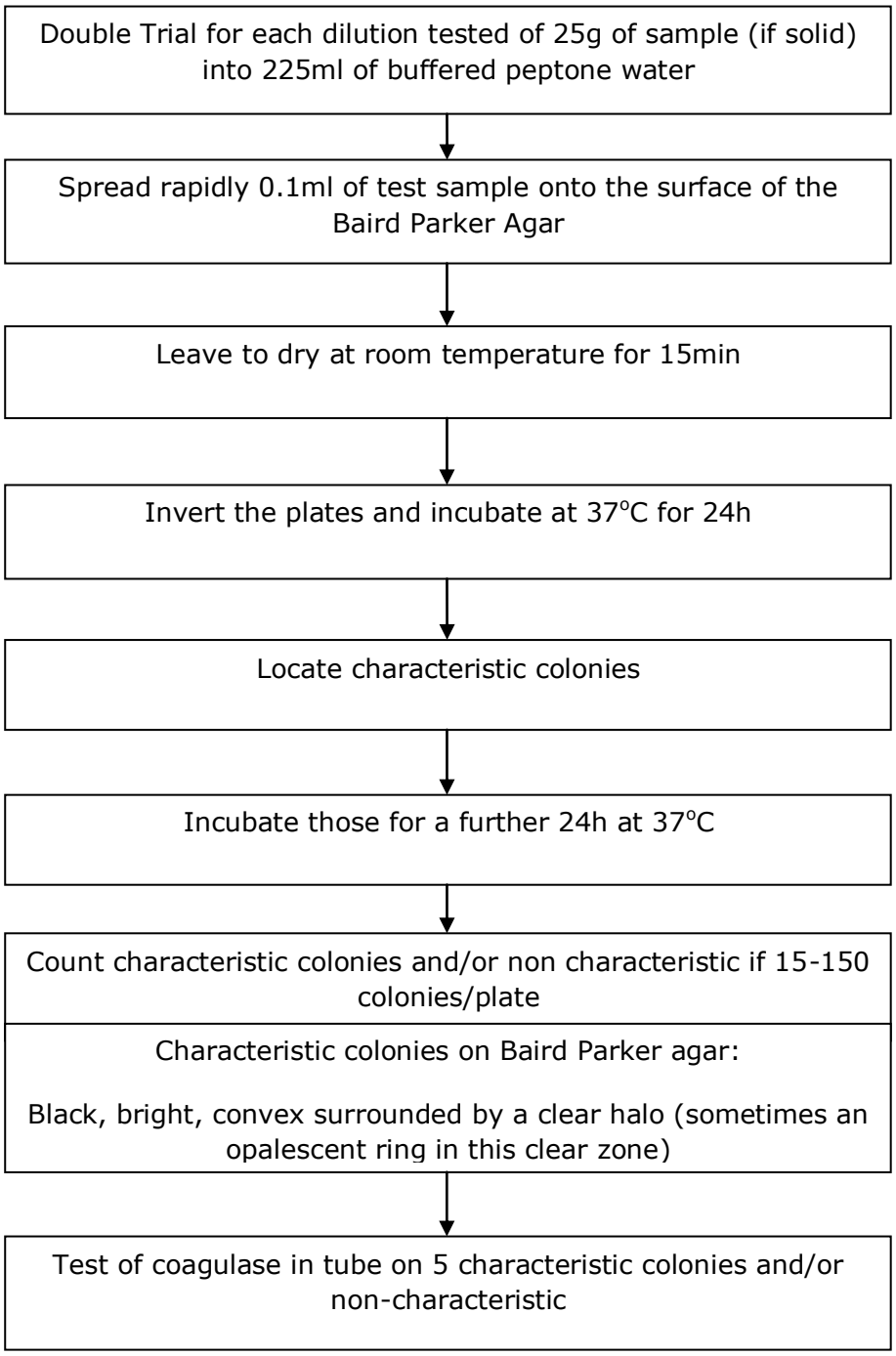
necessary a further 24 h incubation was carried out. Characteristic black, bright, convex colonies surrounded by zone of clearing or 'halo' (Figure 2.7) were considered as presumptive *S. aureus.* However colonies that did not form the halo were still considered positive, until further testing was carried out.

Figure 2.7: Staphylococcus aureus on BP agar



An image showing S. aureus colony surrounded by a 'halo', grown on Baird Parker agar using a digital eye piece on a microscope.

Figure 2.8: Scheme for isolation and detection of *Staphylococcus*



Generalised scheme for isolation and detection of *Staphylococcus aureus* (Biomerieux, 2004). For details of other tests performed see sections 3.2.2.2.

2.2.4.3. Catalase test

The cover slip based method was used to detect catalase activity. A small portion of colony grown on BP agar was transferred onto the centre of a cover slip. The cover slip was then inverted and placed on the drop of hydrogen peroxide from a Pasteur pipette. If vigorous bubbling occurred within 10 s the organism was catalase positive. *Streptococcus pyogenes* (NLTL 1200) was used as a negative control and *S. aureus* as a positive control.

2.2.4.4. Coagulase test

Organisms that were positive for catalase test (section *2*.2.4.3) were tested using the Staphytect plus kit (Oxoid). The latex reagents were brought to room temperature before use and were mixed thoroughly. One drop, from the dropper bottles provided, of the latex solution was placed in the centre of a circle, on a test card provided. One drop of control latex, which is not linked to IgG, was placed onto another circle. As a control, around five colonies from *S. aureus* (R3293/02) was mixed with both latex samples using a sterile loop. The cards were then rocked and rotated for 20 s to mix the samples. After mixing the latex linked to IgG should show agglutination (a grainy appearance) and the negative control latex solution should have shown no agglutination (suspension remains uniform). To test isolates, five colonies of catalase positive *S. aureus* isolates were then tested in the same way. If agglutination occurred then the sample were recorded as coagulase-positive. The cards after use were then disposed of into disinfectant (Virkon, 5 % (w/v) solution).

2.3. GENERAL TESTING METHODOLOGY

2.3.1. Viable Count Determination

The colonies that formed were counted and the cfu/ml was calculated using the formula:

$$\text{cfu/ml} = \frac{\text{Number of Colonies x Dilution}}{\text{Amount Plated}}$$

Where possible, only samples with colony counts in the statistically acceptable range (30-300) were used.

2.3.2. Preparing Powdered Foods for Microbiological Testing

Twenty-five grams of each powdered product was reconstituted with 225 ml of BPW. For the pre-enrichment steps required for *L. monocytogenes* testing, 25 g of each powdered product was also reconstituted into 225 ml of Half Fraser broth.

2.3.3. Determining the Aerobic Plate Count of the Powdered Products

Samples (0.1 ml) of the reconstituted powdered food was spread onto PCA plates and incubated at 37 ^{o}C for 48 h. The number of colonies was counted and the cfu/ml calculated as described in Section 2.3.1.

2.3.4. Long Term Storage of Bacteria

Colonies that grew on the diagnostic agars, whether characteristic or not, were put into frozen storage using Microbank (ProLab Diagnostics). Each vial was labelled with; product, agar used for isolation and colony description. Each separate colony was first streaked for single cells on BHI agar. Under aseptic conditions, the cryopreservation fluid in the vials was inoculated with pure colonies isolated on from a BHI plate up to approximately McFarland Standard No. 3 or 4. The vial was then closed and inverted four or five times to emulsify

the organism. The excess fluid was then aspirated and the tops closed and placed in the freezer at -20 ^{o}C. To recover cultures from these frozen samples one bead was inoculated into BHI broth and incubated overnight at 37 ^{o}C, giving cultures of approximately 10^{8}-10^{9}cfu/ml.

2.3.5. Gram Staining

A loop of maximum recovery diluent (MRD) was placed on a microscope slide. Part of one colony was picked off a fresh overnight plate cultures with a sterile loop. This was mixed with the MRD on the glass slide. The sample on the slide was then heat fixed by passing the slide over a Bunsen flame 15-20 times.

The prepared slides were then placed in a rack and placed into crystal violet solution for 60 s. The slides were then washed with water. Next the slides were placed in iodine for 30 s. Iodine is a mordant that binds with Crystal violet and is then unable to exit Gram-positive cells. The slide is then washed again with water. The slide is then placed in methanol for 60 s. This is a decolouriser that removes any crystal violet from Gram-negative cells. The slides were then placed in safrinin, which acts as a counter stain, for 30 s. The slides were then blotted dry and observed under a 100x oil immersion lens. Gram-positive cells are those that are stain purple and retained the crystal violet, whereas Gram-negative cells are pink from the safrinin.

2.3.6. Wet Mount Observations

A loop of sterile MRD was placed onto a microscope slide and part of a fresh colony was mixed in with the MRD using a sterile loop. A cover slip was then placed on top of the microscope slide. One drop of immersion oil was then put on the cover slip. Using a 100x oil immersion lens, the motility of the organism, morphology and other characteristic features, such as cell arrangement or any pigmentation, was recorded.

2.3.7 Biochemical Identification Tests

2.3.7.1. Haemolysis test

Organisms for testing were recovered from the PALCAM and TSC agar and then streaked for single colonies onto the thin blood agar plates. These were then incubated for 24 h at 37 ^{o}C under either microaerophilic conditions for organisms isolated from PALCAM or under anaerobic conditions for organisms isolated from TSC agar. Two types of haemolysis were recorded: Alpha haemolysis which forms a narrow greenish zone of clearing around colonies, and beta haemolysis which forms a zone of complete clearing round colonies.

2.3.7.2. Inducing Bacillus cereus spores

The method to detect *B. cereus* spores was adapted from Reyes *et al* (2007). 25 g of sample was resuspended in 225 ml BPW. One 10-fold dilution was carried out in a sterile tube. This was then incubated at 80 ^{o}C for 10 min then cooled in an ice bath. Three more 10-fold serial dilutions were then prepared. Samples (1 ml) were then inoculated into 90 ml of tryptone soya broth (TSB, Oxoid), which was supplemented with 1 ml of reconstituted polymyxin B supplement (Oxoid). This was the incubated for 24 h at 30 ^{o}C. After incubation, samples (0.1 ml) were spread in triplicate on MYP agar plates. These were then incubated at 30 ^{o}C for 24 h. After incubation the MYP agar plates that yielded pink colonies (see Figure 2.1), and had the characteristic *B. cereus* traits under the microscope (Gram-positive, motile rods) was considered to be spore formers.

2.3.8. DNA Extraction

2.3.8.1. Extraction of genomic DNA from isolates

Fresh colonies of the bacteria isolated from the powdered foods were grown on BHI agar. A sterile loop was used to resuspend one colony in 1 ml of TE buffer

(10 mM Tris-HCl, pH 7.5, 1 mM EDTA) supplemented with lysozyme (20 µl/ml). The cells were then incubated at 37 ^{o}C for 15 min. The solution was then boiled at 95 ^{o}C for 10 min, then cooled and centrifuged at 10,580 g for 5 min. For each polymerase chain reaction (PCR) a 10 µl sample was used as template DNA.

2.3.8.2. Column purification of template DNA (ZymoResearch)

One colony from an overnight culture was transferred to a microcentrifuge tube containing 20 µl of sterile distilled water (SDW). This was then incubated at 95 ^{o}C for 5 min. Following this, the sample was incubated at 37 ^{o}C for 10 min. The solution was then transferred to a Zymo-spinI column in a collection tube. This was then centrifuged at 6,260 g for 60 s. The flow through was discarded. 200 µl of wash buffer (provided by the manufacturer in the Zymo-spinI kit) was added to the column and it was centrifuged again at 10,000 rpm for 30 s. This step was repeated twice more. The column was then pulse centrifuged at 10,580 g for 10 s. 10µl of water was directly pipetted to the column. The column was then placed in a 1.5 ml microcentrifuge tube and incubated for 60 s at room temperature. This was then centrifuged at 6,260 g for 60 s to elute the DNA. Pure DNA was then ready for use as a template for PCR.

2.3.8.3. GES extraction of DNA from powdered food isolates

A 37 °C overnight culture of each organism was obtained in BHI broth to obtain cultures with between 10^{8} and 10^{9} cfu/ml. 1.5 ml of this was placed in a 1.5 ml microcentrifuge tube and centrifuged at 10,580 g for 60 s. The supernatant was removed and the step above repeated until a clearly visible cell pellet was formed. The pellet was then resuspended in 1 ml of lysis buffer (25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 50 mM sucrose). To aid lysis of Gram-positive cells 50 mg/ml of lysozyme was added to the lysis buffer and the samples incubated for 30 min at 37 ^{o}C. GES solution (5 M guanidine thiocyanate,

0.1 mM EDTA, 0.5 % sucrose; 500 µl) was then added. This solution was then mixed well and incubated at room temperature for 5 min before cooling on ice for 2 min. To this 250 µl of ice cold 7.5 M ammonium acetate was added then mixed by vortex before incubating on ice for 10 min. After incubation, the solution was centrifuged for 10 min at 10,580 g and 850 µl removed from the upper phase and placed in a new microcentrifuge tube. Exactly 0.54 volumes of cold isopropanol (459 µl) was added to the solution. This was then mixed for 60 s, centrifuged for 20 s at 10,580 g and the supernatant removed. The pellet was then washed three times in 70 % ethanol, and air dried in a 37 ^{o}C incubator. After drying, the pellet was resuspended in 50 µl of TE buffer.

2.3.8.4. DNEasy DNA extraction kit

Overnight cultures of isolates were grown in BHI broth to approximately 10^{8}-10^{9} cfu/ml. One and a half millilitre of this was placed in a microcentrifuge tube and centrifuged for 60 s at 10,580 g. The supernatant was then removed and discarded. The pellet was suspended in 180 µl lysis buffer (25 mM Tris-HCl (pH 8.0), 10 mM EDTA, 50 mM sucrose). Lysozyme was then added at a concentration of 20mg/ml and incubated for 30 min at 37 ^{o}C. Fifty milligrams per millilitre was added for the *S. aureus* strains.

After the incubation 25 µl of proteinase K and 200 µl of buffer AL (lysis buffer, no composition given) was added and this was mixed by vortexing. After mixing the tube was then incubated at 95 ^{o}C for 15 min. 200 µl of ethanol was then added to the solution and mixed again by vortexing.

The DNA was then purified from the sample. This was carried out by pipetting the solution into a DNeasy mini spin column. The spin column was then placed in a 2 ml collection tube. This was then centrifuged at 4,007 g for 60 s. The flow through and collection tube were then discarded. The column was then placed in a new collection tube and 500 µl of buffer AW1 (composition not given) was then

added to the spin column. This was centrifuged for 60s at 4,007 g. The flow through and collection tube were then again discarded. The DNeasy spin column was placed in a new collection tube and buffer AW2 (composition not given) was then added on top of the spin column. To dry the spin column membrane, the spin column was then centrifuged at 12,271 g for 3 min. The flow through and collection tube were again discarded.

The spin column was placed in a new microcentrifuge tube. 200 µl of buffer AE (composition not given) was the placed on the spin column membrane. This was incubated for 60 s at room temperature. After incubation the sample was centrifuged for 60 s at 4,007 g to elute the DNA. This was then repeated with 100 µl of buffer AE instead of 200 µl. Purified DNA was left in the microcentrifuge tube, and could be used as template for the PCR reactions (Section *2*.4)*,.x-x).*

2.3.8.5. Nano-Drop to determine the concentration of primers

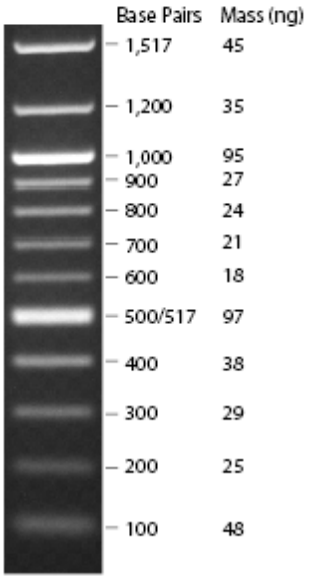
The Nano-drop machine was programmed to read 'nucleic acids.' 1 µl of RO water was placed on the reading platform to blank the Nano-drop. The platform was the wiped clean and 1 µl of each reconstituted primer was placed on the reading platform (see primer list, Section 2.4.2) The concentration on the machine was then noted.

2.4. MOLECULAR IDENTIFICATION METHODS

2.4.1. DNA Molecular Weight Marker

Five micro-litre of 100 bp DNA ladder (BioLabs Inc.) was placed in a well on the gel. The ladder ranges from 100 to 1,500 base pairs. The ladder consists of eleven fragments that range in size from 100–1,000bp in 100bp increments (see Figure 2.9), plus an additional fragment at 1,500bp. The 500bp fragment is present at increased intensity to allow easy identification. A Blue Loading Dye is provided to visualise the migration.

Figure 2.9: 100 bp DNA ladder with each band size



This image shows the bands that form on a 2% agarose gel after 1 h of separation with the bands and 1000 and 500 bp at higher intensities to aid in identifying the size of bands.

2.4.2. Primers

Table 2.1: Primers used in each PCR reaction.

Organism	Primer Name	Sequence (5'---> 3')	Gene Name
Eubacteria	UNI-F	TTAGTGGCGGACGGGTGA	16s rRNA
	UNI-R	GGTATCTAATCCTGTTTGCTC	16s rRNA
B. cereus	B-K1-F	TCACCAAGGCRACGATGCG	16s rRNA
	B-K1-R1	CGTATTCACCGCGGCATG	16s rRNA
C. perfringens	CPA-F	GCTAATGTTACTGCCGTTGA	Plasmid *cpA* DNA
	CPA-R	CCTCTGATACATCGTGTAAG	Plasmid *cpA* DNA
L. monocytogenes	MONO-5-F	GCTAATACCGAATGATAAGA	16s rRNA
	MONO7-Fa	GGCTAATACCGAATGATGAA	16s rRNA
***Listeria* Genus**	LIS-R	AAGCAGTTACTCTTATCCT	16s rRNA
	IVA-F	AGCTTGCTCTTCCAATGT	16s rRNA
	MG-F	GCTTGCTCCTTTGGTCG	16s rRNA
	LIS-F	AGCTTGCTCTTCCAAAGT	16s rRNA
S. aureus	SA-442-F	AATCTTTGTCGGTACACGATATTCTTCACG	*
	SA-R	CGTAATGAGATTTCAGTAGATAATACAACA	*
Staph Toxins	ST-U	TGTATGTATGGAGGTGTAAC	*
	ST-R	TCTTGAACDGTHACHHTTTTYTT	*

* Undefined chromocomal sequence identified by hybridisation (Martineau *et al.,* 1998).

2.4.3. Universal Eubacteria PCR

This was achieved by using 1 µl of two universal primers; UNI-F and UNI-R (see Table 2.1). These were placed in separate 25 µl PCR tubes with 0.1 µl of 10x buffer (BioLabs), 0.1 µl of 0.2 mM mixture of dNTPS (containing dATP, dGTP, dCTP and dDTP) and 1.5 µl of 1.5 mM $MgCl_2$. sterile RO water (9 µl) was then added to the tube. As a template 10 µl of DNA (section 2.3.8) was then added to the tubes and finally 0.1 µl of Taq DNA polymerase was then added to the PCR mixture. The tubes were then pulse centrifuged to bring contents to the bottom of the tube before being placed in a thermo-cycler. The parameters for the thermo-cycler was then set at an initial denaturing step of 95 ^{o}C for 5 min. 25 cycles of; 95 ^{o}C for 45 s, 55 ^{o}C for 45 s and then 72 ^{o}C for 45 s. This was followed by a final extension step of 72 ^{o}C for 5 min. PCR products were resolved on a 2 % agarose gel containing 5 % (v/v) ethidium bromide. 2 µl of 5x loading dye (25 mg bromophenol blue, 4 g of sucrose and H_2O to 10ml) was added to each PCR product and 15 µl of each dyed PCR product was placed in separate wells on the 2% gel and separated using 70 V for 1 h. The gels were then visualised under UV light. The expected product size for Eubacteria was 700 bp.

2.4.4. Molecular Identification of *Bacillus cereus*

A PCR method combined with amplified ribosomal DNA restriction analysis (ARDRA) was used to identify *Bacillus cereus* from the presumptive positive colonies grown on MYP agar. Wu *et al.* (2006) described a reaction of; 10 µl Extracted DNA (section 2.3.8) was used in a 25 µl PCR reaction volume. The mixture consisted of 0.8 mM of dNTPS, 1x Buffer solution (see Section 2.4.3), 1.5 mM MgCl2, 1 µM of each primer; B-K1/F and B-K1/R (see Table 2.1, primer list) and 1 µl of Taq DNA polymerase. The mixture was then pulse centrifuged and placed in a thermo-cycler. The PCR parameters consisted of a denaturing

step of 94 °C for 3 min, followed by; 25 cycles of 94 °C for 30 s, 63 °C for 30 s and 72 °C for 2 min, and a final extension step of 72 °C for 10 min.

The results from both sets of PCR reactions were then visualised on a 1.5 % agarose gel with 5% (v/v) ethidium bromide. Two micro-litres of loading dye was added to each of the PCR reaction tubes and 15 µl of each PCR product was then placed in separate wells on the agarose gel separated at 70 V for 1 h. The band sizes were estimated by comparing with the migration of the 100 bp ladder (Section 2.4.1).

Using the method described in Section 2.3.8, the DNA was purified from bands formed in the gel. 5 µl of the purified DNA was then digested with *Alu*I and *Taq*I separately in a 20 µl reaction volume containing 2 µl 10 x buffer (supplier) and 2 units of enzyme for 4 h at room temperature. The restriction digests, along with 100 bp DNA ladder, was then visualised on a 2 % agarose gel with 5% (v/v) ethidium bromide using electrophoresis for 1 h at 70 V. The bands were then visualised under UV light.

2.4.5. *Clostridium perfringens* Molecular Identification

The method for identifying *Clostridium perfringens* was adapted from a protocol developed by Songer and Bueschel (1999). A 25 µl reaction volume consisted of 10x buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, and 0.01% (w/v) gelatine), 25 mM $MgCl_2$, 0.12 mM dNTPs and 0.5 mM of each *cpa* primer CPA-F and CPA-R; (see Table 2.1). Sterile RO water was then added to 25 µl and then 0.5 µl of Taq DNA polymerase was added. The reaction solution was then pulse centrifuged and placed in a thermo cycler. The PCR parameters were as follows; an initial denaturing step of 95 °C for 5 min, then 35 cycles at; 94 °C for 60 s, 55 °C for 60 s and 72 °C for 60 s and a final extension step of 72 °C for 7 min.

The PCR products were then separated on a 1.5 % agarose gel with 5% (v/v) of ethidium bromide. Two micro-litres of loading dye was added to each of the PCR reaction tubes and then 15 µl of each PCR reaction was then placed in separate wells on the agarose gel. The DNA from the PCR reaction was then separated at 70 V for 1 h. The bands were then visualised under UV light and their size estimated by comparing with the migration of the 100 bp ladder (Section 2.4.1.). The expected bands size for the *C perfringens cpa* gene was 324 bp.

2.4.6. PCR to Identify *Listeria monocytogenes*

The molecular identification of *L. monocytogenes* was carried out using a method described by Somer and Kashi (2003). The DNA was extracted from the colonies that formed on PALCAM agar using methods described in Section 2.3.8. The DNA used was that. A 25 µl multiplex PCR reaction was prepared containing 0.1 µl of 10x buffer, 0.1 µl of 0.2 mM mixture of dNTPS (containing dATP, dGTP, dCTP and dDTP) and 1.5 µl of 1.5 mM $MgCl_2$ in a thin walled microcentrifuge tube. Each primer used in this reaction was then reconstituted to 100 pmol (see Section 2.4.2 for primer list). 0.5 µl of primers IVA-F, MG-F and LIS-F, 1µl of MONO-5 and MONO-7 and 1.5 µl of LIS-R was then added to the tube (see Table 2.1) followed by 6 µl of sterile RO water. Ten micro-litres of extracted DNA (Section 2.3.8) and 0.1 µl of Taq DNA polymerase was then added to the PCR mixture. The tubes were then pulse centrifuged before being placed in a thermo-cycler. The parameters for the PCR reaction were initial annealing temperature of 95 ^{o}C for 5 min. This was followed by five cycles of; 95 ^{o}C for 45 s, 53 ^{o}C for 45 s and then 72 ^{o}C for 45 s. This then followed by 30 cycles of; 95 ^{o}C for 45 s, 58 ^{o}C for 45 s and then 72 ^{o}C for 45 s. There was then a final extension step at 72 ^{o}C for 7 min.

The PCR products were visualised on a 2 % agarose gel. Two micro-litres of loading dye was placed in each PCR and 15 µl of each dyed PCR product was then placed into separate wells of the agarose gel containing 5% (v/v) of ethidium bromide. The products were separated using 70 V for 1 h and the bands visualised under UV light. The expected band sizes were 400 bp for members of the *Listeria* genus and two bands of 400 bp and 287 bp for *L. monocytogenes.*

2.4.7. Molecular Identification of *Staphylococcus aureus* and its Toxins

S. aureus was identified using a PCR method based on that described by Martineau *et al.* (1998). The 25 µl reaction volume consisted on 10 µl of extracted DNA (Section 2.3.8) from organisms that had grown on BP agar. One micro-litre of 50 mM KCl, 2 µl of Tris-HCl (pH 9.0), 2 µl of 0.1% Triton X-100, 2µl of 2.5 mM $MgCl_2$ and 1 µl of each *S. aureus* primers; ST-U and ST-P (see Section 2.4.2, Table 2.1) was added to this. To this 0.1 µl of dNTPs, followed by 0.5 µl of Taq DNA polymerase was added. The volume was then made up to 25 µl with sterile RO water. The solution was then pulse centrifuged before being placed in the thermo-cycler. The parameters for the PCR reaction were; 3 min at 96 ^{o}C to denature the DNA, followed by 40 cycles of 95 ^{o}C for 1 s and 55 ^{o}C for 30s. Then there was a final extension of 72 ^{o}C for 5 min.

The isolates were then tested for all of the known *S. aureus* toxins using a method described by Letertre *et al.* (2003). A 25 µl reaction volume was set up, consisting of; 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 50 mM $(NH_4)_2SO_4$, 4 mM $MgCl_2$, 400 µM of dNTPs and 0.5 µl of Taq DNA polymerase. 1 µl of each toxin primers; ST-U and ST-R (see Section 2.4.2, Table 2.1) and 10 µl of purified DNA (section 2.3.8) was added to the reaction tube and then sterile RO water was added to bring the volume up to 25 µl. The solution was then pulse centrifuged and placed in a thermo-cycler. The parameters for the PCR reaction were as

follows; 94 ^{o}C for 10 min as a denaturing step. This was followed by 35 cycles of; 30 s at 94 ^{o}C, 30 s at 45 ^{o}C and 30 s at 72 ^{o}C and a final extension step of 72 ^{o}C for 7 min.

The PCR products from reactions were then visualised on a 2 % agarose gel with 5% (v/v) of ethidium bromide. 2 µl of loading dye was added to each of the PCR reaction tube and then 15 µl of each PCR reaction was then placed in separate wells on the agarose gel. The DNA from the PCR reaction was then separated at 70 V for 1 h. The bands were then visualised under UV light and band sizes were estimated by comparison with the migration of the 100bp ladder (see Section 2.4.1). The expected band sizes were 108 bp for the *S. aureus* identification PCR and 140 bp for detection of the *S. aureus* toxin genes.

CHAPTER 3

SAMPLING POWDERED FOODS FOR GRAM-POSITIVE PATHOGENS

3.1. INTRODUCTION

Powdered foods are used for a wide variety of reasons and can be aimed at a range of individuals. The focus of the project is to determine whether these powdered food products that are aimed at those who may be immunocompromised are safe enough eat.

Three areas of focus are infant formula milk (IFM) for young children aged between nought to six months, protein based drinks used by individuals during sports (SD) and weight-gain products that are aimed primarily at the elderly (EBU), but may be used by individuals who have lost weight, possibly through serious illness, but may still be immunocompromised. All the people that use these powdered products may be in some way immunocompromised. Babies have a lower immunity if they are not being breast fed, as there is no passive immunity being transferred to the new born baby, which is well-known to give protection from infection (Hanson, 1998). Individuals undertaking sports whilst taking protein-based drinks may be at higher risk. This is because exercise at high intensities has been reported to be responsible for serum immunoglobulin levels to be depressed for up to 2 days after exercise (Niemen and Nehlsen-Cannarella, 1991). The elderly are potentially at risk because as people get older their immune system begins to decline (Ginaldi, *et al.,* 2007), leaving them vulnerable to pathogens that would not normally be a problem for healthy adults.

Forty-eight products were tested (17 IFM, 19 SD and 12 EBU) for the presence of four Gram-positive food borne pathogens, known to be able to survive in harsh environments. Using corresponding ISO methods (Section 2.2) each product was tested for *Bacillus cereus, Clostridium perfringens, Listeria monocytogenes* and *Staphylococcus aureus.* Viable counts were made to determine the extent of contamination (if any). Postulations, depending on which

organism is involved, can then be made to determine how the foods became contaminated.

3.2. RESULTS

3.2.1. Preliminary Results

3.2.1.1. Detection of Gram-positive organisms in powdered milk

The aim of these experiments was to determine whether the bacteria could be isolated from the powdered foods using the appropriate ISO isolation methods (see section 2.2). A 25 g sample of store-bought powdered skimmed milk was reconstituted in 225 ml of buffered peptone water (BPW) and separate 10 ml aliquots of the samples were inoculated with *Listeria monocytogenes, Staphylococcus aureus* and *Bacillus cereus* to approximately 10^4 cfu/ml from overnight cultures of laboratory strains of all of these bacteria (section 2.3). Cultures of *Clostridium perfringens* were not used as it requires Home Office permission to culture this organism and therefore its isolation was not tested using spiked samples. After processing using the recommended ISO method for each bacterium, a series of 10-fold dilutions were carried out (section 2.3) and the samples were then spread on to each of the corresponding diagnostic media. For the isolation of *Bacillus cereus,* mannitol egg yolk polymyxin (MYP) agar was used (section 2.2.1), for *Staphylococcus aureus* Baird Parker (BP) agar (setion 2.2.4) and for *Listeria monocytogenes* PALCAM agar incubated under microaerophilic conditions was used (section 2.2.3). Samples were also plated onto plate count agar for total aerobic count. A negative control of a sample of milk that was not inoculated with any of the test cultures was also plated onto each of the diagnostic agars as well as onto the plate count agar.

After the different incubation periods (see sections 2.2 -2.4) the results showed that each organism had grown on their respective diagnostic agars

(identification in this case was based on presumptive colony morphology alone). However the number of colonies that grew on the different diagnostic media did not correspond to the amount spiked into the milk. This may have been due to competition from all of the spiked bacteria possible out competing in the milk or the milk not being the most suitable environment for growth and therefore some cells may not have survived. The negative control sample plates had no growth on either the diagnostic or the plate count agars showing that any organisms detected were those added to the samples. After incubation the plate count agar had too many colonies to count but showed that the number of organism present in each sample had increased. From these results it can be seen that these organisms can be detected from the powdered milk products, therefore the ISO standard protocols do not have to be adapted to be able to isolate these Gram-positive bacteria. Thus the recovery of all three Gram-positive bacteria was possible. It was assumed that this would also be the case for *Clostridium perfringens*.

3.2.1.2. The effect of sterile tap water on bacteria recovery

In the initial experiment the powdered milk was reconstituted with BPW as recommended by the ISO method. However for consumption at home these foods would normally be reconstituted using tap water at 1g/ml as recommended by the manufacturer. Since tap water is not isotonic with the bacterial cells, the effect of using this to reconstitute the milk was tested. Two samples of the powdered milk were prepared, one reconstituted with BPW and one using sterile tap water both at concentrations of 1 g/ml as recommended by the manufacturer. Ten-fold dilutions were then prepared and the number of organisms enumerated on plate count agar. The results show (Table 3.1) that there was no significant difference (after using a Paired T-test as the numbers

were parametric (median and mean are close) and the sample size was low) between using BPW and tap water when cells were immediately recovered.

3.2.1.3. BPW compared to tap water in cold recovery of organisms

Since the bacteria in the samples could naturally be in the reconstituted milk for longer periods of time, it was decided to investigate the effect of these two different solvents on the recovery of bacteria over a longer period. Therefore a further experiment was performed to investigate the effect of refrigerated storage on recovery. To do this, the samples prepared from the previous experiment were placed in the refrigerator that was set at 4^{o}C for five days and seven days. After each incubation period the samples were removed and viable counts were enumerated on PCA as described in section 2.3. The results (Table 3.2) showed that there was a significant increase in the viable count in the milk after 5 d and 7 d, indicating that growth was possible. However there was no significant difference (using One-way analysis of variance carried out as the mean and median are close enough to consider parametric) between the recovery of cells from the samples when the two different diluents were used. Therefore, even though for consumption the organisms would normally be present in milk reconstituted in water, isolation of each organism using the ISO method would be representative of the numbers of organisms that are likely to survive in the home environment. This was important to establish as one aim of this project was to establish the microbial risk of powdered foods to the general public. If by using BPW recovery of organisms occurred that would normally not survive in the food product that risk would be over estimated.

Table 3.1: Comparison of viable counts from samples reconstituted in tap water and BPW

	Number of Colonies					
Dilution	**Tap Water**			**Mean**	**Median**	**SD**
10^0	5	9	6	6.67	6	2.08
10^-1	0	1	0	0.33	0	0.58
10^-2	0	0	0	0	0	0
	Number of Colonies					
Dilution	**Buffered Peptone Water**			**Mean**	**Median**	**SD**
10^0	4	1	3	2.67	3	1.53
10^-1	0	0	0	0	0	0
10^-2	0	0	0	0	0	0

Paired t test	
P value	0.1946
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.922 df=2
Number of pairs	3

Tap water compared to buffered peptone water to show if there was a significant difference in recovery.

Table 3.2: Effect of cold recovery on viable counts of bacteria from samples reconstituted in sterile tap water and BPW

	Number of Colonies (5d)					
Dilution	**Tap Water**			**Mean**	**Median**	**SD**
10^0	12	17	6	11.67	12	5.51
10^-1	2	0	0	0.67	0	1.15
10^-2	0	0	0	0.00	0	0.00
	Number of Colonies (5d)					
Dilution	**Buffered Peptone Water**			**Mean**	**Median**	**SD**
10^0	21	22	11	18.00	21	6.08
10^-1	1	0	2	1.00	1	1.00
10^-2	0	0	0	0.00	0	0.00
	Number of Colonies (7d)					
Dilution	**Tap Water**			**Mean**	**Median**	**SD**
10^0	44	81	29	51.33	44	26.76
10^-1	18	11	6	11.67	11	6.03
10^-2	1	0	0	0.33	0	0.58
	Number of Colonies (7d)					
Dilution	**Buffered Peptone Water**			**Mean**	**Median**	**SD**
10^0	67	35	51	51.00	51	16.00
10^-1	0	0	0	0.00	0	0.00
10^-2	1	0	0	0.33	0	0.58

One-way analysis of variance			
P value	0.0283		
P value summary	*		
Are means signif. different? (P < 0.05)	Yes		
Tukey's Multiple Comparison Test	**Mean Diff.**	**q**	**Significant? P < 0.05?**
5d- Tap Water vs 5d- BPW	-6.333	0.6804	No
7d-Tap Water vs 7d-BPW	0.3333	0.03581	No

Tap water compared to BPW in the recovery of organisms after refrigeration. The results show there is no significant different on the same day, but there is a significant difference in growth between the two days.

3.2.2. Testing Powdered Foods for Contamination

For this survey 17 different IFM, 19 SD and 12 EBU powdered products were examined for contamination. As outlined in the ISO methods (see Chapter 2 and below) 25 g of each powdered product was reconstituted with 225 ml of BPW. In addition to the selective isolation procedures, 0.1 ml of each reconstituted powdered food was spread plated onto PCA plates to determine the initial aerobic count of each product. The results (Appendices 3.1 to 3.3) show that generally there was very little or no growth after direct plating onto PCA from most of the powdered food products. However one infant formula milk, B-HF, produced 330 cfu/ml, and some of the elderly build up products, (namely NV, NS and NC), yielded colonies from 100 to 700 cfu/ml, or too many colonies to count ($>3x10^7$ cfu/ml). This suggests that not all the products were manufactured to the same hygienic levels.

3.2.2.1 *Isolation of Presumptive B. cereus*

Each powdered food product was reconstituted in buffered peptone water and 0.1 ml samples were spread onto the surface of MYP agar. This was then incubated for 24 h at 37 ^{o}C. After incubation growth on the plates was examined and presumptive *B. cereus* isolates were identified (see section 2.1 and figure 2.1). As manufacturing regulations are not clear and the number of permitted organisms in IFM products aimed at 0-6 month old babies is not defined, when presumptive *Bacillus cereus* were detected in infant formula milk, only growth or no growth results were recorded instead of colony numbers (Table 3.3). Three IFM products (B-AH, B-HPF and B-HPS) yielded colonies which were characteristic pink in colour with a zone of precipitate.

For the EBU powder samples, colony numbers were recorded. All 12 of these products yielded colonies, however only six out of the 12 samples gave colonies with the characteristics of *B. cereus*; these were E-NS, E-NV, E-NC, E-CV, E-CS and E-CO. From the SD products, 11 samples yielded colonies, nine of which were characteristic for *B. cereus*. These products were: S-MMX, S-MMP, S-MEP, S-WP, S-LSP, S-SPP, S-PSP, S-HPC and S-PV. From these data the viable count values were calculated for the elderly build up and sports powders (see Tables 4 and 5).

Table 3.4 shows that there are generally low numbers of cells found in each of the sports products. However, S-MMX and S-MMP both yielded extremely high counts, much larger than the higher limit of 10^4 cfu/ml recommended by Warburton *et al.* (1998). It should be noted that in these cases, where the microbial load was higher than anticipated, the colonies formed were difficult to count and the actual numbers should be seen as a very approximate, but the viable count remain representative of the actual number of microbes present.

Table 3.5 shows how almost every elderly food product that yielded presumptive *B. cereus* organisms also had a very high cfu/ml value. This has a serious implication as each product that had over 10^4 cfu/ml of presumptive *B. cereus* present is in serious breach of guidelines used by the food industry for safe manufacture of these types of foods (Warburton *et al.,* 1998).

Tables 3.3: Presence of Gram positive pathogens in IFM samples

	Bacillus cereus		***Clostridium perfringens***		***Listeria monocytogenes***		***Staphylococcus aureus***	
Product/ Dilution	10^{0}	10^{-1}	10^{0}	10^{-1}	10^{0}	10^{-1}	10^{0}	10^{-1}
B-AD	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
B-AH	**+ve**	-ve	-ve	-ve	-ve	-ve	-ve	-ve
B-AF	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
B-HH	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
B-HN	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
B-W	-ve	-ve	-ve	-ve	-ve	-ve	**+ve**	-ve
B-SH	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
B-S	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
B-SF	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
B-SLF	-ve	-ve	-ve	-ve	**+ve**	**+ve**	**+ve**	-ve
B-HPF	**+ve**	-ve	-ve	-ve	**+ve**	-ve	-ve	-ve
B-HPS	**+ve**	-ve	-ve	-ve	-ve	-ve	**+ve**	-ve
B-CF	-ve	-ve	**+ve**	**+ve**	-ve	-ve	-ve	-ve
B-CH	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
B-CC	-ve	-ve	-ve	-ve	-ve	-ve	**+ve**	-ve
B-CI	-ve	-ve	-ve	-ve	**+ve**	**+ve**	-ve	-ve
B-E	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

Characteristic organisms that have grown from Infant formula milks onto diagnostic agar, each bold is a positive in each given dilution.

Table 3.4: Viable count of presumptive *B. cereus* (cfu/ml) **from reconstituted SD**

Product Identifier	**cfu/ml**
S-LSP	3.00E+02
S-MEP	4.00E+02
S-WP	1.00E+02
S-SPP	3.60E+02
S-PSP	2.00E+02
S-BFW	6.30E+02
S-HPC	1.00E+02
S-PV	4.60E+02
S-BSN	2.00E+02
S-MMP	>1.00E+05
S-MMX	>1.00E+05

Organisms that formed characteristic colonies on MYP agar (pink colonies) cfu/ml were counted as presumptive *B. cereus*.

Table 3.5: Viable count of presumptive *B. cereus* from EBU products

Product Identifier	cfu/ml
E-SAN	4.13E+04
E-C	>1.00E+05
E-CC	>1.00E+05
E-COC	9.60E+02
E-CO	TMTC
E-CV	1.92E+06
E-CS	5.13E+05
E-COF	1.01E+04
E-NC	>1.00E+05
E-NV	>1.00E+05
E-NS	>1.00E+05
E-BC	>1.00E+05

Organisms that formed characteristic colonies on MYP agar (pink colonies) were considered as presumptive *B. cereus.*

3.2.2.2. Isolation of Presumptive Clostridium perfringens

After each product was reconstituted in BPW, 1 ml of each sample was poured into a sterile dish and mixed with cooled TSC agar. After the top layer had been applied and set, the plates were incubated under anaerobic conditions. After incubation the presumptive positive colonies from the infant formula milk were identified (black, with or without a zone of precipitation. See section 2.2.2).

As described in section 3.2.2.1, only presence/absence results were recorded for Infant formula milk samples. From these products only two samples yielded colonies, B-CF AND B-HPS (see Table 3.3). However only B-CF produced black colonies that did not have a zone of precipitation. B-HPS formed white colonies, which are not characteristic for *C. perfringens.* Therefore only further confirmatory tests were carried out on the isolates from product B-CC.

For the sports drinks the viable count from the triplicate TSC agar plates were calculated. Appendix 3.4 shows that five of the sports drinks produced colonies on the agar, however only three of these (S-LSP, S-MMX and S-MMP) formed characteristic black colonies, with or without a zone of precipitation. Table 3.6 shows the cfu/ml recorded for these products and that all of them were above the recommended viable count of 10^2 cfu/ml that are allowed in these powdered foods (Warburton, *et al.,* 1998).

From the elderly build up powders, three products yielded characteristic *C. perfringens* colonies. These products were E-NS, E-NC and E-BC. Table 3.7 shows the numbers of cells detected (cfu/ml) for each of these reconstituted products. Both E-NS and E-NC were above the accepted level of contamination for these foods of 10^2 cfu/ml (Warburton *et al.*1998). For E-BC the colony numbers were too low to count accurately (below 3-300) and therefore were recorded as being below the 10^2 acceptable threshold.

Table 3.6: Presumptive *Clostridium perfringens* counts (cfu/ml) **from reconstituted SD**

Product Identifier	cfu/ml
S-LSP	1.17E+02
S-MMX	9.55E+04
S-MMP	9.55E+05

Organisms that formed characteristic colonies on TSC agar (black colonies formed with or without a zone of precipitation) were considered as presumptive *C. perfringens* colonies and counted.

Table 3.7: Presumptive *Clostridium perfringens* counts (cfu/ml) **from reconstituted EBU products**

Product Identifier	cfu/ml
E-NC	1.12E+03
E-NS	2.22E+04
E-BC	>2.00E+02

Organisms that formed characteristic colonies on TSC agar (black colonies formed, with or without a zone of precipitation) were counted as presumptive *C. perfringens* colonies in elderly build-up powders.

3.2.2.3. Isolation of Presumptive Listeria monocytogenes

For the IFM products, it was anticipated that the numbers of *L. monocytogenes* present would be very low and so the isolation of *L. monocytogenes* was carried out following enrichment (see Section 2.3). After enrichment, samples were plated onto PALCAM agar for isolation of presumptive *L. monocytogenes*. Black or dark green colonies that formed on the agar with a black 'halo' were considered to be presumptive *L. monocytogenes.* Presence/absence results were then recorded.

Table 3.3 shows that three infant formula milks tested positive for presumptive *L. monocytogenes.* These products were: B-SLF, B-HPF and B-CI. Two other products, B-SD and B-SH, also produced colonies on this agar but these had atypical colonies morphologies and were discarded as probable *Bacillus spp.* organisms.

For sports and elderly build-up products direct plating onto PALCAM agar was carried out rather than an enrichment allowing estimation of presumptive *L. monocytogenes* viable counts. From the sports products, six out of 19 yielded black or dark green presumptive *L. monocytogenes* colonies, these products were: S-MEP, S-SPP, S-BFW, S-MMX, S-MMP and S-BSN. Table 3.8 shows that the counts from these products are quite high, especially from S-MEP, S-MMX and S-MMP where there was over 10^4 cfu/ml detected in the sample.

From the elderly build up products, four produced characteristic colonies on PALCAM agar. These products were E-CS, E-NC, E-CV and E-NS. However compared to the sports drinks, the numbers were generally lower (Table 3.9). However E-NC had levels towards the upper limit of acceptable contamination and so could still pose a threat after human consumption, especially if the product was stored for any length of time after rehydration before consumption.

Table 3.8: Viable counts (cfu/ml) **of presumptive *L. monocytogenes* from reconstituted SD**

Product	cfu/ml
S-MEP	1.24E+04
S-SPP	4.00E+02
S-BFW	9.47E+02
S-BSN	3.53E+02
S-MMX	1.03E+05
S-MMP	2.24E+04

Organisms that formed characteristic colonies on PALCAM agar (formed black or dark green colonies with a black 'halo') were counted as presumptive *L. monocytogenes* colonies detected in sports powders.

Table 3.9: Viable counts (cfu/ml) **of presumptive *L. monocytogenes* from reconstituted EBU products**

Product	cfu/ml
E-CV	4.60E+01
E-CS	6.06E+03
E-NC	5.00E+01
E-NS	1.26E+02

Organisms that formed characteristic colonies on PALCAM agar (formed black or dark green colonies with a black 'halo') were counted as presumptive *L. monocytogenes* colonies detected in EBU products.

3.2.2.4. Isolation of Presumptive Staphylococcus aureus

After the reconstituted products had been spread onto BP agar and incubated, colonies were identified as being presumptive *S. aureus* if they were the characteristic round, black, shiny convex colonies that are surrounded by a 'halo.' It must be noted that colonies that do not have the halo may still be *S. aureus,* but are coagulase negative, so these may also considered presumptive positive isolates.

With infant formula milk, again only a presence/absence result was recorded (see Table 3.3) as described in section 3.2.2.1. Products B-W, B-SLF, S-HPS and S-CC all produced colonies indicative of both coagulase-positive and coagulase-negative *S. aureus.* Grey and irregular colonies that formed on the BP agar were dismissed as representing contaminating *Bacillus ssp.*

Number of characteristic presumptive *S. aureus* (cfu/ml) colonies from sports and elderly build-up foods was determined. Seven sports powders produced characteristic *S. aureus* colonies (Table 3.10). These were S-LSP, S-WP, S-PSP, S-BFW, S-PV, S-MMX and S-MMP. Both S-MMX and S-MMP were above the recommend levels of 10^4 cfu/ml for these foods stated by Warburton, *et al.* (1998), and of particular note was product S-MMX where a count greater than 10^6 cfu/ml was detected of the presumptive colonies.

Nine elderly build-up samples produced colonies on the BP plates and six of these formed characteristic colonies for *S. aureus*. These products were E-CC, E-CV, E-CS, E-SAN, E-NV and E-NS. Table 3.11 shows the viable counts recorded for each of these reconstituted products, and E-CC and E-NV formed large numbers of colonies that were above the upper limit of allowed organisms recommended by Warburton *et al.* (1998).

Table 3.10: Viable counts (cfu/ml) **of presumptive *S. aureus* from reconstituted SD products**

Product	cfu/ml
S-LSP	1.03E+02
S-WP	2.33E+01
S-PSP	1.17E+02
S-BFW	2.80E+02
S-PV	5.67E+01
S-MMX	1.51E+06
S-MMP	4.53E+04

Organisms that formed characteristic colonies on BP agar (formed black round shiny convex colonies, with or with a 'halo') were counted as *S. aureus* colonies detected in SD products.

Table 3.11: Viable counts (cfu/ml) **of presumptive *S. aureus* from reconstituted EBU products**

Product	cfu/ml
E-SAN	7.60E+01
E-CC	1.92E+06
E-CV	3.00E+01
E-CS	3.56E+02
E-NV	1.83E+04
E-NS	4.66E+03

Organisms that formed characteristic colonies on BP agar (formed black round shiny convex colonies, with or with a 'halo') were counted as *S. aureus* colonies detected in EBU products.

3.2.3. Analysis of Type of Powdered Food and Patterns of Contamination

Each product was divided up into milk or non-milk based on the basis of their ingredients (see Appendix 3.5). From this survey there were 36 products that were based on milk, either having milk or proteins derived from milk in them, and 12 products that did not have milk at all. Thirty-six percent of all the milk-based products and 33 % of the non-milk based products were found to be contaminated with any one of the four Gram-positive organisms under investigation (see Appendix 3.6). From these results, there was seemingly no difference between the two sets of results for the different product types, suggesting that the source of the powdered food does not have an impact on the contamination. Analysing the data for the types of organisms found in either the milk or non-milk based foods (Appendix3.6) showed that there was no discernable difference in the likelihood of particular organisms being associated with a particular product type, which suggests that the products that have milk in them do not have an increased chance of contamination with some types of organisms.

3.3. DISCUSSION

3.3.1. *Bacillus cereus* in Powdered Foods

As a spore forming organism, the presence of *Bacillus cereus* in these powdered foods is not unexpected. The spores can survive pasteurization in the dairy plant and *B. cereus* can thus be present in the milk (Svensson, *et al.,* 2006). The spores also have the ability to adhere to, and germinate on, dairy equipment, e.g., silo tanks (Svensson, *et al.,* 2004). Therefore the ability for these organisms to contaminate the powdered food is not uncommon. The milk-based products are particularly at risk as raw milk is readily contaminated through cows eating grass or straw contaminated from the soil or in faeces. From this study 36 % of the milk based products tested contained presumptive *Bacillus cereus* colonies. However this compares well to Becker *et al.* (1994), where they found the incidence of *Bacillus cereus* in milk-based foods to be 54 %. They also found in Germany that in 1992, 70 % of their infant formulae tested, was contaminated with *B. cereus,* whereas only 18 % of the infant formula milk tested in the current study was contaminated with presumptive *Bacillus cereus.* This suggests that the practices at the farm during milking or in food processing factories are improving as the amount of contamination in this type of sample is decreasing. However the sampling range and the region where the products were collected for this study is very small and may not be indicative of the overall contamination of these infant formula milks.

The safety measures applied to treating the milk into becoming safe to eat actually can be the trigger in *Bacillus cereus* contaminating the milk products. The initial heat treatment step applied in the production of dried milk is very important for the activation and germination of *B. cereus* spores. Whereas raw milk does not support the germination of spores a high temperature short time treatment renders the milk a good germination medium (Wilkinson and Davies,

1973). However when the products is then spray dried and the water content would be too low for germination, and germinated cells would be far more susceptible to desiccation. Thus cells that may form spores again during spray drying possibly enabling them to be primed after further treatment to germinate once the product is reconstituted.

Non-milk based foods in this study have also been seen to be contaminated with this organism. Even though these foods may not be traced back to raw milk, as an ubiquitous organism, it can be found on range of products, that may have been used in making the non-milk based foods. The drying process can involve a lot of equipment and can be difficult to clean depending on which methods for drying is used. A quarter of the non-milk based foods tested in this project were found to be positive for presumptive *Bacillus cereus* (Appendix3.6). This compares with Becker *et al.* (1994), where they found over half of their non-milk based products tested positive for *Bacillus cereus.* This again may suggest that there are beginning to be improvements in the way these foods are processed, however again it must be mentioned how small the sample size was and the region they were tested is small when compared to Becker *et al.* (1994) where samples for their study came from sources worldwide. This could be of some importance, as the majority of the products used in this study came from the UK or the EU, which may have higher manufacturing standards. This compares to products sourced in Becker, *et al.* (1994) study where they sourced products from many different countries outside this highly regulated zone.

3.3.1.1. Bacillus cereus colony counts from MYP Agar

As mentioned above the infant formula milk was only based on presence or absence testing. Three infant formula milks tested positive for presumptive *Bacillus cereus* spores which is extremely worrying. Neonates are particularly

vulnerable to any pathogen due to their underdeveloped immune system and as they may not be receiving any immunity through breast feeding, the potential implications of this are severe. The sports and elderly foods were enumerated for viable count. The *Bacillus cereus* on MYP agar does not form discrete colonies and they were sometimes difficult to count, as merging and larger groups forming a large dome over the colonies made it difficult to read some of the results. In these cases counting was based on the areas that formed the characteristic colonies.

The majority of the sports products that gave characteristic *Bacillus cereus* colonies on this agar were below the recommended viable count limit of 10^4 cfu/ml give the actual count described by Warburton *et al.* (1998) for the limits in 'health foods.' However two sports drinks S-MMP and S-MMX yielded more than 10^4cfu/ml, whereas all but two elderly foods that tested positive for presumptive *Bacillus cereus* had colony counts higher that 10^4cfu/ml, some being as high as 10^6cfu/ml (see table 5). According to Warburton *et al.* (1998) any counts above 10^4cfu/ml constitutes a breach of the highest threshold for health foods contaminated with *Bacillus cereus.* The infective dose required to cause *Bacillus cereus'* diarrhoeal syndrome is thought to be as low as 10^3-10^4 cful/ml (Andersson *et al.,* 1995). Therefore products that yield colony counts as high as these could potentially cause problems for the consumers, depending on the strains contaminating the products. Where 10^3-10^4 cfu/ml is enough to cause the diarrheal syndrome, 10^5 cfu/ml and above, can cause the emetic syndrome (Granum and Lund, 2006). Therefore if large numbers of these bacteria are ingested and they survive defences in the gut, proliferation and synthesis of diarrhoeal toxin to a high level may occur. Therefore it all depends on the strain consumed to what illness may be seen.

3.3.2. *Clostridium perfringens* in Powdered Food

As a spore former *Clostridium perfringens* would be thought to be as proficient as *Bacillus cereus* at contaminating these powdered foods, as their ability to survive the processing can lead to food contamination. As this organism is ubiquitous within the environment it can be assumed that the organism could be present on any of the food sources being processed or on equipment. Like *Bacillus cereus,* it has been investigated that the practices put in place to reduce the microbial load in food may in fact provide the organisms with the ability to survive further processing and contaminate food. Juneja *et al.* (2003) determined that heat shocked *C. perfringens* that had been heat shocked and then sporulated exhibited higher heat resistance and survived longer than non-heat shocked cells that then formed spores. Thus when the milk based products are undergoing treatments to destroy pre-formed spores, instead of killing the organism, mechanisms inside them may be triggered to enable survival for longer under extreme conditions further down the processing line such as during spray drying. The heat treatment and spraying process may therefore not kill any surviving *Clostridium perfringens* spores, but enable its survival.

In this study, 17% of the foods sampled tested positive for presumptive *C. perfringens.* There has not been many published results on the contamination rates of *C. perfringens* in powdered foods. However Barash *et al.* (2010) had performed experiments to determine the numbers of *Clostridium* species found in infant formula milk. Their results found that 30 out of 39 products tested contained *Clostridium* species including presumptive *C. perfringens.* This shows how the infant formula milk can contain a wide range of *Clostridium* species including *C. perfringens* however it cannot be compared to the results found in this project as Barash *et al.* (2010) focussed on clostridial species, and did not use standard ISO methods which involved using diagnostic media to

presumptively identify *C. perfringens* and based all the results on haemolysis and subsequent API 20A identification.

Only one of the 12 non-milk based powdered food contained presumptive *C. perfringens.* As there are no published data it is difficult to compare the results with other studies. However, as mentioned above, the generally low numbers when compared to *B. cereus* is strange. As a spore former it would be acceptable to expect similar high numbers of this organism in powdered foods especially with *C. perfringens'* ability to form spores and survive extreme conditions. A reason for the lower counts found in the milk based products may be because *C. perfringens* is normally associated with meat based products. Although it can contaminate and survive in milk it generally prefers to reside in meat due to its demand of 13 different amino acids it cannot synthesise (Boyd, *et al.,* 1948). The sporulation tends to occur hand in hand with enterotoxin production inside the gastrointestinal tract (Zhao and Melville, 1998). Another reason this organism may be not as prevalent in these products, is because of its obligate anaerobic nature. Although the organism is able to survive in an aerobic environment (but mostly because of its spores), it thrives in anaerobic conditions posing the reason for the organism not growing and forming large viable counts in these open environments. Therefore where *B. cereus* can be found everywhere in soil and on crops which may be fed to cows and contaminate their milk, *C. perfringens* that would be more likely to be found in deep soil or in faeces. As cows would not tend to forage deeply for food and would probably avoid eating their own faeces the lower contamination rates of this organism compared to *B. cereus* may start to be understood.

3.3.2.1. Clostridium perfringens colony counts from TSC Agar

The infant formula milk was not evaluated for colony counts, just presence or absence. One infant formula milk product formed characteristic *Clostridium perfringens* colonies which as described in the previous section is not a surprising result as this organism does may not contaminate milk based foods to an infective amount, where as it does in meat products which are not stored appropriately and outbreaks occur and are reported.

Three products from both the sports and elderly build ups foods formed characteristic colonies for *Clostridium perfringens.* Two from each of these products were above the upper threshold of 10^2 cfu/ml described by Warburton *et al.* (1998). The infective dose is related to when CPE is produced in the small intestine. This generally occurs when around 10^7 cfu/ml has been ingested (Brynestad and Granum, 2002). Warburton *et al.* (1998) may have published 10^2 cfu/ml as the upper limit due to the food, although not contaminated to an infective amount, may spoil the food and make it inedible. However none of the products tested here managed to breach this number. Although the numbers found in the powdered products were below those of the infective dose normally required to cause illnesses, potential problems could still arise from consuming these products, especially in immunocompromised individuals such as neonates. This is because they may not have developed a normal gut flora, which could then become imbalanced from the increased number of a pathogenic organism such as *Clostridium perfringens* resulting in the food borne illness as the environment in the gut is very favourable for this organism.

3.3.3. *Listeria monocytogenes* in Powdered Food

This organism is a non-spore former, which would reduce its ability to survive during pasteurisation and in very extreme conditions compared to the *B. cereus* and *C. perfringens* that have the ability to form spores. However *L. monocytogenes* is a very hardy bacterium with the ability to survive in relatively harsh conditions. In this study seven out of 48 samples proved positive for presumptive *Listeria monocytogenes* on PALCAM agar. All of these seven presumptive positive results came from milk based products.

Heat treatment is the main control measure used in the food industry to reduce or eliminate non-spore forming organisms from food. Pasteurisation is thought to be an efficient and effective method of killing *Listeria monocytogenes* in powdered food. However it has been described, that if not heated properly bacteria can increase their heat resistance when they are exposed, for a short time, to moderately elevated temperatures, normally above their maximum growth temperatures, before actual heat treatment is applied (Jorgensen *et al.,* 1999). If this true, then with *L. monocytogenes'* may have the ability to survive at high temperatures if exposed to sub-lethal temperatures (Farber and Brown, 1990).

A different way contamination may be happening is through contaminated surfaces on equipment used during food processing. Wilks *et al.* (2006) found that viable *Listeria monocytogenes* cells could be detected on stainless steel after 24 h incubation at room temperature. This shows that this organism can survive for extended periods in equipment or in food processing plants. Indeed it has been reported that outbreaks in 1988 and 2000 could be traced back to the same food plant with the same subtype of the organism (Farber, 2000). This suggests the persistence of *L. monocytogenes* in an environment over many years.

Humans can also act as carriers for this bacterium (Welshimer and Donker-Voet, 1971) meaning that if poor hygiene practices are carried out with people who handle food or contact surfaces, then this could result in contamination.

3.3.3.1. Colony counts of presumptive Listeria monocytogenes from PALCAM agar

Three infant formula milks produced characteristic *Listeria monocytogenes* colonies following enrichment. This frequency of isolation relates to how *L. monocytogenes* is adaptable to a wide range of difficult environments. This suggests that this organism is eluding cleaning processes enabling it to contaminate food during processing or it is entering the food through human contact and surviving in the dry environment.

Six sports products and four elderly build-up powders formed characteristic colonies for *Listeria monocytogenes* on PALCAM agar. The majority had colony counts ranging between 10^1 and 10^2cfu/ml. However three sports and one elderly build-up product had colony counts higher than these, the highest being 10^5cfu/ml. The infective dose for *Listeria monocytogenes* is not known, as it can vary between strain and the individual infected. However there is an agreement that it can be as low as or lower than 10^2cfu/ml. As the foods under examination in this project are generally aimed at individuals that may be immunocompromised this lower estimation of the infective dose would be valid. Therefore the products where colony counts were above 10^2cfu/ml is extremely serious as such high counts are rare with this organism. Especially as these products are aimed at individuals who are susceptible to these organisms.

3.3.4. *Staphylococcus aureus* in Powdered Food

This study found that nine different food products out of 48 were contaminated with presumptive *Staphylococcus aureus.* Seven were milk based products whereas two were from non-milk based alternatives. The lower frequency of isolation of this organism in the food is not surprising as *Staphylococcus aureus* is not a spore former and cannot survive like *B. cereus* and *C. perfringens* can. However *Staphylococcus aureus* has the ability to survive for long periods of time in dry, desiccated environments, which pose the obvious risks for consumers of these powdered foods (Portocarrero *et al.,* 2002).

From the study, two out of the nine products tested positive in the non-milk products, whereas seven out of nine tested positive in the milk-based foods. This is not an unexpected result as cows are commonly infected with *Staphylococcus aureus,* which causes mastitis, although if visible contamination is observed the milk is discarded, however infection can occur asymptomatically in cows as well as the surface of the udder and the teat canal harbouring the organism. Thus *S. aureus* can contaminate the milk with large fluctuations in counts ranging from zero to 10^8 CFU/m (Asperger and Zangerl, 2003). However *S. aureus* is readily killed by pasteurisation temperatures, and so the presence of the organisms in these products suggests either faulty heat processes have taken place or post-process contamination has occurred such as from food handlers, that can have the organism on their skin, just like the cows can, and have gone on to contaminate the products.

3.3.4.1. Colony counts from Baird Parker Agar

Four infant formula milks tested positive for presumptive *Staphylococcus aureus.* This may have been from milk that has come from cows that have the

organism on their skins surface as mention in the section above. However this may only be the case if heating processes are proved to be faulty. Food handlers have also been seen as a possible route to contamination as much of the population can be carriers of this organism. However *S. aureus* has a particular niche that many organisms cannot survive in. Where cleaning and hygiene protocols in food processing factories may be enough to eliminate the majority of pathogens *S. aureus* may not reside in areas that are thought to be uninhabitable, which can explain their incidence in food stuffs that require processing (Loir *et al.,* 2003).

Relatively a large proportion of sports and elderly build-up products tested positive for presumptive *Staphylococcus aureus.* Seven of the sports and six of the elderly build up products had presumptive *S. aureus* in it. According to Warburton *et al.* (1998) the upper limit allowed for health foods containing *S. aureus* is 10^4cfu/ml. One sports and one elderly build up product breached this upper limit. The infective dose of this organism is around 10^5cfu/ml, however very little toxin is required to cause staphylococcal food poisoning (Loir *et al.,* 2003). Therefore these products may be able to cause food poisoning to the consumers of these products. However the presence of these organisms alone may not be indicative of the product's ability to cause food poisoning as it is the enterotoxins made by *S. aureus* which cause the illness. These are formed during growth so if not already expressed by the cells would require a short period for production once the product was rehydrated. However the very high numbers of cells found would allow a toxic level to form rapidly. In milk products another concern is that the toxins are heat stable and may be able to survive heat treatment and drying during processing. Therefore products may have enterotoxin contamination with the ability to cause food poisoning without having viable *Staphylococcus aureus* present. However in this instance viable cells were present.

CHAPTER 4

FURTHER IDENTIFICATION OF PRESUMPTIVE ISOLATES

4.1. INTRODUCTION

The colonies formed on the diagnostic agars (Chapter 3) may not definitely be the organisms that the agar is selective for, even if they have the characteristic colony morphology. Therefore further identification of the isolates needed to be carried out. In this study microscopy, biochemical and molecular methods were used in parallel to identify the organisms to the species level with a degree of confidence.

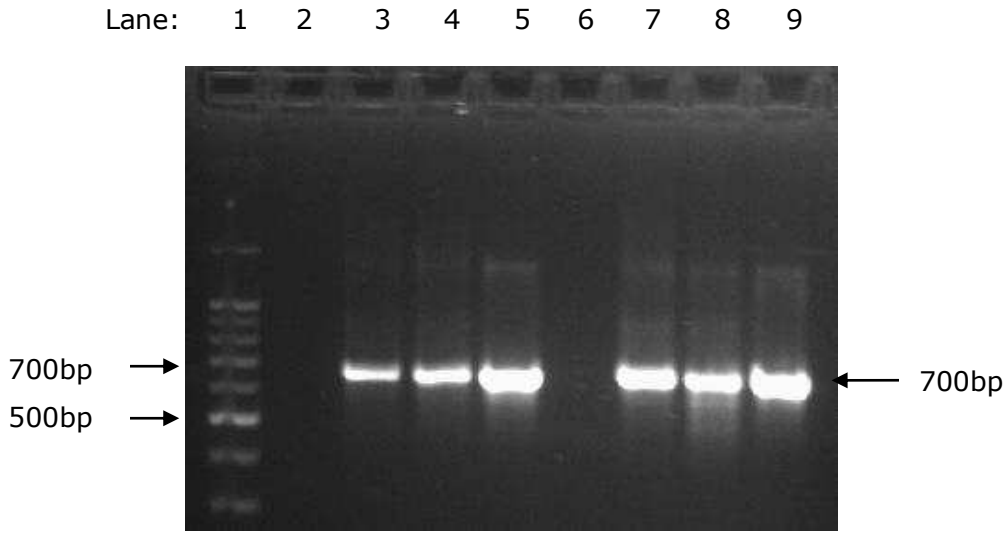
Each of the presumptive isolates from the different diagnostic agars was further characterised using classical techniques such as microscopy to determine motility, cell morphology and Gram-stain reaction. Appendices 7 to 10 show the results from each medium that yielded characteristic colonies from each diagnostic agar. This was followed by specific biochemical testing and finally molecular identification tests based on PCR amplification of signature sequences. Surprisingly, there are no specific PCR assays that are recommended as part of standard isolation and identification methods. Hence in each case for the work presented here previously published PCR identification methods were used.

4.2. TESTING PURIFIED DNA FOR PCR INHIBITION

As Polymerase Chain Reaction (PCR) assays were going to be used as a major diagnostic test in this study DNA purified from each organism was tested by performing a universal PCR assay that should amplify DNA from all members of the Eubacteria group. This was achieved by using primers designed against conserved 16S ribosomal DNA regions found in all bacteria and should produce a band of 700 bp (Somer and Kashi, 2003). This was used as a positive control for the further PCR reactions carried out after this to show that the purification of the DNA for PCR was successful. Figure 4.1 shows the results of examples of these PCR experiments after testing each laboratory strain of *B. cereus, L.*, the *monocytogenes* and *S. aureus. C. perfringens* DNA could not be tested due to

Home Office restrictions on culturing this organism so that pure DNA could not be purified.

Figure 4.1: Example results of control Eubaateria PCR reaction



Two per cent TAE agarose gel separated for 1 h at 70 V(Section 2.4.3) with PCR amplification products amplified from representative examples of different bacteria tested using primers specific for Eubacteria (section 2.4.2). Lane 1 is marker (Promega 100 bp ladder; section 2.4.1). Lane 2 and 6 are negative controls containing only water and DNA from a baker's yeast cell as templates, respectively. Lanes 3-5 and 7-9 are PCR products amplified from, sequentially, laboratory strains of *L. monocytogenes* (ATCC 23074 and NCTC 7973), *S. aureus* (RN3293/02 and 12100) and *B. cereus* (211b).

4.3. *BACILLUS CEREUS* IDENTIFICATION

The most commonly isolated organism was the presumptive *B. cereus.* Which had been found to be present in 18 if the products tested after pure culture on BHI agar one colony from the pure culture was tested.

4.3.1. Microscopic Analysis

Cell morphology characteristics of these bacteria are: Gram-positive, motile rods. After microscopic analysis 89 % (16/18) of the isolates were considered to be *B. cereus* as they had these attributes.

4.3.2. Examination of spore forming ability

Further identification of the organisms that had characteristic colony and cell morphology from MYP Agar was achieved by examining if they had the ability to form spores, which is a key trait of *B. cereus.* The method to detect *B. cereus* spores was adapted from Reyes *et al.* (2007; Section 2.3.7.2). Briefly colonies from the MYP agar plates were resuspended in BPW, and incubated at 80 ^{o}C for 10 min. The cells were then cooled and inoculated into TSB supplemented with polymyxin B and incubated for 24 h at 30 ^{o}C. After incubation, samples were plated onto MYP agar and samples that yielded pink colonies (see Figure 2.1), were confirmed to be spore-forming organisms. The results therefore indicated that each of the 16 colonies that had characteristic traits of *B. cereus* (Gram-positive, motile rods) were also able to form spores and survive the heat treatment. These organisms had all the expected traits of *B. cereus* and had the characteristic colony morphology on the diagnostic agar.

As this organism has so many close relatives, it is very difficult to confirm its identity solely using biochemical or physiological methods. However further biochemical tests could be carried out to increase the certainty that these

bacteria were in fact *B. cereus.* The researchers who developed the MYP Agar listed further tests which were found invariably positive for *B. cereus* that could be used to confirm the identity of *B. cereus.* These were the anaerobic dissimilation of glucose, gelatin liquefaction, nitrate reduction, and profuse growth on chloral hydrate agar (Mossel *et al.,* 1967). Many of these tests are incorporated into the API 50 CHB kit, which can be used as an alternative. However, even after all these tests, it can be difficult to prove whether or not isolates are definitely *B. cereus.*

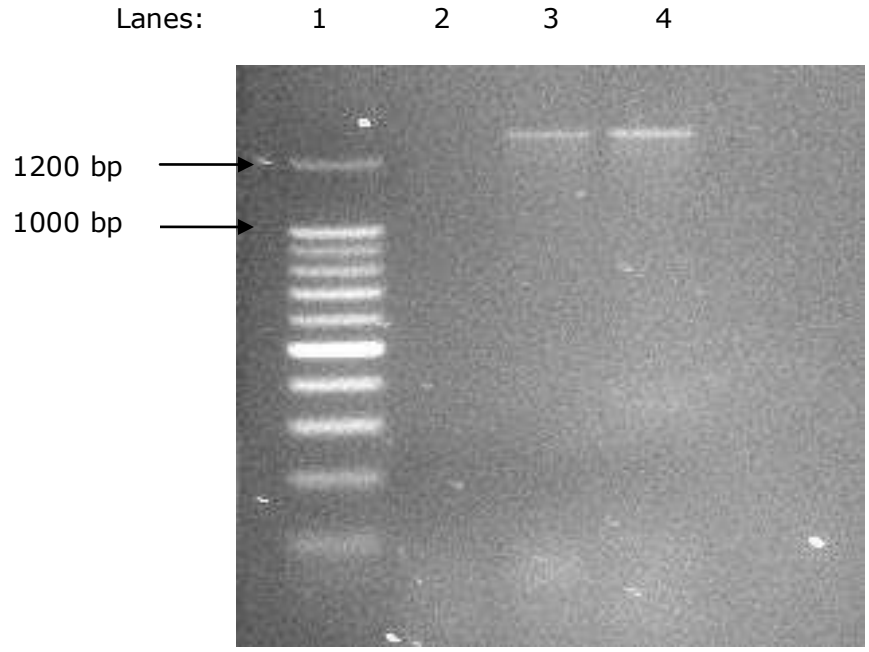
4.3.3. Molecular Identification of Isolates

PCR assays have been described by a wide range of workers to confirm the identity of *B. cereus* isolates. For instance the use of PCR combined with ARDRA has been developed by Wu *et al.* (2006). Previously two 16S rRNA universal primers were designed that allowed detection of a range of *Bacillus* species (Liu *et al.,* 1997). In the assay described by Wu *et al.* (2006) a PCR reaction was carried out using these primers and then the PCR products were subjected to restriction using several enzymes (ARDRA; amplified ribosomal DNA restriction analysis). This allows a range of different restriction patterns to be produced on a gel and each pattern can be matched to a known band pattern of the different known species. This is reported to allow good identification of the organisms isolated (Wu *et al.,* 2006).

In conjunction with the findings from the phenotypic analysis, and to enable a positive identification of the presumptive organisms grown on MYP agar, one attempt was made to use this PCR method of identification. The methods described in Section 2.3.8 were used to purify DNA from the presumptive positive colonies grown on MYP agar and used as a template for the PCR reaction (Section 2.4.4). DNA was amplified using primers K-B1/F and B1-K/R1 (section 2.4.2) and this produced a band of at least 1200 bp (see Figure 4.2). This was

greater than the predicted 1200 bp described by Wu et al. in 2006, however this PCR product was purified from the gel and the purified DNA was then digested with two separate enzymes, *Alu*I and *Taq*I (Section 2.4.4). The method proved to be quite time consuming and, due to time restraints and availability of the required enzymes to carry out the restriction digest, this work was not completed. Further work would be needed to accurately size this PCR product, and compare the restriction patterns matching those shown in Appendix 4.1. However until this method is better optimised, and the results fully analysed, at this stage we cannot conclusively identify the isolates as *B. cereus.*

Figure 4.2: PCR showing the amplification of a 16S rDNA site for the *Bacillus species*



A 2 % TAE agarose gel separated for 1.5 h at 70 V (section 2.4.4) with PCR amplification products specific for the *Bacillus* genus (section 2.4.2). Lane 1, molecular weight marker (Promega 100bp ladder; section 2.4.1). Lane 2 is a negative control (sterile RO water) Lanes 3 and 4 contain PCR products gained from a laboratory strain of *B. cereus* (ATCC 211B). These were larger than the 1200 bp marker, and were estimated to be at least 1350 bp but were not sized accurately.

4.3.4 Summary of *Bacillus cereus* Results

Together the results gained showed that out of the 18 products that yielded characteristic pink colonies on MYP Agar, 16 of them could be considered to be contaminated with *B. cereus.* This was because the organisms isolated from these 16 products, all had the features that are characteristic to *B. cereus* (i.e. Gram-positive, motile, spore-forming rods giving characteristic growth on MYP agar). However the final PCR and ARDRA identification was not completed and thus the identification could not be completely certain. However the fact that 89% of the organisms that formed characteristic colonies on a very good diagnostic medium (Guven *et al.,* 2006), and had the correct cell morphology and spore forming capability, means that one could be fairly sure of their identity.

The *Bacillus* genus is a very large and some members are very closely related. For example it has been noted that *B. cereus* and *B. thuringiensis* cannot be differentiated on the basis of biochemical characteristics (McKillip, 2000). There are also strong genetic links between *Bacillus anthracis* and *Bacillus cereus* making them difficult to distinguish (Ivanova *et al.,* 2003). However confirming that it was part of the *B. cereus* group and not another *Bacillus* spp. was good enough for the purposes of this study.

The diverse nature of the *Bacillus* genus also makes it difficult to find distinctive conserved regions within *B. cereus* isolates. PCR-based assays have been developed to identify the different toxin genes the organism possesses that give rise to the emetic and diarrhoeal syndromes. However even with these there is possible risk of false positives. Yang *et al.* (2007) developed a real-time PCR to detect the *nhe* gene which encodes the diarrhoeal toxin NHE. Although this gene is present in 100% of toxigenic strains of *B. cereus* it is also present in 100% of *B. thuringiensis* strains. Emetic toxin genes are harder to detect and at

this time there is no simple PCR assay that enables detection of all the genes involved in the emetic syndrome of *B. cereus.*

Overall the level of identification gained for this organism in this project was enough to assume that the isolates from 16 of the powdered foods were indeed *B. cereus.*

4.4. *CLOSTRIDIUM PERFRINGENS* IDENTIFICATION

To identify this organism tests such as the reverse CAMP test can be carried out which can, in conjunction with colony morphology descriptions, yield good confirmatory identification results for *C. perfringens* (Eisgruber *et al.,* 2000). Another alternative method used to confirm the identification of the toxins of this organism is the enzyme linked immunosorbent assay (ELISA) which can be applied without further culture. Although the ELISAs allow reliable identification of *C.* perfringens enterotoxin, the use of PCR to identify isolates is still the better option at this time. For example, so far no ELISA is available to detect the β_2-toxin. In addition high levels of enterotoxin have been shown to be present during sporulation only (Baums *et al.,* 2004) and therefore false-negative results may occur due to the cultivation method used. However this group have developed a reliable species-specific multiplex PCR for the detection of the *cpa*, *cpb*, *cpb*2, *cpe*, *etx* and *iap* toxinogenic genes of *C.* perfringens isolates in a single reaction that does not require DNA purification and therefore is feasible and easy to apply.

4.4.1. Microscopic Analysis

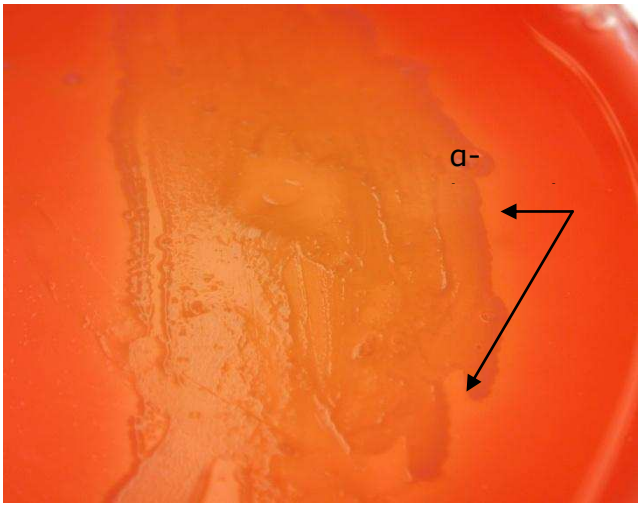
From the TSC Agar, seven products gave colonies which had the characteristic colony morphology to assume they were presumptive *C. perfringens.* Characteristic cell morphologies for this organism are: Gram-positive, non-motile, spore-forming rods which only grow under anaerobic conditions. All the

colonies formed on this medium grew in an anaerobic environment. However only 57 % (4/7) of the presumptive organisms had the characteristics attributed to *C. perfringens.*

4.4.2. Haemolysis

Colonies isolated on TSC Agar that had the characteristic cell morphologies were examined to determine whether they produced the alpha-toxin which has the ability to break down red blood cells (Section 2.3.7.1) After streaking onto blood agar, it was found that all four organisms exhibited haemolysis, however only three of them exhibited the characteristic *beta*-haemolysis associated with *C. perfringens*, adding further information to aid their identification. One of the organisms produced *alpha*-haemolysis (described in Section 2.3.7.1), giving a greenish zone of haemolysis suggesting it was not *C. perfringens*. Figures 4.3 and 4.4 show the *alpha*- and *beta*-haemolysis results, respectively, that were detected. However, Hall *et al.* (1963) have shown that different *C. perfringens* isolates may display varying strengths of haemolysis activity but it is always present at some level therefore this result does not definitively exclude the isolate that exhibited *alpha*-haemolysis being *C. perfringens.*

Figure 4.3: *Alpha*-haemolysis on sheep-blood agar exhibited by organism that formed on TSC Agar



Images showing green *Alpha*-haemolysis (arrows) produced on blood agar plates from an organism thought to be presumptive *C. perfringens* isolated from product E-BC

Figure 4.4: *Beta*-Haemolysis on sheep-blood agar exhibited by organism that formed on TSC Agar

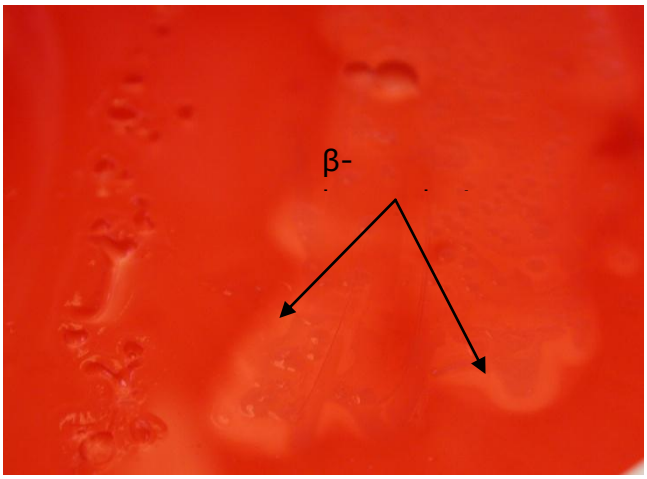


Image showing weak but definite characteristic 'clearing' *Beta*-haemolysis (arrows) from a presumptive *Clostridium perfringens* organism isolated from product E-NC.

4.4.3. API Tests for *Clostridium perfringens*

API 20A is a biochemical testing kit that can be used to help identify anaerobic bacteria in conjunction with the tests carried out above (microscopic analysis and haemolysis). Using the method described in Section 2.2.2.3, a pure culture of the colonies that were isolated from the TSC agar was tested. The results also provide information about what the organism may be if it is not *C. perfringens.* Table 4.1 shows the results from the API 20A (see Appendix 4.2 for API strips).

The results from the API 20A test showed that only one organism, from the powdered food product E-NS, was quite certainly *C. perfringens.* This corresponds with the findings from the haemolysis and colony and cell morphology results. Isolates from E-NC and S-LSP, which also had characteristics of *C. perfringens* were found to have an unacceptable profile (*i.e.* the results did not correspond to anything in the API database) and a very good identification as *Clostridium difficile,* respectively. The others isolates, which had already been ruled out as being *C. perfringens,* produced a range of unacceptable and acceptable profiles including non-pathogenic *Clostridium* species.

Table 4.1: API 20A identifications of colonies formed on TSC Agar

API20A Results	Identification	1st ID	2nd ID
B-CC	Not Valid	*Peptostreptococcus* group (76.6%)	*Clostridium spp*. (11.8%)
S-LSP	Very Good	*Clostridium difficile* (99..9%)	*Clostridium spp.* (0.1%)
S-MMX	Unacceptable	*Clostridium beijerinkii/butyricum* (n/a)	*Clostridium septicum* (n/a)
S-MMP	Very Good	*Clostridium beijerinkii/butyricum* (99.9%)	*Actinomyces israelii* (0.1%)
S-WP	Very Good	*Clostridium beijerinkii/butyricum* (99.9%)	*Clostridium spp.* (0.1%)
E-NC	Unacceptable	*Clostridium beijerinkii/butyricum* (n/a)	*Clostridium septicum* (n/a)
E-NS	Very Good	*Clostridium perfringens* (99.3%)	*Clostridium botulinum* (0.6%)
E-BC	Good	*Clostridium spp.* (93.8%)	*Clostridium botulinum* (4.6%)

This table shows the API 20A results from APIWeb, with percentage of similarity for each recording indicating the closest identification match and whether the results are reliable or not. Acceptable values are those above 80% similarity to a named organism in the data base.

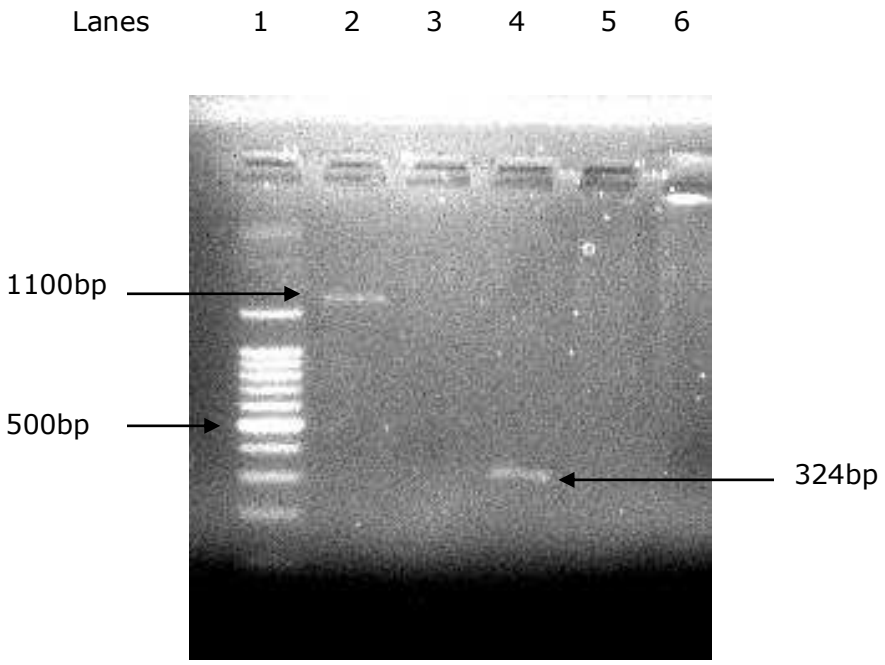
4.4.4. *Clostridium perfringens* Identification Using PCR

Molecular identification was carried out to determine whether the results found from the biochemical tests could be supported by PCR identification. The method for identifying *C. perfringens* was adapted from a protocol developed by Songer and Bueschel (1999). The method targets the *cpa* toxin gene which is present in all strains, including food poisoning strains, of *C. perfringens* (Section 2.4.5). In this assay a band at 324 bp confirms that the strain is a *C. perfringens* isolate capable of causing food poisoning. The isolates from the four products that yielded colonies with characteristic cell and colony morphology and were positive on the API tests, as well as those that had inconclusive results were tested (S-MMX, S-LSP, E-NS andE-NC). Figure 4.5 shows that only one out of these four isolates (from E-NS) gave a positive PCR result and could be confirmed to encode the *C. perfringens cpa* toxin gene. Since the API tests identified this as *C. perfringens,* and this isolate did have the *cpa* gene required for the synthesis of the enterotoxin associated with *C. perfringens*, therefore there is a reasonable certainty that this product was contaminated with *C. perfringens.*

The presumptive *C. perfringens* colony isolated from E-BC, which was confirmed using API 20A as being *Clostridium* spp. did not appear to be *C. perfringens.* However it must be noted that there was no positive control carried out for this organism, due to Home Office restrictions that require a license to handle pure cultures of such organisms and so no genomic DNA purification could be carried out. However as the positive internal control yielded bands as well as the isolate suspected as being *C. perfringens* having a band at the right position the gel, it can be assumed that the PCR was working. Therefore just a negative control was used for the PCR. Isolate S-MMX produced an anomalous band at over 1 kbp which should not have been produced from *C. perfringens*. As the identity of the PCR product was not investigated further, no information

can be gained from this result; however the API result for this isolate was also inconclusive and together these results would suggest this is not a strain of *C. perfringens*.

Figure 4.5: PCR showing presence of the *cpa* toxin gene of the presumptive *C. perfringens* isolates formed on TSC Agar



A 1.5 % TAE agarose gel separated for 1.5 h at 70 V (section 2.4.5) with PCR amplification products specific for *C. perfringens cpa* toxin (section 2.4.2). Lane 1, molecular weight marker (Promega 100bp ladder; section 2.4.1). Lanes 2-5 contain PCR products gained from isolates (2) S-MMX, (3) S-LSP, (4) E-NS and (5) E-NC. Lane 6 is a negative control (sterile RO water).

4.4.5. Summary of *Clostridium perfringens* Results

Therefore out of the seven presumptively positive organisms detected on the TSC Agar, only one of them was confirmed as being *C. perfringens.* Hauschild and Hilsheimer (1973) have noted, after comparing a range of media used to detect *C. perfringens,* that black colonies had grown on TSC agar that were not this organism. However it must be said that these organisms did not form characteristic colonies. In the current study colonies were characteristic and therefore this demonstrates the need for secondary testing of isolates to confirm the presence of *C. perfringens* in a product.

4.5. *LISTERIA MONOCYTOGENES* IDENTIFICATION

In addition to those methods described in Chapter 2, other methods of confirming the identity of *L. monocytogenes* isolates involving genotyping or immuno-assays have been employed (Fluit *et al.,* 1993 and Gravesen *et al.,* 2000, respectively), and these can yield faster and more accurate results than conventional biochemical testing. Covalent linking of specific monoclonal antibody (MAb) or polyclonal antibody to paramagnetic beads offers a valuable means to capture *Listeria* bacteria from clinical, food and environmental samples and can be used to reduce the isolation of non-specific organisms (such as *Bacillus* spp.) on diagnostic agars and to eliminate PCR-inhibitory factors (Liu, 2008). However many researchers and, now food companies, are employing a cheaper and faster combination of genotyping methods to confirm the identity of the strain of this organism. Random amplification of polymorphic DNA (RAPD) together with multiplex-PCR serotyping allowed rapid discrimination of *L.* monocytogenes (Aurora et al., 2009). However it should be noted that although these methods are cheaper than using an immuno-assay, the use of the RAPD assay is difficult to perform and requires specialist training.

4.5.1. Microscopic Analysis

From the PALCAM agar, originally 13 products were considered to contain presumptive *L. monocytogenes.* However after further investigation and observation of colony morphology three isolates were discarded as not being this organism. Out of the 10 remaining samples, 90 % (9/10) had the characteristic cell morphology of this organism, being Gram-positive rods. These organisms should also be motile at 25 ^{o}C, but not at 37 ^{o}C. However out of the nine samples tested only three were motile at 25 ^{o}C.

4.5.2. Haemolysis

Colonies isolated from PALCAM agar were examined to determine whether they produce enzymes with the ability to break down red blood cells. To do this each isolate was grown on blood agar plates and their ability to perform haemolysis was examined (see Section 2.3.7.1).

Seven out of the nine colonies that exhibited a positive presumptive result on PALCAM which had the cell morphology attributed to *L. monocytogenes* produced *Beta*-haemolysis on blood agar, which provides another indicator for positive identification of the colonies recovered from the powdered food as *L. monocytogenes.* Some of the isolates produced a strong positive haemolytic result, where haemolysis was clearly visible, whereas others displayed a weaker haemolysis characteristic of *L. monocytogenes*. Figures 4.6 and 4.7 show examples of the strong and weaker haemolysis detected

Figure 4.6: Weaker *beta*-haemolysis exhibited from organism isolated from PALCAM agar

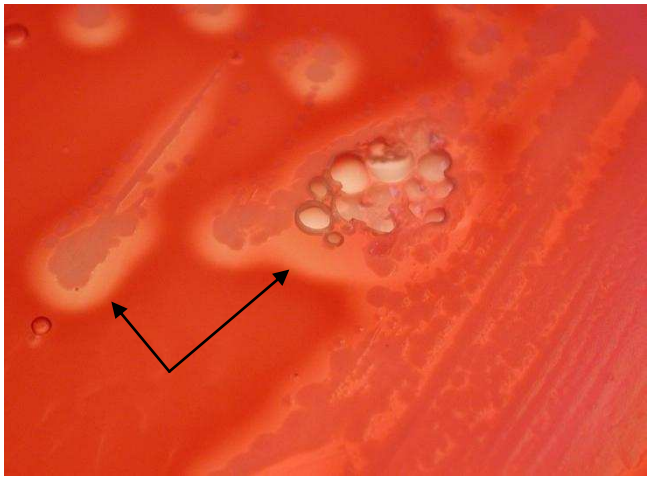


Image showing the weaker haemolysis produced by presumptive *L. monocytogenes* isolate from E-NC CC, on sheep-blood medium with clearing indicated by the arrows.

Figure 4.7: Stronger *beta*-haemolysis exhibited from an organism isolated on PALCAM agar

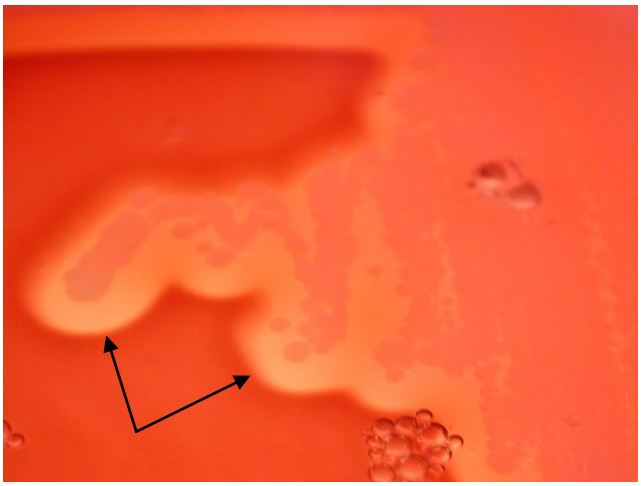


Image showing strong haemolysis produced by presumptive *L. monocytogenes* isolate from E-CC , on sheep-blood agar with clearing of the areas formed around the colonies are indicated by arrows.

4.5.3 API Listeria results

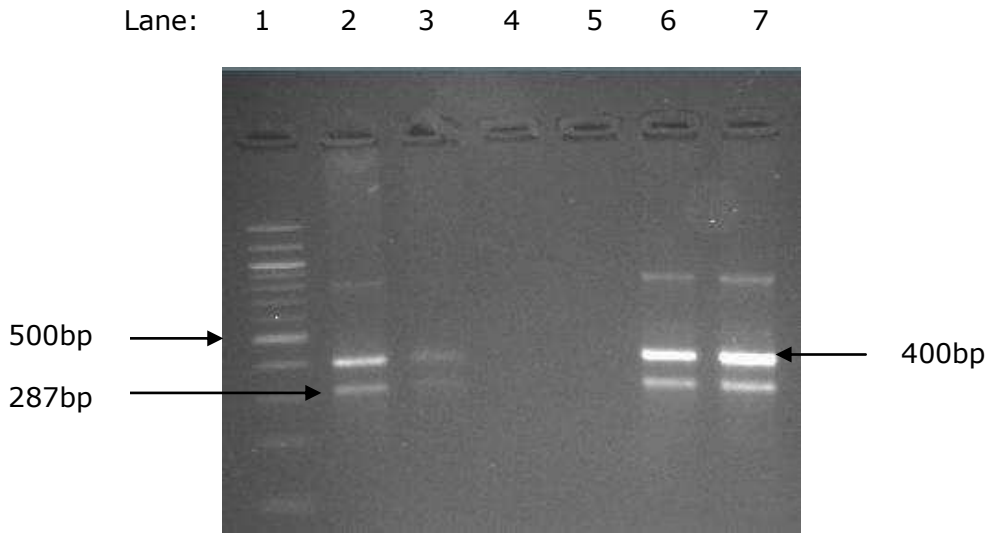
Using the method described in Section 2.2.3.1. API Listeria was used to identify whether the presumptive organisms from PALCAM agar were *L. monocytogenes*. Each test performed confirmed that the organisms belonged to *Listeria* spp. However when it came to a positive identification as belonging to the *L. monocytogenes* species, each produced an unacceptable profile and therefore the results did not give a definitive result. Thus further molecular identification techniques were used to confirm the identity of these isolates.

4.5.4. *Listeria monocytogenes* PCR Identification

The molecular identification of *Listeria monocytogenes* was carried out using a method described by Somer and Kashi, (2003). This multiplex PCR reaction involves using primers that target conserved DNA regions in the *Listeria* genus to produce a band of 400 bp and regions of the DNA only found in *L. monocytogenes* to produce a band of 287 bp.

Seven haemolytic presumptive *Listeria monocytogenes* isolates were examined with the PCR. The DNA was extracted from the pure cultures of colonies that formed on PALCAM agar using methods described in Section 2.3.8. DNA from a laboratory culture of *L. monocytogenes* (NCTC 7973) was used as a positive control. As a negative control, isolates that did not have characteristic colony and cell morphology features of *Listeria* spp. were chosen and also a reaction containing no DNA template was prepared. Figure 4.8 shows an example of the results gained, indicating the diagnostic bands that should appear on an agarose gel. The results gained showed that three of the seven presumptive isolates were *L. monocytogenes.* These were isolates from products B-HPF, S-MMX and E-NV. Three, S-MMP, S-BSN and E-NC, were identified as *Listeria* species. One isolate tested, B-SLF, did not appear to belong to the *Listeria* genus.

Figure 4.8: PCR to distinguish *L. monocytogenes* spp. from *Listeria* genus



A 2% TAE agarose gel separated for 1.5 h at 70 V (Section 2.4.6) with representative PCR amplification products amplified from *L. monocytogenes* isolates (section 2.4.2). If isolates were part of the *Listeria* genus a band of 400 bp alone is produced. For *L. monocytogenes* an additional band of 287 bp is produced. Lane 1 is the molecular weight marker (Promega 100bp ladder; section 2.4.1). Lanes 2-3 and 6-7 are laboratory strains of *L. monocytogenes* (ATCC 23074 and NCTC 7973, respectively). Lanes 4-5 are negative controls (water only and *B. cereus* – 211b template DNA, respectively).

4.5.5. Summary of *Listeria monocytogenes* Results

Originally 13 powdered food samples gave growth on the selective agar used for *L. monocytogenes* isolation. However three were discarded because the colony morphology was not characteristic for *L. monocytogenes* leaving 10 samples for further characterisation. After microscopic analysis nine of these were considered to have the attributes expected of *L. monocytogenes.* Seven of these were haemolytic however haemolysis produced by *L. monocytogenes* and *L. seeligeri* on blood agar is frequently difficult to interpret (Rodriguez *et al.,* 1986). This is because these organisms generally give a weak haemolysis reaction. However there were some strong haemolysis reactions seen with some organisms that may indicate that they were not *L. monocytogenes.* The ruminant pathogen *L. ivanovii* differs from *L. monocytogenes* in that it produces strong, bizonal haemolysis (González-Zorn *et al.,* 2002). Therefore the organisms that produced a strong haemolysis may have been *L. ivanovii,* which has been identified as being able to cause rare cases of listeriosis in humans.

As mentioned in Section 4.5.3 the API Listeria test did not yield any definable results. This may have occurred due a range of reasons. In particular the ZYM-B, a reagent used in the DIM test that differentiates *L. monocytogenes* from other *Listeria* spp.*,* is a very sensitive reagent and if it is exposed to light or not properly refrigerated, then it can lead to false negative or anomalous results.

Molecular identification was carried out using a PCR-based assay that was able to distinguish both *L. monocytogenes* and all other *Listeria* species. From the seven haemolytic organisms, three of them were confirmed by PCR as being *L. monocytogenes,* three were part of the *Listeria* genus and one was not part of the *Listeria* genus. It can be assumed that the strongly haemolytic organisms that were part of the *Listeria* genus, but were not positively identified as *L. monocytogenes,* were in fact *L. ivanovii,* which is still a concern as it has been

implicated in human cases of listeriosis (Guillet *et al.,* 2010). However further tests would be required to confirm the identity of these isolates.

Therefore out of 10 samples that were presumptively positive for *L. monocytogenes*, only three of them have been confirmed as being this organism. PALCAM agar may support growth of other organisms and Pinto *et al.* (2001) described how the PALCAM and Oxford media do not allow a distinction between colonies of *L. innocua* and *L. monocytogenes.* Therefore this lack of selectivity could cause high presumptive isolation, many of which were subsequently identified as members of the *Listeria* genus but not the *L. monocytogenes* species.

4.6. *STAPHYLOCOCCUS AUREUS* IDENTIFICATION

This organism has two distinctive features that allow relatively quick and accurate identification. Provided that the organism has the characteristic cell and colony morphology, two tests are normally enough to identify whether or not the organism is *S. aureus*. The first of these tests is for catalase production. This determines whether the organism can break down hydrogen peroxide in to water and oxygen. The second is the coagulase test. It detects the organism's ability to synthesise a protein used in clotting blood. Positive results from both tests are sufficient to identify the organism as *S. aureus.* However it must be noted that some strains may be atypical and do not produce coagulase (Moroni *et al.,* 2009). PCR-based methods for the detection of *S. aureus* and its toxins have been described by Martineau *et al.* (1996) and Letertre *et al.* (2003), respectively. Martineau and co-workers have designed primers that are conserved within the *S. aureus* genome that can positively identify the organism. In addition Letertre *et al.* (2003) designed universal primers to determine whether or not the isolates are toxigenic. This is particularly important within this project as finding toxigenic strains of the *S. aureus* is very important.

4.6.1. Microscopic Analysis

From the BP Agar, 18 products were considered to contain presumptive *S. aureus*. Of these 66 % (12/18) of the isolates had characteristics that are attributed to this organism, that is they were; Gram-positive, non-motile coccoid cells, that were clustered together.

4.6.2. Biochemical Identification of *Staphylococcus aureus*

The cover slip based method was used to detect whether or not the colonies isolated from BP agar were catalase positive. A small portion of colony grown on BP agar was spotted onto the centre of a cover slip which was then inverted and placed on a drop of hydrogen peroxide. If vigorous bubbling occurred within 10 s the organism is recorded as catalase-positive. A laboratory strain of *Streptococcus pyogenes* (NLTL type strain) was used as a negative control and *Staphylococcus aureus* (NTCC 12100) as a positive control.

To confirm whether isolates were coagulase positive or negative a Staphytect Plus kit (Oxoid) was used. Using a sterile loop, around five colonies from the suspect *S. aureus* isolate was mixed with one drop of test latex solution. If agglutination occurred then the sample was scored as coagulase-positive.

Table 4.2 shows the result of the coagulase and catalase tests for the 12 isolates with characteristic cell and colony morphology for *S. aureus*. Nine of them were positive for both coagulase and catalase tests. However one, S-WP, was catalase-positive but coagulase-negative. This may be a coagulase negative species of *Staphylococcus,* such as *S. epidermidis.* However atypical coagulase negative *S. aureus* can come from cows, so this may be where the food is being contaminated.

Table 4.2: Catalase and coagulase tests results for presumptive *S. aureus* isolates

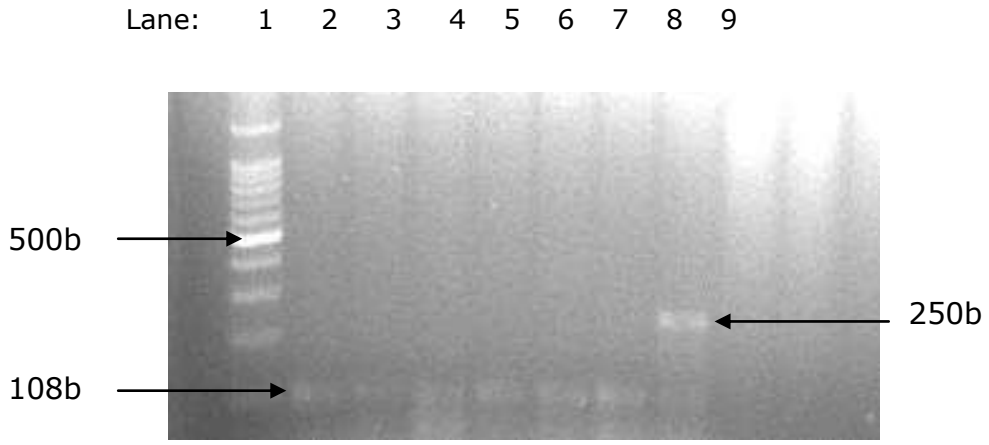
Product	Catalase	Coagulase	Indicative of *S. aureus*?
B-E	+ve	+ve	Yes
B-SLF	+ve	+ve	Yes
B-SW	+ve	+ve	Yes
S-WP	+ve	-ve	Confirmed as *Staphylococcus* species*
S-PV	-ve	N/A	No
S-BFW	+ve	+ve	Yes
S-PSP	+ve	+ve	Yes
S-LSP	+ve	+ve	Yes
S-MMX	+ve	N/A	Yes
S-MMP	-ve	N/A	No
E-NV	-ve	N/A	No
E-CC	+ve	+ve	Yes
+ve Control	**+ve**	**+ve**	**Yes**
-ve Control	**-ve**	**N/A**	**No**

Results for organisms isolated from BP Agar, which had characteristic colony and cell morphology, for catalase and coagulase tests. Results are scored as positive (+ve) or negative (-ve). N/A not applicable as test only appropriate for catalase positive Gram positive cocci. * This organism is coagulase negative but may still be *S. aureus* or another coagulase negative *Staphylococcus.*

4.6.3 *Staphylococcus aureus* Species and Toxin Identification

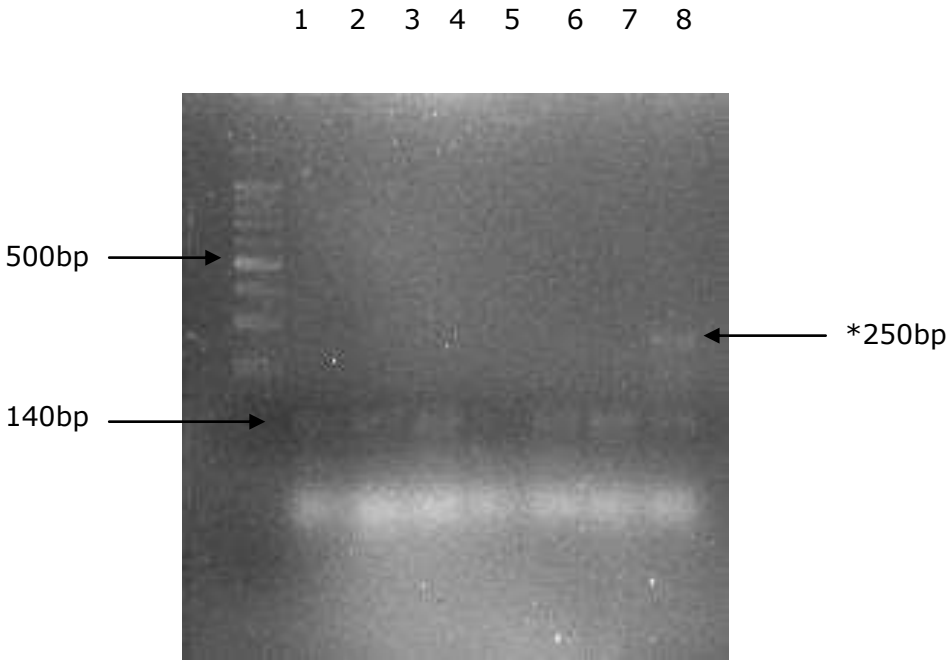
S. aureus was identified using a PCR method based on one described by Martineau *et al.* (1998) that uses primers (see Table 2.1) that bind to conserved regions of a fragment of chromosomal DNA found within all *S. aureus* that produces a band of 108 bp. The isolates were then tested for all of the known *S. aureus* toxins using a method described by Letertre *et al.* (2003) that uses primers that are able to bind to conserved regions found in the *S. aureus* toxins genes producing a band of 140 bp. Figures 4.9 and 4.10 show representative results of these PCR reactions.

Figure 4.9: Species-specific PCR of presumptive *S. aureus* organisms



A 2% TAE agarose gel separated for 1.5 h at 70 V (section 2.4.7) with PCR amplification products specific for *S. aureus* (section 2.4.2). Lane 1 is molecular weight marker (Promega 100bp ladder; section 2.4.1). Lane 2 is positive control (*S. aureus*, strain-12100 DNA) lane 3-8 consist of PCR products amplified from isolates from products S-WP, S-PSP, B-E, S-PSP, B-SLF and S-LSP and lane 9 is negative control (sterile water).

Figure 4.10: Toxin gene-specific PCR of presumptive *S. aureus* organisms



A 2% TAE agarose gel separated for 1.5 h at 70 V (section 2.4.7) with PCR amplification products specific for *S. aureus* (section 2.4.2). Lane 1 is the molecular weight marker (Promega 100bp ladder; section 2.4.1). Lanes 2-8 contain PCR products after amplification of DNA from isolates from products E-CC, S-BFW, S-PSP, B-SLF, B-SW, S-MMX and S-LSP.

The results show that most of the catalase and coagulase positive isolates were indeed *S. aureus,* including S-WP, which was found to be coagulase-negative. This is an interesting finding as coagulase negative *S. aureus* normally isolated from cows and so there could have been a problem in the dairy industry as this was milk based product. However S-LSP, which was coagulase-positive, yielded irregular results of a band of approximately 250 bp (Fig. 4.10, lane 8). B-SLF also did not produce a band at 108 bp (see Figure 4.9). However in Figure 4.10, after testing the same product again using the PCR a band was seen at 108 bp. Thus the organism contaminating B-SLF was *S. aureus* and highlights the need for internal controls when carrying out PCR reactions as negative results caused by PCR inhibition can be misleading (Murphy *et al.,* 2007).

An attempt was made to detect the toxin genes from the isolates using the multiplex PCR linked with the *S. aureus* genes as described in Section 2.4.7. However time restraints and optimising the assay proved difficult and when tried only very faint and difficult to read results were seen (Appendix 4.3). Therefore toxin identification was not completed.

The PCR reaction used to identify the organisms as *S. aureus* was used to confirm the results from the biochemical analysis. From the results, eight of the nine samples were confirmed as *S. aureus,* including the coagulase-negative isolate. However the isolate from product S-LSP, which was coagulase and catalase positive, produced anomalous results. In both PCR reactions this organism gave a faint band at 108 bp which is a positive reaction. However it also gave a band at 250 bp. This did not happen with any of the other samples, where only one band was seen. However as there was a band at 108 bp it suggests that the organism has the conserved region found in *S. aureus* and therefore produces a positive result using this test. Another anomalous result was produced by the isolate from B-SLF, where in one reaction no bands were

seen however in the second PCR reaction a band was seen, thus it can be considered a positive result.

4.6.4 Summary of *Staphylococcus aureus* Results

Eighteen powdered foods were considered contaminated by presumptive *S. aureus.* After microscopic identification 12 of the isolates had cell morphologies typical of that found for *S. aureus.* Two very standard tests that are used widely to biochemically confirm the identity of potential *S. aureus,* are the catalase and coagulase test. Positive results from these coupled with the Gram stain and cell morphology are usually enough to distinguish this organism from anything else isolated from a food source. After performing these tests eight samples were both catalase and coagulase positive. One sample was only catalase positive. However coagulase negative *S. aureus* can occur (Moroni *et al.,* 2009) and so it was treated as a positive result until further tests can prove otherwise.

These results are the only results that show the same readings from both, the biochemical and molecular identification, the minor exceptions being with anomalous readings from the PCR, with regards to the extra band seen from the organism isolated from S-LSP, and with coagulase negative *S. aureus.* Therefore one can say with a high degree of certainty that nine of the samples were positively contaminated with *S. aureus.*

4.7. SUMMARY OF PRODUCT CONTAMINATION

Out of the 48 products tested, 24 of them were contaminated with different combinations of the four pathogens under investigation in this project. Figure 4.11 shows which products were contaminated and whether they were contaminated with one or more of these Gram-positive pathogens.

From the results, six of the infant formula milks were found to be contaminated with one organism tested for, and one (B-HPF) was contaminated

with two of these pathogens. Two out of the 11 sports drinks were found to contain three out of the four pathogens tested; these were S-LSP and S-MMX. Three others were contaminated with two of the pathogens and the rest were contaminated with one. Out of the seven elderly products that were found to be contaminated, three were contaminated with two of the pathogens under investigation, these were E-NC, E-NV and E-NS.

Table 4.3 shows how *B. cereus* contaminated most of the products. The problems escalate when both *B. cereus* and *S. aureus* are found in the same product. If the powdered food was reconstituted and left at room temperature, on their own, both organisms may not be at high enough numbers to cause food poisoning. However if both produce toxins together a synergistic effect may occur, where less numbers of organism together may cause food poisoning. As well as this, the data shows that some products are contaminated with three of the pathogens under investigation in this study. This shows extremely poor GMP.

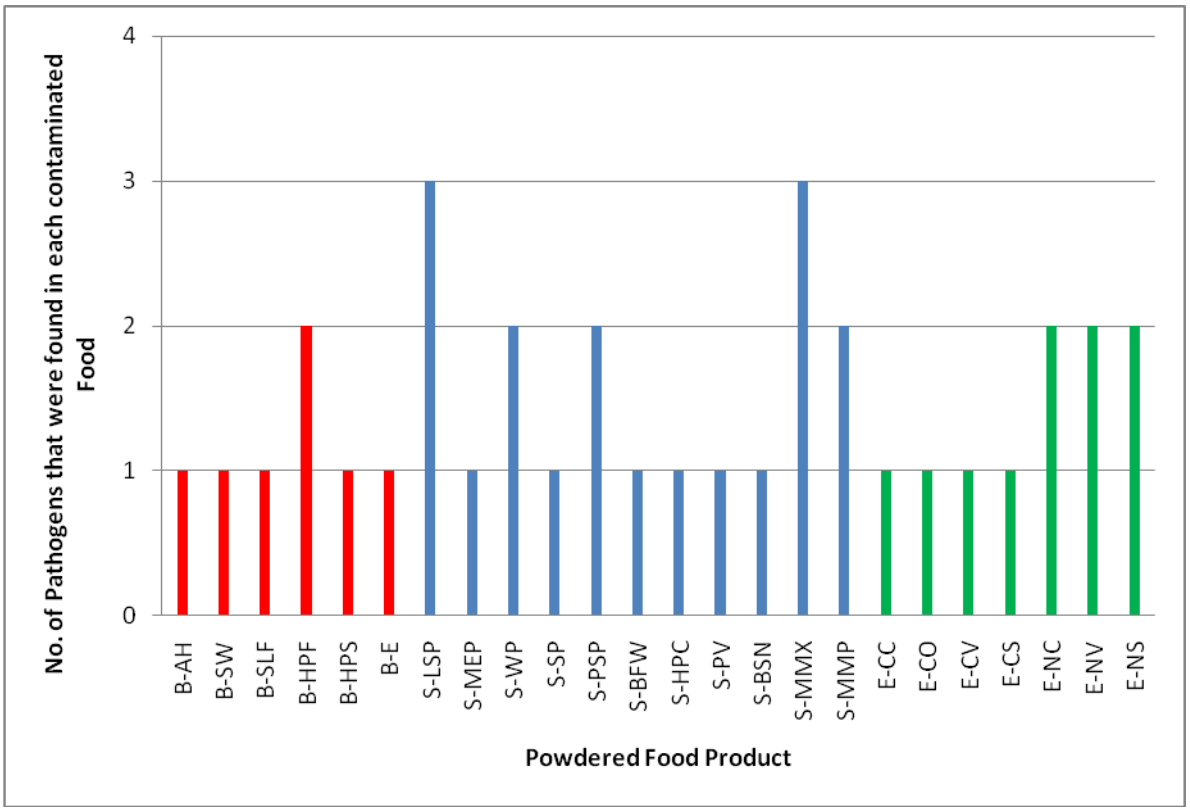
Table 4.3 also shows how all the products that were contaminated with more than one of the pathogens always had *B. cereus* in it. This could then be useful as general indicator of GMP in food production.

Table 4.3: Pathogens that contaminated each product

	Positive Contmination with:				
Contaminated Product	***B. cereus***	***S. aureus***	***L. monocytogenes***	***C. perfringens***	**Total**
B-AH	**X**				1
B-SW		**X**			1
B-SLF		**X**			1
B-HPF	**X**		**X**		2
B-HPS	**X**				1
B-E		**X**			1
S-LSP	**X**	**X**		**X**	3
S-MEP	**X**				1
S-WP	**X**	**X**			2
S-SP	**X**				1
S-PSP	**X**	**X**			2
S-BFW		**X**			1
S-HPC	**X**				1
S-PV	**X**				1
S-BSN			**X**		1
S-MMX	**X**	**X**	**X**		3
S-MMP	**X**		**X**		2
E-CC		**X**			1
E-CO	**X**				1
E-CV	**X**				1
E-CS	**X**				1
E-NC	**X**		**X**		2
E-NV	**X**		**X**		2
E-NS	**X**			**X**	2

Table shows which powdered food products were contaminated and by what pathogen. With the totals that correspond to Figure 4.12.

Figure 4.11: Product contamination patterns



A diagram showing the products that were contaminated with either, *Bacillus cereus, Clostridium perfringens, Listeria monocytogenes* or *Staphylococcus aureus.* The red bars show infant formula milks, the blue; sports drinks and the green; elderly build up products.

4.8. CONCLUSIONS

With regards to *Bacillus cereus,* as there was no molecular identification step carried out reliance was on the diagnostic ability of the medium and microscopic analysis. This suggested that 89% (16/18) of the samples that had characteristic colonies were contaminated with *Bacillus cereus.* This is not a certain result, because without performing more biochemical tests and molecular analysis, definitive readings cannot be made. However even as preliminary results this demonstrates a significant potential from these products to cause food borne disease. Further understanding of the toxigenic potential of the isolates would allow an understanding of whether the risk is of the emetic syndrome through the production of a pre-formed toxin (which would require a growth period prior to consumption) or from diarrhoeal disease which would just require a large number of cells to be present. As in a number of products (13) the levels were at 10^6cfu/ml then these products present a serious risk of causing food borne disease.

Clostridium perfringens also had its problems in identification with regards to its restraints on storing and culturing. However the results from the PCR coupled with performing an API showed that 14% (1/7) of the presumptive organisms were found to be *C. perfringens.* Another interesting result is the detection of *Clostridium difficile* which was also isolated from the TSC Agar, and identified through the API test. Although this organism is not related to food poisoning it can cause gastrointestinal disease and the fact that it is in the food is still worrying. The most important risk factor for *C. difficile*–associated diarrhoea is prior antibiotic use (Johnson and Gerding, 1998). The foods being tested in this project are foods that are aimed at people who are immunocompromised, and therefore they may be on antibiotics. Thus food contaminated with this organism that finds its way into the gut of a person who is being treated with antibiotics, may increase their risk of contracting the illness.

Thirty percent (3/10) of the samples giving presumptive colonies on PALCAM agar were shown to be *Listeria monocytogenes.* Initially after biochemical and microscopic analysis, 70% (7/10) of the isolates tested were considered *Listeria* spp. This coupled with the PCR analysis enabled identification of three *L. monocytogenes* and three with the potential to be *Listeria ivanovii,* which has been seen to infect humans. However further tests on these organisms would need to be carried out to determine whether or not they are *L. ivanovii*.

Staphylococcus aureus was presumptively identified in 18 products. However after microscopic and biochemical analysis it was found that 50% of products (9/18) were in fact contaminated with *Staphylococcus aureus.* This was then confirmed with molecular identification that enabled good positive identification of all but one of the isolates, with one which yielded an irregular band. However a PCR to identify toxins in the isolates was not carried out due to time restraints. Therefore whether the organisms were toxigenic or not remains unknown.

Half of the infant formula milks that were investigated were contaminated with at least one pathogen. B-HPF was contaminated with two which were identified as *B. cereus* and *L. monocytogenes*. Five of the infant formula milks that were identified as being contaminated were not a major product produced by that company. Kandhai *et al.* (2004) have shown that organisms were found on equipment used in the manufacture of infant formula milk post-pasteurisation, demonstrating that there is potential for bacteria to contaminate products if the equipment is not well maintained. Thus where the product is the main focus of a company, a well maintained and managed production line that is regularly used may result in lower levels of contamination. Whereas products that are produced off this main production line – possibly sporadically or by a sub-contractor - may result in higher levels of contamination.

There could be similar reasons for the contamination of each of the sports powders and elderly build-up foods. However it must be noted that the elderly

build-up products that were shown to be contaminated with one or more pathogens were all produced by the same company. *Bacillus cereus* was the main contaminant, but in E-NC and E-NV, *Listeria monocytogenes* was also present. This suggests that different brands of foods may be produced on the same production line, leading to cross-contamination if equipment is not adequately cleaned, or that common source materials were the cause of the contamination. To try and understand it there was one source of contamination, the serotypes and molecular subtypes of the *L. monocytogenes* detected could be determined. This would identify if the products were contaminated by one persistent organism or randomly by different isolates.

It must be noted that in this project only four pathogens are under investigation. Chapter 2 describes how many other organisms grew on the different diagnostic agars used. Therefore to further this project, identification of the other contaminants would be prudent to determine whether any other pathogens were present in the powdered foods rather than just these four that were the focus of this study.

CHAPTER 5

DETERMINING THE HEAT TOLERANCE OF POWDERED FOOD ISOLATES

5.1. INTRODUCTION

It is important to the food industry to determine when, or where, in a food production process contamination of a food product may have taken place. Since pasteurisation or other heat treatments are often used as critical control points in food processing, knowing the D-value of organisms may help answer this question. As spore forming organisms, *B. cereus* and *C. perfringens* can survive pasteurisation heat treatment, and so it can be difficult to identify where they entered the product and their presence in these powdered products is not unexpected. However as *L. monocytogenes* and *S. aureus* are non-spore forming bacteria, they should be killed readily by heat treatments, such as pasteurisation, therefore it is most likely that the contamination of the products took place after heat treatment processes. However both *L. monocytogenes* and *S. aureus* are known to be thermoduric organisms and research has shown that when *L. monocytogenes* was grown in dairy products, and particularly butter, the heat resistance of some strains was enhanced (Casadei *et al.,* 2002). Hence it is important to determine the heat resistance of these organisms in the food products themselves.

Therefore an examination of the heat resistance of the strains that were isolated on PALCAM and Baird Parker agars in this study was carried out to see if the bacteria, present in the powdered food, were able to survive pasteurisation temperatures. This would therefore give an indication of whether contamination of the product took place before or after the heat treatment processes.

5.2. DETERMINING THE HEAT SENSITIVITY OF *LISTERIA* AND *STAPHYLOCOCCUS*

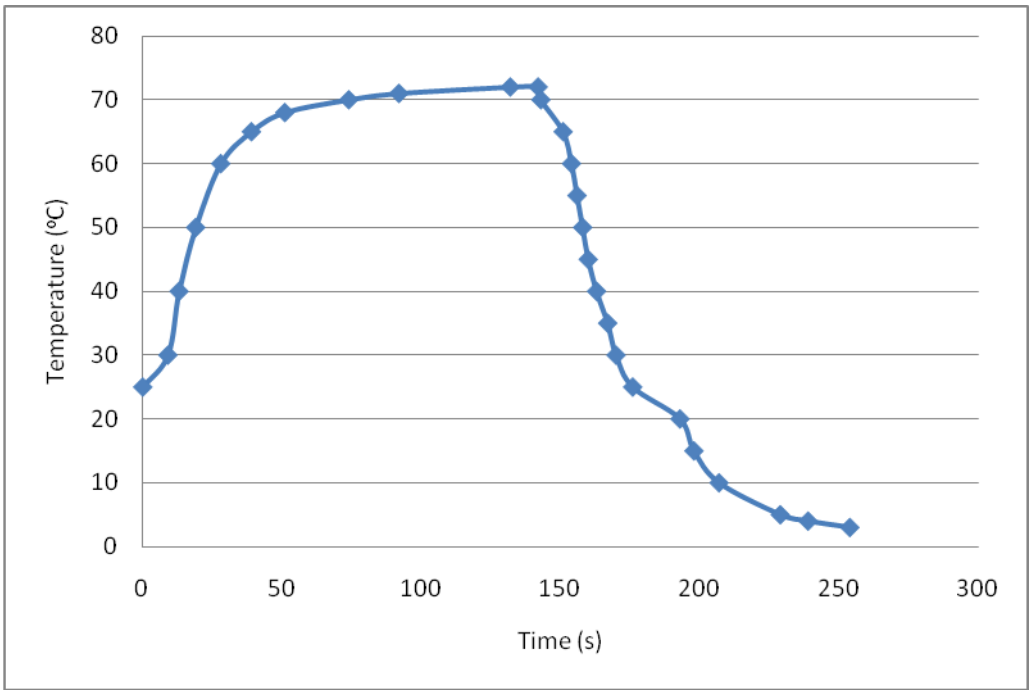
Isolates of *L. monocytogenes* and *S. aureus* from the powdered foods were recovered from cold storage and overnight cultures prepared in BHI broth to produce cultures containing approximately 10^8-10^9 cfu/ml (see Section 2.3).

These cultures were used to inoculate one of the powdered milk products (E-CCO). This powdered product was chosen as it had been shown not to contain detectable levels of viable *L. monocytogenes* or *S. aureus*. To make this sample, 25 g of powder was reconstituted in 225 ml of buffered peptone water. This was spiked with the different test bacteria (Section 2.3) and samples were then used to determine the D-values of the different bacteria at 72°C (standard pasteurisation temperature). This temperature was chosen as there are published data that could be used for comparison with the results obtained.

5.3. DETERMINING THE TEMPERATURE PROFILE OF THE HEAT TREATMENT

The method chosen to determine the heat sensitivity was based on that published by Farber *et al.* (1989), which used 1 ml samples in small sealed glass vials (Wheaton vials). These were heated by placing in a 72 °C water bath and then rapidly cooled by removing the vial from the water bath and placing it in iced water at the end of the heat treatment. Before starting the experiment it was necessary to determine the time taken for the samples to reach 72 °C. To do this 1 ml of the reconstituted powdered food was placed into a Wheaton vial. This was then placed in a water bath set at 72 °C and a temperature probe inserted into the liquid. The temperature of the samples was recorded until the sample in the Wheaton vial reached 72°C, and then the time taken for the sample to cool to below 5 °C was also recorded. The resulting heating profile of the sample is shown in Figure 5.1. This shows that it took 132 s for the sample to reach 72 °C from room temperature and that after being placed in iced water it took 87 s for the sample to get below 5 °C.

Figure 5.1: Thermal profile of heating and cooling phases



The graph shows the time taken for 1 ml of the reconstituted powdered milk product to reach 72 ºC and then to cool to below 5 ºC. The length of holding time was varied during the D-value experiments.

5.4. DETERMINING D-VALUES IN POWDERED MILK

The powdered milk sample was then inoculated separately with each overnight culture of *L. monocytogenes* and *S. aureus* (isolated from E-NS and S-MMX respectively) to an approximate concentration of 10^5 cfu/ml and ten 1 ml samples of each organism transferred into 10 separate Wheaton vials. For comparison, cultures of laboratory strains of both *L. monocytogenes* (ATCC 23074) and *S. aureus* (RN3293/62) were also tested. As the powdered milk was not sterile, an uninoculated sample of the reconstituted powdered food product was also heat treated.

According to Farber (1989) who performed similar tests using a similar 'glass tube' method, the D-value of *L. monocytogenes* at 72 ^{o}C was 7s. Therefore once the vials had reached 72 °C, a vial was removed from the water bath every second and placed in iced water. The total time of heat exposure used in this experiment was 10 s and this was carried out with each organism.

Once all the samples were collected, the powdered milk samples in each vial were then serially diluted using maximum recovery diluent (MRD) and the Miles and Misra technique (five 20 µl samples; Miles and Misra, 1938) was used to determine the viable count. For samples inoculated with *Listeria*, samples were plated on BHI (total viable count), TSYE (recommended recovery medium for D-value determination, Chenoll *et al.*, 2006) and PALCAM agar. For samples inoculated with *Staphylococcus*, samples were plated on BHI, TSYE and BP agar. The BHI and TSYE agar plates were then incubated at 37 ^{o}C for 24 h or 48 h for PALCAM and BP agar. The plates with *L. monocytogenes* samples were incubated under microaerophilic conditions, whereas the plates with *S. aureus* were incubated under aerobic conditions. After the incubation, colonies were counted and the number of cells surviving at each time point in the experiment (cfu/ml) calculated (see Section 2.3.1).

5.4.1 Control Experiments

For the controls experiments, samples were just removed from the 72 °C water bath at three different time points; t = 0, t = 5 s and t =10 s and placed in iced water. Once all the samples were collected, the powdered milk samples in each vial were then serially diluted using maximum recovery diluent (MRD) and the Miles and Misra technique (five 20 µl samples). This was used to determine the viable count using the agars and incubation conditions described above. After the incubation, colonies were counted and the number of cells surviving at each time point in the experiment (cfu/ml) calculated (see Section 2.3.1)

As expected the negative control yielded no colonies typical for *Listeria* or *Staphylococcus* on their respective diagnostic plates at any of the three time points, indicating that the reconstituted powdered milk did not contain these organisms (panel 2, Tables 5.1 and 5.2,). The counts on the non-selective agar at T= 0 gave an indication of the levels of the background flora present in the reconstituted milk (2.3 – 2.7 $\log_{10}$ cfu/ml and 1.98 -2.36 $\log_{10}$ cfu/ml for the *Listeria* and *Staphylococcus* experiments, respectively). Since these numbers decline slowly during the experiment it can be expected that the majority of these organisms are quite thermoduric and could be spore formers.

The inoculum used in each case can be calculated as the difference between the T=0 values in the inoculated and uninoculated samples (3.4 – 3.6 $\log_{10}$ cfu/ml for *Listeria* and 3.4 – 3.5 $\log_{10}$ cfu/ml for *Staphylococcus*), and these numbers correspond approximately to the numbers of these organisms recorded in the T=0 samples on their respective selective media.

In the control experiments cell numbers of both of the laboratory strains of *L. monocytogenes* and *S. aureus* were reduced significantly by the heat treatment producing a 3.1 $\log_{10}$ cfu/ml drop in *Listeria* counts on PALCAM plates and a 3.3 $\log_{10}$ cfu/ml drop in *Staphylococcus* counts on BP plates showing that these strains are sensitive to the heat treatment. The *L. monocytogenes*

seemed to be more heat sensitive than the *S. aureus* strain tested as, in the latter case, cells were recovered from the inoculated sample but not from the uninoculated sample.

Table 5.1: Heat treatment of control samples for the *L. monocytogenes* experiment

Panel 1

	Positive Control		
Time	**PALCAM**	**BHI**	**TSAYE**
0	3.651	5.692	6.362
5	1.123	2.034	4.013
10	0.562	0.212	2.569

Panel 2

	Negative Control		
Time	**PALCAM**	**BHI**	**TSAYE**
0	0	2.32	2.70
5	0	1.65	1.60
10	0	1.12	0.42

Tables showing the $\log_{10}$ cfu/ml of the viable count of experiment using *L. monocytogenes* (ATCC 23074) at the beginning of the experiment (T=0) and after 5 and 10 s of heat treatment (Panel 1) and the viable count recorded from the uninoculated sample (Panel 2). Data represent the results of an average of 5 replica samples.

Table 5.2: Heat treatment of control samples for the *S. aureus* experiment

Panel 1

	Positive Control		
Time	**BP**	**BHI**	**TSAYE**
0	4.36	5.78	5.48
5	2.46	4.6	4.59
10	1.001	2.114	3.023

Panel 2

	Negative Control		
Time	**BP**	**BHI**	**TSAYE**
0	0	2.36	1.98
5	0	1.26	0.85
10	0	0	0

Tables showing the $\log_{10}$ cfu/ml of the viable count of experiment using *S. aureus* (RN3293/62) at the beginning of the experiment (T=0) and after 5 and 10 s of heat treatment (Panel 1) and the viable count recorded from the

uninoculated sample (Panel 2). Data represent the results of an average of 5 replica samples.

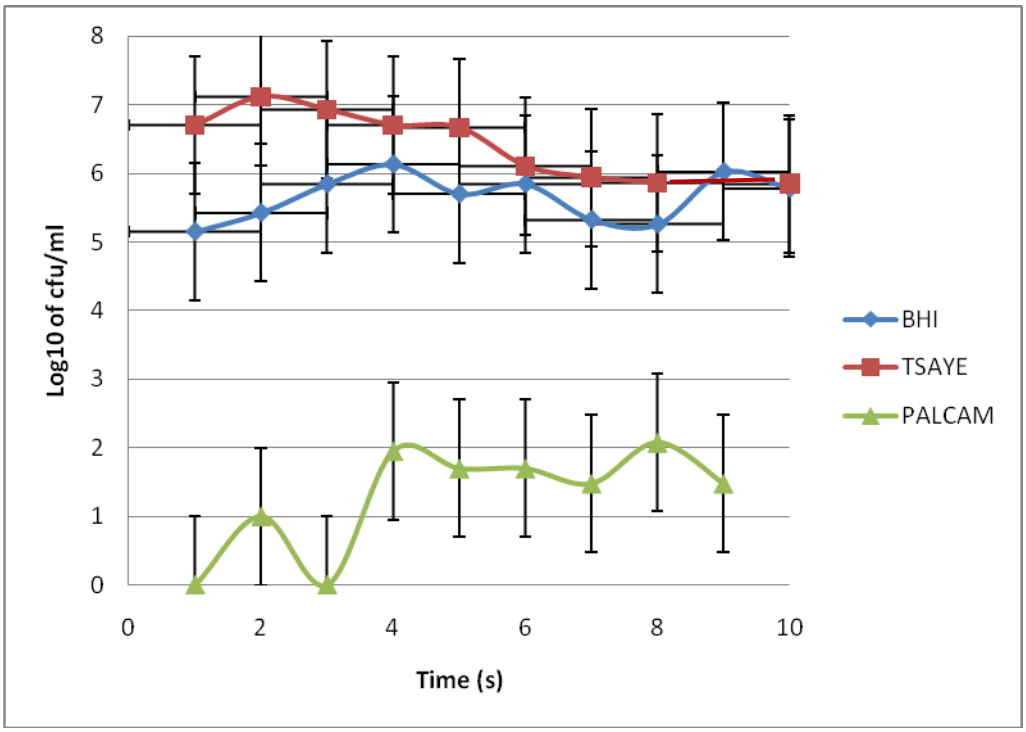
5.4.2. Heat Treatment of Isolates from Powdered Foods

In these experiments, samples inoculated with powdered food isolates were removed from the water bath at 1 s intervals and held on ice until all the samples were collected. As before these were then serially diluted using MRD and the viable count determined using the Miles and Misra technique (five 20 µl samples). *Listeria* samples were plated on BHI, TSYE and PALCAM and *Staphylococcus* samples were plated on BHI, TSYE and BP Agar and incubated as described in Section 5.4.

5.4.2.1. Heat sensitivity of L. monocytogenes

For this experiment the *L. monocytogenes* isolated from product E-NS was used. Figure 5.2 shows the viable count results gained on all three types of agar after 10 s of heating. From the results gained on the BHI agar plates it is clear that the numbers of the background flora in the reconstituted powdered product that grew on these plates did not reduce after the 10 s of heating (initial inoculum of *L. monocytogenes* in the sample was 10^5 cfu/ml). A 1 $\log_{10}$ drop in cell numbers was seen on the TSYE suggesting that this agar selects for the growth of more of the heat-sensitive population. In contrast there was a significant 4-5 $\log_{10}$ reduction in the number of cells that were grown in the PALCAM agar. In this case only the growth of *Listeria* cells is detected and suggests that those added to the product were very heat sensitive or that they had difficulty surviving when plated onto this selective media when they had been heat treated.

Figure: 5.2. Heat inactivation of *L. monocytogenes*

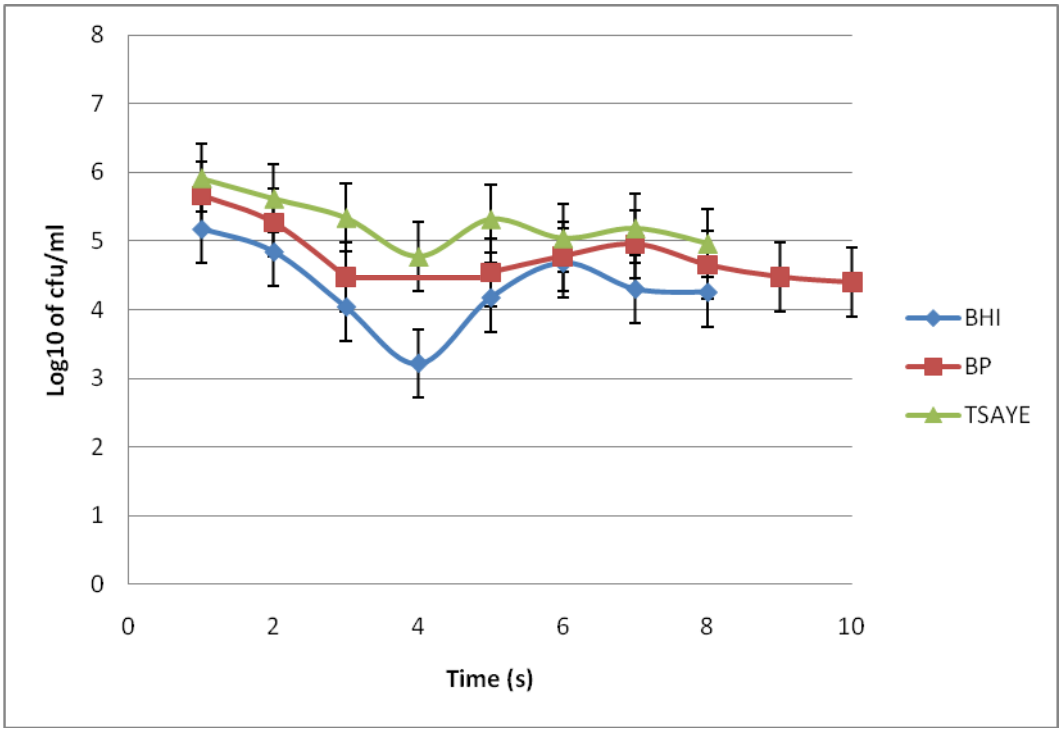


Graph shows number of *L. monocytogenes* cells recovered on different agars after heating for up to 10s at 72 °C in reconstituted powdered milk. Data represent the average of 5 replica samples.

5.4.2.2. Heat sensitivity of S. aureus

The results for the experiment with *S. aureus* isolated from S-MMX are shown in Figure 5.3. In this case a 2 $\log_{10}$ reduction in cell numbers (cfu/ml) was detected on the BHI agar and approximately a 1 $\log_{10}$ drop seen in cell numbers on the TSYE and selective BP agar (Figure 5.3) after the 10 s of heating. This is in contrast to the *L. monocytogenes* results, where the viable count was reduced rapidly after the heating, again suggesting that the *S. aureus* strains are more heat resistant than the *Listeria* isolates or that the BP agar allows the growth of more heat injured cells.

Figure 5.3: Heat inactivation of *S. aureus*



Graph shows number of *S. aureus* cells recovered on different agars after heating for up to 10s at 72 °C in reconstituted powdered milk. Data represent the results of an average of 5 replica samples.

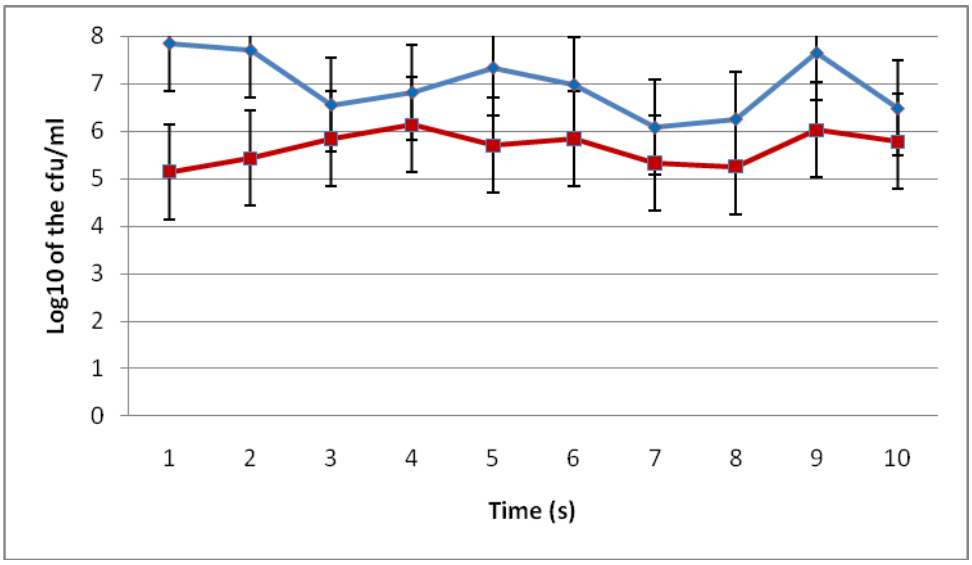
5.4.3. Determining Effect of Cold Incubation on Recovery of Heat Treated Cells

The dramatic difference in the viable counts achieved on the non-selective and selective agars in the *Listeria* experiment may have been explained by the fact that selective media such as PALCAM are known to select against the growth of sub-lethally injured cells. Cold recovery of cells has been previously reported (Dabbaha *et al.,* 1969) and so this was used to try and investigate this result further.

After plating the original samples from the experiment described in Section 5.3.2, the dilution samples in MRD were the placed in the refrigerator for 3 d. After the refrigerated incubation these samples were then plated out again on all three agar types using the same Miles and Misra method. These were then incubated using the same conditions as the original samples before the colonies were counted and again the number of cells recovered (cfu/ml) was calculated.

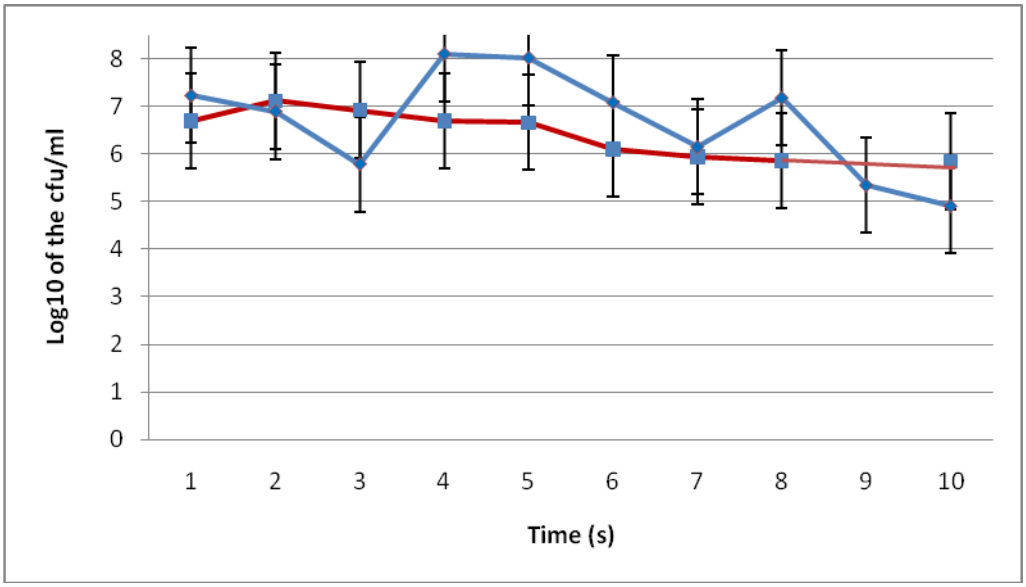
The results for both BHI and TSYE agars did not change very much, as there was barely one $\log_{10}$ difference between each of the data sets, although the cold recovered samples were generally slightly higher than the results gained when the samples were directly plated, however with the standard error bars, it can be seen that the results overlap and so generally the background flora stayed the same (Figures 5.4 and 5.5). In contrast there was a clear rise in the numbers of viable cells detected on the PALCAM agar with a minimum of a 5 $\log_{10}$ cfu/ml increase in the viable count recorded after cold recovery (Figure 5.6). As this agar is selective for *Listeria* species this shows that after refrigeration of the heat treated samples the *L. monocytogenes* cells recovered during the refrigeration period.

Figure 5.4: Viable count of *Listeria*-spiked samples plated on BHI agar after heating and cold storage



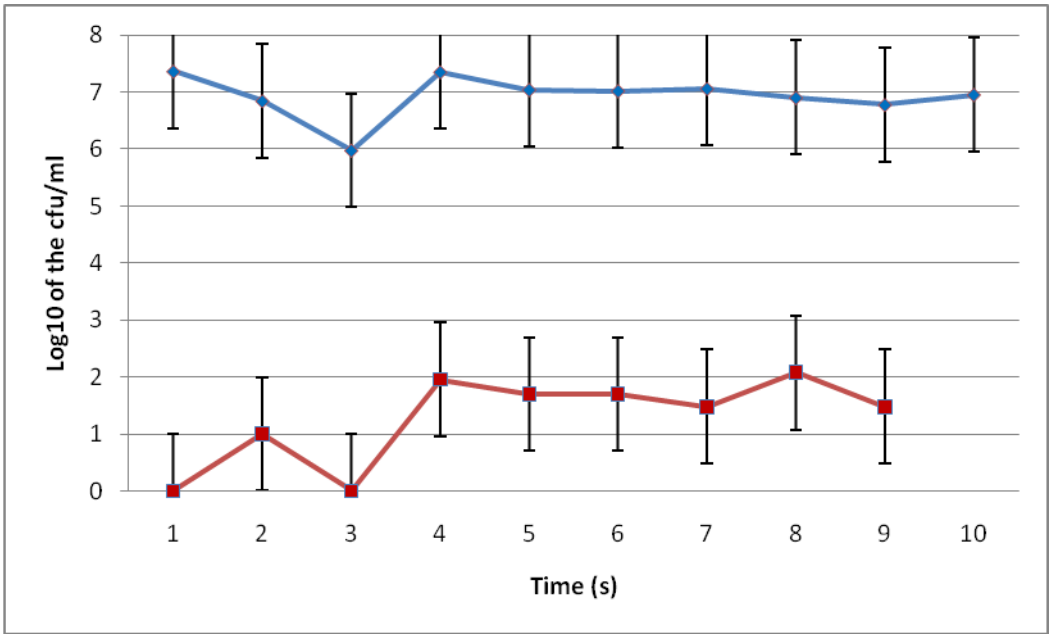
Graph shows the $\log_{10}$ of the viable count of *L. monocytogenes* and background flora after heating (red line) and after heating plus refrigeration (blue line). Data represent the results of an average of 5 replica samples.

Figure 5.5: Viable count of *Listeria*-spiked samples plated on TSYE agar after heating and cold storage



Graph shows the log10 of the viable count of *L. monocytogenes* and background flora after heating (red line) and after heating plus refrigeration (blue line). Data represent the results of an average of 5 replica samples.

Figure 5.6: Viable count of *L. monocytogenes* from spiked samples after heating and cold storage

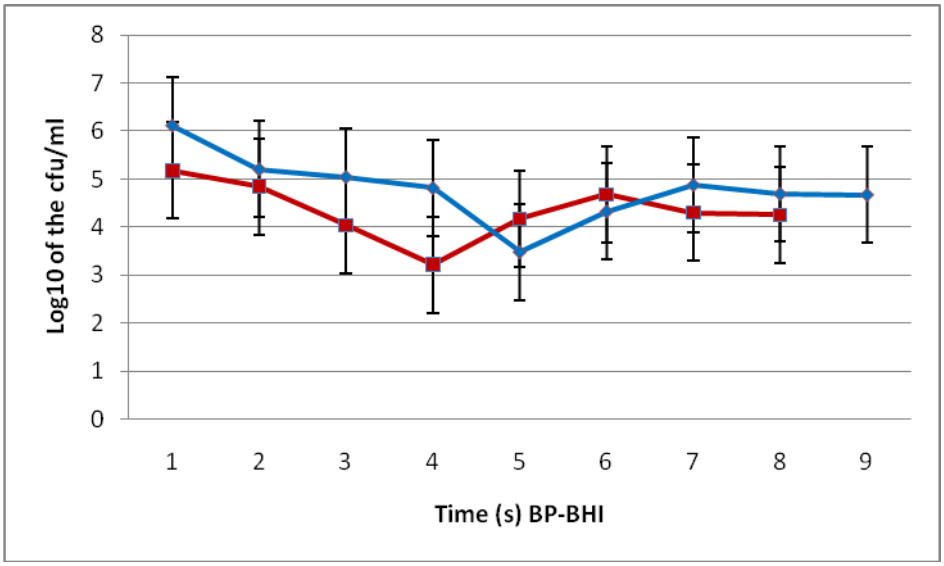


Graph shows the log10 of the viable count of *L. monocytogenes* on PALCAM agar after heating (red line) and after heating plus cold storage (blue line). Data represent the results of an average of 5 replica samples.

5.4.4. *S. aureus* after heat treatment and cold storage

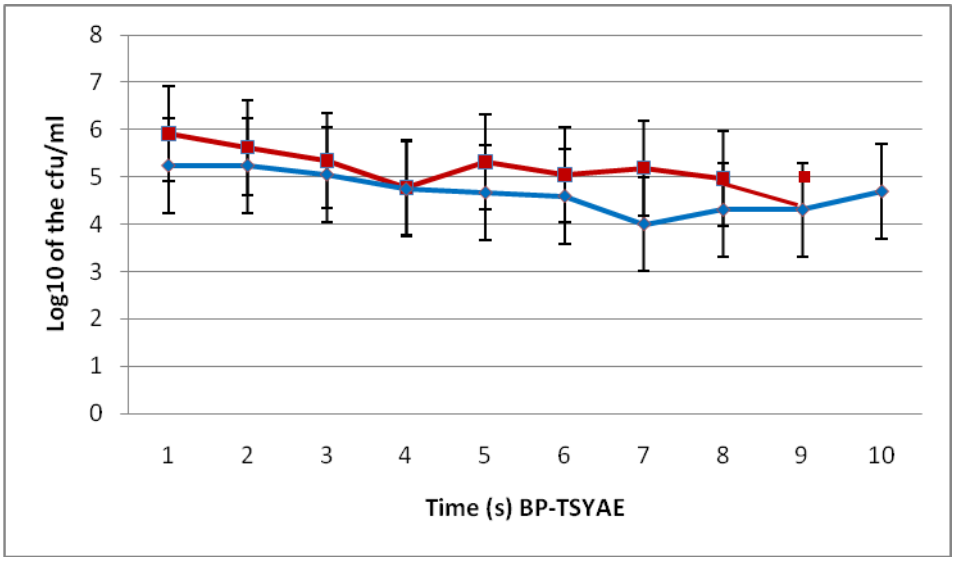
The cold recovery experiment was also carried out using *S. aureus* isolated from S-MMX. In contrast to the results gained for *L. monocytogenes* after cold storage, Figure 5.9 shows that the *S. aureus* cell numbers did not increase during refrigeration, and indeed cells seemed to die or become damaged as the viable count was reduced to undetectable levels in the samples treated for 8 s or more. In this case the counts seen on TSYE and BHI agar were not very different before or after cold storage and no cold recovery effect could be seen at all.

Figure 5.7: Viable count of *S. aureus*-spiked samples plated on BHI agar after heating and cold storage



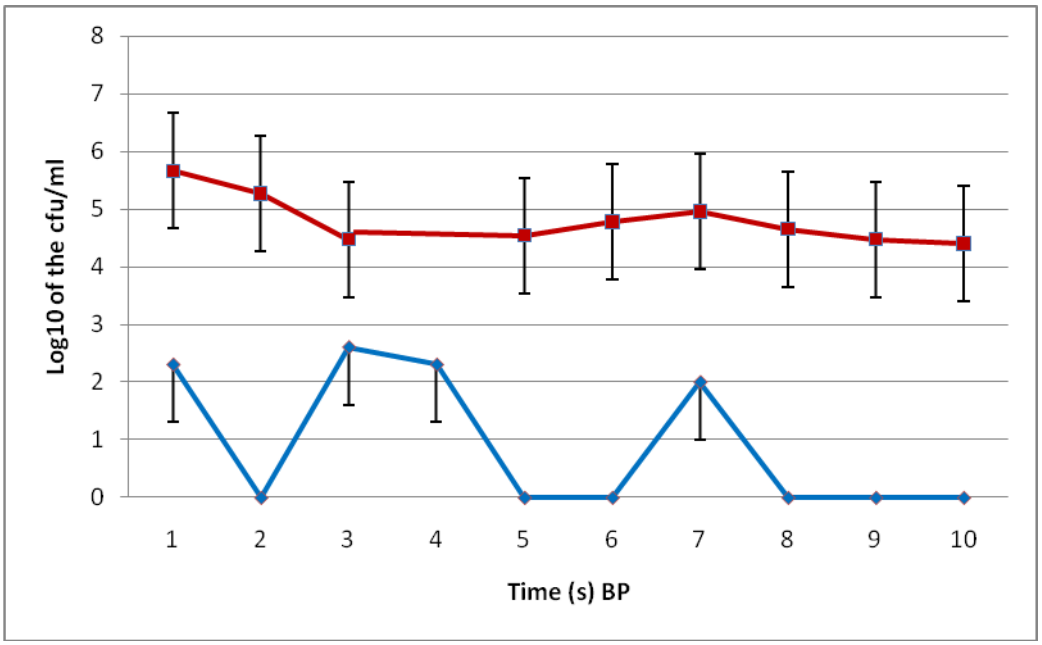
Graph shows the log10 of the viable count of *S. aureus* and background flora after heating (red line) and after heating plus refrigeration (blue line). Data represent the results of an average of 5 replica samples.

Figure 5.8: Viable count of *S. aureus*-spiked samples plated on TSYE agar after heating and cold storage



Graph shows the log10 of the viable count of *S. aureus* and background flora after heating (red line) and after heating plus refrigeration (blue line). Data represent the results of an average of 5 replica samples.

Figure 5.9: Viable count of *S. aureus* from spiked samples after heating and cold storage



Graph shows the log10 of the viable count of *S. aureus* on BP agar after heating (red line) and after heating plus cold storage (blue line). Data represent the results of an average of 5 replica samples.

5.5. DISCUSSION

5.5.1. Performing Heat Treatment Experiments

Each organism tested was isolated from the powdered food products as this might give an insight into whether the organisms found in the powdered foods have particular attributes that allowed them to survive the rigorous processing and the powdered food environment. A temperature of 72 ^{o}C was chosen to perform the tests as this is the standard pasteurisation temperature. This type of pasteurisation is called High Temperature Short Time (HTST) pasteurisation. Normally it lasts for 15 s however Farber (1989) had determined that *L. monocytogenes* has a D-value at 72 ^{o}C of 7 s and so this heating regime would only be expected to reduce the *Listeria* cell numbers by 2 $\log_{10}$ cfu/ml.

The samples spiked with the organism were plated onto three separate agars, the appropriate diagnostic medium for the organism under investigation and then BHI and TSYE agars. TSYE and BHI agars have the ability to aid in the recovery of organisms that have been heat treated. This was shown by Ellin and Alejandro (2000), where they investigated thermal resistance in *Salmonella spp*. As the powdered food product being tested was not sterilised beforehand (autoclaving would alter the consistency and filter sterilising was almost impossible because the small pore size filters block because of the high protein content) so any background flora would be recovered on both BHI and TSYE agars. In these experiments similar counts from both BHI and TSYE plates were gained and ANOVA statistical analysis showed that there was no significant difference in the numbers recovered. Diagnostic agars were used to determine whether the bacteria under investigation that were added to the samples had grown, by eliminating the ability of the background flora to grow. However this raised the problem that, whereas TSYE and BHI agars aid in the recovery of injured cells, the selective agents in the diagnostic agars may affect the viability of injured cells (Pinto *et al.,* 2001).

5.5.2. *Staphylococcus aureus* Response to Heat Treatment

After heating the *S. aureus* for 10 s at 72 °C the organism did not seem to die as expected from previous reports, as there was not even a one $\log_{10}$ reduction in the number of cells surviving in the sample. This is in contrast to the results of Jackson and Woodbine (2008), when an enterotoxigenic strain of *S. aureus* was subjected to heat treatment and subsequently inoculated into nutrient broth and incubated at 37 °C and a fall in viable numbers was seen. At 72 °C the *S. aureus* should have been killed, since the sub-lethal heat treatment used by Jackson and Woodbine (2008) was only at 65 °C and they saw a fall in viable count. However their experiment was different and the use of broth over agar may have made a difference in the results compared to those in this study.

It is possible that there may have been problems with the heating within the Wheaton vials. Even though the probe reached 72 °C in the test experiment, each sample did not contain a probe and the reconstituted product may not have reached the appropriate temperature. In addition, heating was only carried out for 9 s, whereas pasteurisation at this temperature is for 15s. So it could be expected that a further drop in viable cells detected could occur after a longer length of heating. The ingredients in the powdered milk as well may offer protection from heat treatment. Increased milk-solid levels have been shown to affect a significant increase in the heat resistance of *L. monocytogenes* (Dega *et al.,* 1972). The fact that the reconstituted powdered food had a very large increase in solids levels compared to just milk alone, possibly suggests that protection from heating may have occurred.

After refrigeration in MRD however, it was expected that relatively similar numbers of bacteria would be recovered on the agar plates as, in that cold, low nutrient environment, growth would not be encouraged, but at the same time it was not expected to kill the organisms. However from figure... the viable count of the *S. aureus* dropped dramatically. This suggests that during cold storage the

S. aureus may have become further damaged rendering them unable to grow. Another possibility is that a response to the cold-shock has put the organism into stasis and then removing them from the refrigerator and incubating them at 37 ^{o}C creates a heat-shock for the organism. This would then injure the cells further and lead to death or inactivation. As well as this the combination of being plated onto a selective medium, which is a much harsher medium t be grown on, may affect the way the cells come out of stasis (if they are in stasis, maybe dead). Therefore although the *S. aureus* may have survived the 72 ^{o}C heat treatment, the succession of heat and cold shocks may have reduced the viability of the cells, and therefore killing or sub-lethally damaging the cells.

5.5.3. *Listeria monocytogenes* Response to Heat Treatment

After heating *L. monocytogenes* at 72 ^{o}C in the reconstituted powdered milk, a significant reduction in colonies formed on the PALCAM agar was noted. Farber (1989) demonstrated that *L. monocytogenes* had a $D_{72^{o}C}$ value of 7 s when plated onto TSYE agar. The dramatic drop seen here, even after only 1 s of heat treatment suggests that the organism had died or been damaged enough not to be able to grow. This also suggests that after the full 15s of heat treatment that enough of the organism would be killed to make the milk product safe. This is also supported by Beckers *et al.* (1987) whose experiments showed that *L. monocytogenes* inoculated at a level of 1.8×10^{4}/ml did not survive heating at 67°C for 20 s or more. They therefore concluded that normal pasteurisation of milk would prevent the contamination of *Listeria monocytogenes*. However Doyle *et al.* (1987) did report that pasteurisation may not always work at the minimum temperatures, as he found viable growth of *L. monocytogenes* after treatment at 72^{o}C for 15s. In the experiment performed here the rapid inactivation seen when plating on PALCAM agar suggests that *Listeria* cells recovered from food products

that had been pasteurised (and are therefore heat injured) may not be detected if plated on selective media to look for the presence of the organism.

The results become more interesting after the samples were refrigerated for 3 days at between 3-5 ^{o}C. The results show a significant increase in viable *L. monocytogenes* cells and the opposite effect was seen on the viability of *S. aureus* cells which decreased significantly when grown on their respective selective agars. The high numbers that grew on the PALCAM plates following the refrigeration and subsequent 37^{o}C incubation suggests that the heat treatment at 72 ^{o}C did not kill the *L. monocytogenes,* but only sub-lethally damaged them and these showed recovery under refrigeration conditions. This may help explain why different authors have seen different results when studying the heat inactivation of this bacterium.

L. monocytogenes is a psychrotrophic food-borne pathogen and therefore has the ability to adapt to and grow at both 37 °C and refrigeration temperatures. At least twelve cold shock proteins are induced by cold shocking *L. monocytogenes* from 37 ^{o}C to 5 ^{o}C (Bayles *et al.,* 1996). These cold shock proteins are known to promote the survival and then proliferation of the organism at low temperatures. Therefore being placed in the cold does not prevent this bacterium from metabolising while in the MRD, and recovery from sub-lethal damage would then be very possible. When the organisms were returned again to 37 ^{o}C, they would now therefore be better able to withstand the heat shock experienced. However compared to Doyle *et al.* (1987), whose results were the reverse from this experiment. He showed that holding milk contaminated with *Listeria monocytogenes* at refrigeration temperatures for 4 d reduced the ability of the organism to grow after heat treatment. Using this model the results gained for *S. aureus*, which is mesophilic and cannot grow at low temperature, could be explained if it would not be able continue metabolising and carrying out repair at 5 °C. Hence these cells – which also experienced a cold shock - could not

recover the damage and then slowly died resulting in a decrease in the viable count on selective agar after cold storage.

5.5.4. Conclusions

The time available for this work only allowed one experiment with one strain each of *L. monocytogenes* and *S. aureus* isolated from one powdered food product. However this has produced an interesting result which will require a much more intense study to investigate it fully. This would require an increase in sample size and the number of strains tested to determine the validity of the conclusions drawn, especially as some of the results conflict with results reported from experiments performed by other researchers. However they do give rise to the idea that standard tests of pasteurised products may not be effective in identifying sub-lethally injured bacteria that have survived the process. If foods are cooled following the pasteurisation step, this may in fact give *L. monocytogenes* the ability to survive harsh treatment further down the processing line e.g in cheese production or even here in the desiccation from spray drying. Therefore investigating the appropriate storage of products used during processing could be just as important as studying the heat treatment alone when trying to design safe food products.

CHAPTER 6

DISCUSSION AND FUTURE DEVELOPMENTS

6.1. DISCUSSION

Powdered foods generally have a fairly low microbial load, due to the drying processes and other physical treatments that occur during their production, which can be more severe than standard pasteurisation (University of Guelph). Despite this these foods have been implicated in outbreaks of illness. Most significantly, there was an outbreak among new born babies. Ten out of the 12 neonates, who became ill, were fed orally with the same brand of powdered milk formula. *Enterobacter* (now *Chronobacter*) *sakazakii* was isolated from the implicated prepared formula milk as well as from several unopened cans of a single batch (Acker *et al.,* 2001). This organism had the ability to survive in unopened cans of powdered infant formula milk. This poses the question of what else may be able to survive in such harsh conditions for such a long time?

The four Gram-positive pathogens under investigation in the work are well known food borne pathogens. *B.cereus* and *C. perfringens* have the ability to form spores that enable them to survive harsh environmental conditions often used as food preservation measure to keep food safe to consume. *L. monocytogenes* and *S. aureus* are unable to form spores, however it has been widely documented, for example by Mead *et al.* (1999), that both of these organisms are implicated in food borne disease and that they are very hardy and have the ability to survive in relatively harsh environments. A review of the literature at the beginning of this project identified the fact that all these Gram-positive bacteria had been found associated with powdered foods and therefore these formed the further focus of this study. In parallel to this work, samples were analysed for the Gram-negative pathogens *Salmonella enterica, Chronobacter sakazakii* and *Escherichia coli* but these results are not reported here as they did not form part of this thesis work.

6.2. SAMPLING

Forty eight powdered food products aimed at individuals, who may be immunocompromised, were tested for the Gram-positive organisms mentioned Section 5. The choice of products was based on the fact that they would be those consumed by at risk groups, and where no clear regulations existed for the allowable levels of these organisms. Testing was carried out using ISO methods of identification, so that the results would be representative of those gained by the manufactures carrying out their own product testing. Each powdered food product was reconstituted and sampled using diagnostic agars to determine how many products were contaminated with these agars. However further tests were then performed to confirm the identity of the presumptive organisms which are not always undertaken during routine food sampling.

Bacillus cereus can make protective spores that enable survival through harsh conditions and environments and therefore its presence in powdered products is not that unexpected. Through the sampling of the 48 powdered foods, it was found that 38% (18/48) of the products were contaminated with presumptive *Bacillus cereus.* This organism has the ability to survive and contaminate a wide range of foods, especially milk. It has been reported that *Bacillus cereus* is the most important of the spore-forming microorganisms for this product because its spores are ubiquitous in raw milk and can survive the pasteurisation process, and also produces different enterotoxins which may cause food poisoning of the diarrhoeal or emetic types (Granum *et al*. 1993). However a concerning result from this study is the levels that were found in the foods. In some cases, especially in the elderly build-up products, there were counts higher than 10^6 cfu/ml recorded on the selective agar (see Section 3.2.2). This shows that even if all of these presumptive isolates did not turn out to be *B. cereus,* the high numbers found in the powdered foods are still concerning and above recommended cell numbers. For instance the HPA guidelines for microbiological

safety of RTE foods states that consumption of foods containing large numbers of Bacillus (10^5 to 10^9 cfu/g or more) can result in food borne illness (Health Protection Agency, 2009). Since these organisms grow over a wide range of temperatures, it is possible that once the product is rehydrated significant growth will occur allowing them to reach these critical levels.

As *C. perfringens* is a spore former, one might have expected similar levels of contamination to those seen for *B. cereus.* However although spores of the bacterium are found in the environment, its presence in foods is mostly indicative of faecal contamination as it is more commonly found in the gut. Hence good hygienic production standards should limit the amount of this organism being found. Colonies whose morphology had the characteristics of *C. perfringens* on TSC Agar indicated contamination of seven foods as with this organism - but only one of them was actually *C. perfringens* and one was *C. difficile*. This is a considerably lower frequency than found with *B. cereus,* however this organism is an obligate anaerobe, and its ability to form spores is not as prominent as that of *B. cereus* and therefore manufacturing conditions do not favour its survival. In fact it is very difficult to obtain a satisfactory degree of sporulation for many strains of *C. perfringens* even under optimum laboratory conditions (Duncan and Strong, 1968). This may be why there are not as high contamination rates of this organism compared to *B. cereus*. However the food with contamination did yield above the recommended levels of contamination for powdered foods, which is 10^4 cfu/ml (Warburton *et al,* 1998). This again is serious as these products are designed to be used by individuals who may be susceptible to these pathogens, and could develop food poisoning. In this respect the presence of *C. difficile,* which can colonise the gut and cause acute diarrhoeal disease, is perhaps even more worrying.

The two non-spore forming bacteria have the ability to survive in harsh environments experienced, but should have been killed by heat treatments such

as pasteurisation. Therefore their isolation from these food products may constitute poor manufacturing processes. Indeed in 2007 there was an outbreak of *L. monocytogenes* associated with a milk processing plant. Swabs from around the plant after product recall had found that the company had not implemented an environmental monitoring programme that is considered GMP (MarlerBlog, 2010). Thus the *L. monocytogenes* was found presumptively in 10 powdered foods and confirmed in three. Levels of contamination were fairly low, except in three implicated sports products, where levels of above 10^4 cfu/ml were seen which is above the infective dose required to cause illness. A concern here is that even if low levels of organisms are present, this bacterium can start to grow and increase numbers even if the reconstituted product is stored chilled.
S. aureus was presumptively identified on BP Agar in 18 powdered food products, which is the same levels as that found for the spore-forming *B. cereus.* This is surprising given that pasteurisation is meant to be used to inactivate *S. aureus* (Evrendilek *et al.,* 2004). This then suggests that again poor manufacturing processes may have led to the contamination of the range of food products. As well as this, colony counts from one elderly build-up product and one sports product were higher than 10^4 cfu/ml. This suggests a serious problem as these are very high cell counts and could potentially lead to food poisoning.

All the products contaminated, whether with spore formers or not, suggest that there is something wrong with the production process of these foods, especially as they are marketed as RTE products for vulnerable people who may become seriously ill if they ingest contaminated products. Particularly surprising is that the regulations for foods aimed at 0-6 months are not clear, regulations are only aimed at older babies over 6 months old rather than newborns under 6 months. Neonates are a high risk group as they have an under developed immune systems, especially if they are not being breast fed. The rates of

contamination indicate the need for better regulations regarding the production of infant formula milk.

6.2.1. Further Development of Sampling Practices

The methods used for isolating these pathogens from the powdered food were ISO standard methods. These can be long and laborious, especially in the case of *L. monocytogenes* which requires lengthy pre-enrichment steps. However these methods are internationally recognised and it would be useful to always use them when trying to identify pathogens from any food product. However some ISO methods can use different diagnostic agars or protocols, for example, a number of culture media specifically formulated for the detection and enumeration of *B. cereus* have been described. All have certain limitations on their use. Problems of detection of atypical strains of *B. cereus,* and poor suppression of the growth of background organisms are some of the problems that can be encountered (Netten and Kramer, 1992). This suggest a review of sampling methods that used by different researchers should be undertaken so that the best media for particular uses can be identified. Until this is done there may be either under or over estimating the number of colonies formed on the media leading to inaccurate reporting or unnecessary product recall.

However the results gained from these isolation studies only covered a very small sampling size of 48 products. Only one product from each type of powdered food was tested with no opportunity to look at a number of batches of the same product. This constitutes a very small sample size that may not be indicative of contamination over the whole range of products. The products tested in this project may have been from a bad batch or just one unfortunate product. To increase the reliability of these results, multiple items from different batches of each product would need to be taken at different times of year. If this then led to similar contamination results, then it would add more strength to the

conclusions drawn. One factor that should be considered is that it has been reported, especially for milk based products, that the time of year at which the milk has been collected may affect the microbial load. Slaghuis *et al.* (1997) found that during the winter months, if the cows are housed, the quantities of *B. cereus* spores in the milk decreased. Thus time of the year when the products are made may have an impact on what is in the products and therefore manufacturing controls may need to be different at different times of the year.

Overall, six IFM, 11 SD and seven EBU powdered foods were positively identified as being contaminated with one of the pathogens under investigation in this study (Section 4.7), with some of these products being contaminated with more than one organism, suggesting a serious lack of GMP.

6.3. IDENTIFICATION

Identification of the presumptive isolates was essential to determine whether or not the organisms, isolated on the diagnostic agars, were the bacteria under investigation. Biochemical and molecular based methods of identification were used to further characterise organisms that exhibited characteristic features of the bacteria in question under microscopic analysis. This was used because the positive identification gained from two different methods would give rise to a conclusive identification of the organisms that contaminated these foods.

However this was not successful for *B. cereus* as time limits meant that the molecular identification was not completed. One factor that caused this is that there are no ISO PCR identification protocols available and all the PCR assays had to be developed working only from published papers. Therefore reliance on the cell and colony morphology on diagnostic agar, coupled with Gram stain and the ability to form spores, were used to identify the organism. Eighty nine percent (16/18) of the characteristic colonies had the features attributed to *Bacillus cereus.* The two products that were ruled out as containing this

organism were two infant formula products, B-AH and B-HPF. The 16 products most probably were contaminated with *B. cereus;* however it cannot be proved conclusively due to the lack of molecular identification.

Problems with the identification of *C. perfringens* after basic characteristics had been identified (black colony, Gram positive non-motile rod, *beta*-haemolytic). Three samples were identified as *C. perfringens* but after biochemical analysis using API 20A, out of the three samples that had the characteristic attributes of this bacterium, one organism, from product E-NS, was found to be *Clostridium perfringens*. That from product S-LSP, after API analysis, was found to be contaminated with *Clostridium difficile* and one product E-NC contained a strain which was unidentifiable. The API tests are widely accepted when used for identification. Therefore the assumption can be made that only one of the seven products that yielded colonies was contaminated with *C. perfringens*. This shows how the diagnostic medium used to identify *C. perfringens* may be flawed as only one sample was positively identified. This suggests that possibly two of the diagnostic mediums used together (TSC and SFP) may have to carried out to reduce the chances of false positive results.

To identify the organism using molecular methods, primers were used that target conserved regions in the organism's DNA that are involved in making the toxins responsible for food poisoning, and are found in all the toxigenic species of *C. perfringens*. One organism, again which was from E-NS, was found to have the toxin genes. This again supports the API identification as well as showing that this product is contaminated with toxigenic *C. perfringens* and therefore presents a risk of causing food borne disease. However as described in Section 4.4.4, no positive controls were used for the experiment. This is due to restrictions on culturing and storing these bacteria. However the biochemical results alone would suggest contamination of this organism and therefore be a cause for concern.

L. monocytogenes did prove simpler to identify as both the molecular and biochemical identification methods are relatively simple to carry out. Ten samples were presumptively identified as being contaminated with *L. monocytogenes.* Seven of these were then, after microscopic and haemolytic analysis, considered to have the attributes of *L. monocytogenes.* It must be mentioned that this organism normally exhibits a weak *beta*-haemolysis; however several of the isolates had a strong haemolytic reaction which is more characteristic of *Listeria ivanovii.* Therefore to determine which organisms were indeed *L. monocytogenes,* API Listeria testing was carried out. The results from these tests proved inconclusive as they were all unidentifiable *Listeria* spp. Identification using PCR was then carried out on these and this test was able to easily distinguish between the different species. As this assay is a multiplex reaction, the production of the Listeria genus-specific band provides a natural internal PCR control, as all bacteria identified as members of the group should produce a band. In this case one organism did not, which again illustrates the need for independent internal PCR controls as this negative result could simply indicate PCR failure (Murphy *et al.,* 2007).

The PCR analysis showed that three out of the seven isolates from products: B-HPF, S-MMX and E-NV were *L. monocytogenes* and three were *Listeria* spp (those from products S-MMP, S-BSN and E-NC). One from product B-SLF was apparently no part of the *Listeria* genus. Interestingly B-HPF, which exhibited a strong haemolytic reaction, was identified as *L. monocytogenes,* whereas B-SLF that gave a weaker *beta*-haemolytic reaction was not part of the *Listeria* genus at all. Therefore the basic biochemical analysis that was carried out is liable to yield false positive results, even if interpreted results are characteristics linked to *L. monocytogenes*.

S. aureus was the simplest organism to identify. There is a breadth of research on identification methods as this is also a major human nosocomial

pathogen, which enables identification to be very simple and normally accurate. Biochemical analysis of organisms showing characteristic colony and cell morphologies using catalase and coagulase tests were carried out. It showed that out of the 18 products that seemed to be contaminated with *S. aureus* (black shiny colonies, with or without halo), eight were tested positive for catalase and coagulase and one was just catalase positive, which suggest it is a coagulase negative *Staphyloccus*. Therefore less than half of the presumptive organisms were confirmed to be *S. aureus.* This is quite a reduction in number of presumptive organisms to those fully identified. However BP agar does allow certain *Enterococcus* spp. to grow on it. This, coupled with the occurrence of coagulase negative *Staphylococcus,* which does not form a halo, means that false negatives are likely to form.

To confirm the identity of the nine isolates, a PCR reaction, testing for *S. aureus* and its toxins was carried out. The results showed that all, except that isolated from product S-LSP, was identifiable as *S. aureus.* For some isolates additional bands were seen at different positions on the gel (albeit there was also a band of the correct size produced). This may have been because the PCR temperature was not exactly right as variation from PCR block to PCR block does occur, and this allowed the primers to bind at the wrong position. However the reaction was carried out twice, with the same result. Coagulase negative *S. aureus* are uncommon but interestingly when they do occur they are found in dairy products from cows. This seems to be the case from this study as the molecular identification confirmed that this organism was an atypical *S. aureus.*

6.3.1. Further Identification

As explained before, *B. cereus* is not easy to distinguish from its nearest genetic neighbours. The diagnostic features of the MYP medium rely upon the failure of *B. cereus* to utilise mannitol and the ability of most strains to produce

phospholipase C, which does act as a good indicator of *B. cereus* (Mossel *et al.,* 1967). More biochemical analysis should have been carried out although a positive identification would still give some doubts about its true identity, because of the similar characteristics of organisms genetically closely related to it. Therefore carrying out the ARDRA analysis would be beneficial in identifying the organism as definitively as possible. Given more time this would be executed to identify the organism as accurately as possible. However if the only difference between *B. cereus* and *B. thuringiensis* is the presence in the latter of a plasmid encoding the delta toxin, while the toxin genes for food poisoning are on the chromosome hence as far as human disease is concerned it does not really matter that they cannot be differentiated as they both behave as the same organism.

Alternative identification using immunoassays may be a beneficial route to pursue. Again it is difficult to pinpoint the proteins or structures on *B. cereus* that are not shared by it close relatives, however its toxins can be targeted. *B. cereus* Enterotoxin-Reversed Passive Latex Agglutination kit (Oxoid) detects the L2 component from haemolysin BL, which is involved in causing diarrhoea in individuals (Beecher and Wong, 1994). However this only detects one toxin and there are several toxins associated with diarrhoeal disease and *B. cereus* has the ability to produce an emetic toxin (cereulide) as well. Therefore the need to use two sets of kits, especially antibody based, can become quite expensive for routine diagnostics.

C. perfringens again is a difficult organism to identify because of its restrictions regarding holding the cultures as well as the organism being difficult to identify. The API 20A kit gave a fairly good and reliable identification. Coupled with the result from the PCR identification, the isolate from E-NS was shown definitely to be contaminated with *C. Perfringens* and it is notable that the method developed for this molecular test does not require the growth of large

cell numbers for DNA purification. However the API 20A kit gave results that were inconclusive for the other isolates and as the PCR only identifies toxigenic strains of *C. perfringens.* This then leaves several organisms that were unidentified although the API did identify some as *Clostridium beijerinckii* but this again that the medium is not very specific for *C. perfringens.*

To confirm the presence definitively of *Clostridium difficile* further identification of the sample that was identified by the API 20A test would also have to be carried out.

The tests carried out in this project for *L. monocytogenes* were adequate to confirm their identity. However the API Listeria did not give any definitive results. As described before, faulty reagents from the kit may have been to blame. However normally the haemolysis and microscopic analysis would be enough to confirm the results from the PCR analysis. However further identification of the non-*L. monocytogenes* isolates would need to be carried out. This is because identification of the possible *Listeria ivanovii* is important due to it being identified as a rare but potential pathogen of humans. Therefore the PCR protocol designed by Bubert *et al.* (1999), where they used primer sets specific for seven different species of *Listeria* to help differentiate them. In the current study there were also, initially, problems with optimising the PCR reaction. This was because it was difficult to gain the two bands from the control. Changes in annealing temperature, $MgCl_2$, primer or DNA concentration may all be factors affecting the PCR reaction. However the most common problem was temperature. If sampling again, a temperature gradient would be set up. This would then be used to find the optimum annealing temperature for the PCR reaction when performing the PCR reaction in the PCR blocks available in this laboratory.

S. aureus identification was the easiest to carry out. The only problem came with the presence of coagulase negative organisms and the optimising of the

PCR reaction. Again like the *Listeria monocytogenes* PCR, a temperature gradient could be set up initially to determine the correct annealing temperature to optimise the PCR. Determining whether the strains were toxigenic or not could also be carried out, as this is important in determining whether the presence of this organism would be likely to cause food poisoning. However as described before *S. aureus* should not survive pasteurisation but its toxins can. Therefore it may be prudent to test all samples for the toxins using the molecular method described in Section 4.6 even if no growth was seen on BP Agar. This is because toxins may still be present even if viable cells are not.

6.4. HEAT TREATMENT

Heat treatment of the non-spore forming organisms was carried out to give an idea about when the contamination of the food products may have occurred. *Listeria monocytogenes* and *Staphylococcus aureus* should not be able to survive heat treatment. Therefore results that show viable numbers of *Staphylococcus aureus* were seen directly after heating and that *Listeria monocytogenes* seemed to recover after 3 d of refrigeration suggests that heating at 72 ^{o}C may not be sufficient in killing the bacterial cells completely. However this is only one heat treatment experiment. Repeats would need to be carried out to see if this holds true each time the organisms are subjected to heat. As well as this pasteurisation at 72 ^{o}C should last for 15 s. However in this experiment it only lasted 10 s to act as a comparison with other published work, however if the curve is extrapolated to the 15 s, a one log reduction is still not seen. There are also other temperatures used for pasteurisation, therefore performing experiments that incorporate different parameters should be considered so that they can be compared.

6.5. CONCLUSIONS

The aim of this project was to determine whether organisms, other than those already known to be associated with powdered foods, posed a risk to human health. The products studied were chosen as they are ones that are likely to be consumed by at risk, immunocompromised groups, and therefore even low levels of contamination are more likely to result in disease. Given the natural source of this bacterium in raw milk and the fact that it forms spores, the finding that products were contaminated with *B. cereus* was not surprising. However the high levels of contamination found suggest that insufficient care is being taken during the manufacturing process. *C. perfringens* contamination rates were low, but given the small sample size again this finding was especially as some products were found to be contaminated with multiple pathogens.

Neither of the non-spore forming bacteria *L. monocytogenes* and *S. aureus* were expected to be found in these powdered products and therefore, again given the small sample size studied, there does seem to be a high risk of food-borne disease being caused by these products that are not heated again before ingestion. Their presence may be due to post-process contamination, as recently reported in an outbreak case in the US (MarlerBlog, 2010). However initial heat inactivation studies completed as part of this thesis work may suggest another explanation for their presence. Work is currently being undertaken to investigate this further.

The other part of this project focussed on the generation of web-based material designed for CPD of staff working in the food industry. If the workers in these fields are better trained and informed then it may help prevent outbreaks of disease caused by breakdown of GMP or other failures in the manufacturing process. This would result in a lower risk of food-borne illness, not only caused by these powdered foods, but by manufactured food products in general.

CHAPTER 7

BIBLIOGRAPHY

8.0. BIBLIOGRAPHY

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CHAPTER 8

APPENDICES

Appendix 1
Appendix 1.1: Web text for *B. cereus* for CPD scheme

Background/History

Organism name – *Bacillus cereus* is a facultative anaerobe that in some strains are harmful to humans, causing food poisoning. This organism can also produce protective spores that can allow them to survive harsh environments.
Outbreaks – It is difficult to keep records of this organism. This is because its emetic symptoms are similar to that of *Clostridium perfringens.* Therefore misdiagnosis and people not reporting the illness may be a factor in why this organism may seem to have low rates of infection. Nevertheless, some large outbreaks have occurred; for example, in 1989 over 100 people were affected in the USA (Slaten *et al.,* 1992).
U.K Outbreak –There have been isolated incidents in fast food restaurants where large quantities of rice are prepared and insufficient temperatures are used when storing. In a UK survey of take-away restaurants found that 10% and 3% of cooked rice meals were of unsatisfactory and unacceptable microbiological quality, respectively (Little *et al.,* 2002).
European Outbreaks – between 2001 and 2002 three separate outbreaks of *B. cereus* was recorded in hospitals in Germany. There were no fatalities; however the fast spread of the organism through the wards was a major cause for concern.
Uses – Non-toxic strains of *B. cereus* can be used in probiotic supplements in animal feed, as it competes with other pathogenic organisms in the gut therefore lowering the overall pathogenic bacterial load in the intestines.
Where found – *B.* cereus is found in a variety of places. The organisms have been detected in soil, water and on food crops. Starchy foods, such as rice or potatoes, are commonly associated with B. cereus emetic (vomiting) toxin outbreaks, whereas the diarrhoea-causing strains have been found in a wider selection of foods. Common sources include meat and vegetable items, soups and milk products (Gilbert, 1979).

Table 1: Classification of *B. cereus*

Classification	
Kingdom	*Prokaryotae, (Bacteria)*
Phylum	*Firmicutes*
Class	*Bacilli*
Order	*Bacillales*
Family	*Bacillaceae*
Genus	*Bacillus*
Species	*cereus*

Genome Size

Figure 1. *Bacillus cereus* complete genome (BacMap,

The size of the *B. cereus* genome is approximately 5,224 kbp with approximately 5,736 genes found on the genome.

Morphology

Organism characteristics:

- Rod shaped bacillus
- Gram positive
- Facultative anaerobe
- Beta-haemolytic
- endospore formation
- Toxin production in some strains, (two types emetic and diarrhoea causing)
- 1 µm wide, 5-10 µm long.
- Gram's stain – *B. cereus* are Gram positive arranged singly or in short chains.
- Spore staining - Malachite green stain is forced into the spore by heating the cells. Vegetative cells are then decolorized with water and stained pink with safranin counterstain (see Figure 1)
- **Growth –** *B. cereus* has an optimum growth temperature between 30°C and 37°C. However the organism has been shown to be able to grow at much lower (4-5°C) or higher temperatures (55°C)**.** The organism can also survive a pH range between 4.5 and 9 (New Zealand Ministry of Health, 2001).
- **Stress –** When introduced to high temperatures, *B. cereus* vegetative bacteria will produce endospores that are more resistant to heat. The spores also allow the bacteria to survive in extremely dry conditions (dried/powdered food).
- **Toxin Stress Resistance –** *B. cereus'* emetic toxin can survive extremely high temperatures and a pH range of 2-11. The diarrhoeal toxin can survive high temperatures as well.

Reproduction/Life cycle

- Transmission – is through the eating of contaminated food. As the organism is found ubiquitously throughout the environment sources are plentiful.
- The emetic syndrome occurs mainly from starchy foods such as rice, where *Bacillus cereus* spores have survived cooking and begin to activate if left at poor storage temperatures making the emetic toxin that can survive stomach acid.
- The diarrhoeal syndrome occurs with the ingestion of the spores or cells and they subsequently make the toxin inside the gut causing abdominal pains and diarrhoea.

Clinical Disease

- There two forms of illness derived from *B. cereus.*
    - The first is emetic, which has a very short incubation time of around 1-6 hours after eating the contaminated food.
    - The second is diarrhoeal, which has a longer incubation time of 10-12 hours.
- The emetic syndrome is caused by the ingestion of a pre-formed toxin which occurs often in rice dishes that have to been stored at

appropriate temperatures. This syndrome causes vomiting and nausea and sometimes diarrhoea.

- Diarrhoeal symptoms results from ingestion of vegetative organisms or spores and their growth and therefore toxin production within the intestinal tract. This mainly causes after its longer incubation period; abdominal pain, watery diarrhoea and occasional nausea. These symptoms are very similar to *C. perfringens* which is why miss diagnosis can occur.

- The infective dose of this organism is generally between 10^6 and 10^8 cells/g.

- This organism can infect anyone at any age, however this is mainly a self limiting organism and recovery after infection is normally within 24 hours.

Pathogenicity

- There are three toxins that *B. cereus* uses to cause food poisoning in humans*;* an emetic toxin and two enterotoxins. Different strains of *B. cereus* may have the ability to produce combinations of the two enterotoxins and emetic toxins.
- The two enterotoxins cause the diarrhoeal syndrome, which is similar to that of *C. perfringens.*
    - The first toxin is called Hemolysin BL (HBL). This is a well-characterized *B. cereus* toxin composed of three components (B, L1, and L2) that together possess hemolytic, cytotoxic, dermonecrotic, and vascular permeability activities (Beecher *et al.,* 1995).
    - The second enterotoxins is nonhemolytic enterotoxin (NHE). This toxin has been found to be involved in the host cell receptor however its exact role has not been established (Bhunia, 2007).
- The emetic toxin causes vomiting and nausea, and has a very short incubation period. The emetic type is caused by a toxin called cereulide. This toxin acts as an ionophore through mitochondrial membranes and interferes with oxidative phosphorylation (Hoton, *et al.,* 2005).
- *B. cereus* also produces three types of phospholipase C. This chemical is supposed to be a virulence factor of many pathogenic bacteria. It is supposed to contribute to tissue damage by inducing the degranulation of human neutrophils (Wazny, *et al.,* 1990).

Epidemiology

- Anyone of any age can be infected with *B. cereus*. Cases have been described of increased infection rates after antibiotics that may have disrupted the flora in the gut allowing growth of pathogenic strains of *B. cereus.*
- Place of Infection – The emetic infections are most commonly the result of eating poorly cooked and stored rice or starchy products. Diarrhoeal infections come can occur from a wider range of foods, such as milk or meat products.
    - Geographically, Japan has much higher incidents of the emetic syndrome attributed to *B. cereus,* compared to that of European

and North American countries where the Diarrhoeal form is more prevalent.

- Transmission – *B. cereus* is ubiquitous in nature and can be found in a wide variety of soil samples and is naturally occurring on many foodstuffs. Transmission occurs from improper treatment of these foods through handling, storing, or insufficient heating and cooling methods.
- It is difficult to get definitive numbers for the number of cases of food poisoning attributed to *B. cereus* as it is generally non reportable organism. It can also be difficult to diagnose due to similarities in symptoms with other organisms.
- There are very few recorded fatalities related to being infected with *B. cereus.*

Reported Cases of *B. cereus* in the UK from Health Protection Agency

Year	***B. cereus***	
	Number outbreaks	**Number affected**
1996	4	118
1997	6	14
1998	0	0
1999	1	14
2000	0	0
2001	4	10
2002	3	8
2003	2	41
2004	0	0
2005	1	33
2006	0	0

Laboratory Diagnosis

- The ISO method to detect *B. cereus* involves a series of enrichment and plating on Mannitol-Egg Yolk-Polymyxin (MYP) agar. However this can yield false positives due to other subspecies of *Bacillus* being able to grow in the agar.
    - Therefore after presumptive colonies have grown on the MYP agar, biochemical or morphological tests are carried out to determine whether they are *B. cereus* or not.
    - Confirmatory tests can then be carried out by using, H-serotyping or phage-typing, or using PCR based methods of detecting.
- The ISO method above is considered fairly slow and labour intensive therefore other methods have been developed to improve timing and sensitvity.
    - PCR based detection methods have been used that detect the 16s rRNA from the isolates, this coupled with Restriction fragment

length polymorphisms (RFLP) can provide a fast and accurate method for genotyping the *Bacillus cereus.* However false positives have occurred using this method as some *Bacillus* species are very similar to each other.

- Multiplex PCR's have also been carried out to detect the toxins produced by the organism. This then enables extremely rapid and sensitive identification of the toxins. However this method is expensive.
- Immuno-assays have also been developed, have can yield highly specific and sensitive results within a day. However there is great expense in obtaining the antibodies (especially monoclonal). Immuno-assays that can be used are:
    - Enzyme Linked ImmunoSorbent Assay (ELISA)
    - Magnetic Immuno Assays (MIA)
    - Latex Agglutination Test

Recent Developments

- In August 2008 the AFGU (department within the Health Protection Agency) have developed the use of Denaturing High Performance Liquid Chromotography (DHPLC) which allows the detection of sequences such as *rpoB*, *gyrA*, *gyrB* and *rnpB,* which are specific to *Bacillus cereus,* as well as allowing rapid screening of a large number of targets for novel mutations (HPA, 2008).
- As stated above developments in the spread or incidents of this pathogen are difficult to determine as this disease is rarely reported, or it is misdiagnosed.

Prevention/Control

- As mentioned above, *B. cereus* is found in many places in the environment and so is difficult control when sourcing foods such as rice. However good hygienic food handling practices should be employed, which would limit the amount of bacteria on the food stuff before cooking.
- Although the spores of the pathogen are heat resistant and cooking may activate the spores, as long as short cooling times are applied to the food and then good refrigeration is applied low numbers of *Bacillus* would be likely to survive.
- Heating the food above 56^{o}C will destroy the diarrhoeal enterotoxin but not he emetic toxin.

Treatment

- There is not a wide range of treatment for *Bacillus cereus,* as it is a very short lived infection period (normally with 24 hours) many people would recover before antibiotic treatment was necessary. However replacing lost fluids is vital especially after diarrhoea.

Acknowledgements

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Appendix 1.2: Web text for *C. perfringens* for CPD scheme

Background/History

Organism name – *Clostridium perfringens* is a gram positive, anaerobic, rod shaped bacterium. This organism also has the ability to form spores. Food poisoning is normally associated with type A.

Outbreaks – These are normally associated with meat that has been cooked but is kept warm for extended periods of time, which may allow spores to activate and begin making toxins. Although this organism is found within the normal flora of the gut imbalances leading to larger amounts of *C. perfringens* will lead to food poisoning. A severe form of food poisoning can occur called Clostridial necrotizing enteritis. This can cause a threatening sequence of severe abdominal pain, vomiting, bloody stool, ulceration of the small intestine with leakage (perforation) into the peritoneal cavity and possible death within a single day due to peritonitis, this is associated with type *C. perfringens*.

U.K Outbreaks – A major UK outbreak of *C. perfringens* occurred 1989, where 58 elderly people in a long stay hospital developed diarrhoea and two fatalities were noted. It was found that inadequately reheated minced beef was the culprit (Pollock and Whitty, 1991).

Worldwide Outbreaks – A major outbreak in 2008 was reported in a county jail in America where 200 inmates were treated for nausea, vomiting and diarrhoea. *C. perfringens* enterotoxin was detected in food samples and large amounts of the pathogen were detected in a casserole that had just been eaten the evening before. It was thought that the food was improperly stored during the time taken to feed large numbers of inmates.

Where found – *C. perfringens* is ubiquitous in nature, being found in soil, decaying material (including animal/human) as well as being found in the intestinal tract of humans and animals. However they are found in low numbers and are often competing with other bacteria and so no illness is caused. The majority of cases come from meat prepared in advance, and that are kept warm over a period of time. This allows spores that have survived to cooking process to activate.

Table 1: Classification of *C. perfringens*

Classification	
Kingdom	*Prokaryotae, (Bacteria)*
Phylum	*Firmicutes*
Class	*Clostridia*
Order	*Clostridales*
Family	*Clostridiaceae*
Genus	*Clostridium*
Species	*perfringens*

Genome Size

Figure 1. *Bacillus cereus* complete genome (BacMap,

The size of the *C. perfringens* genome is approximately 3,031kbp with approximately 2,785 genes found on the genome.

Morphology

Organism characteristics:

- Rod shaped bacillus
- Gram positive
- Anaerobe
- Can form spores under stress
- Forms a protein enterotoxin (CPE) that causes damage to the small intestine

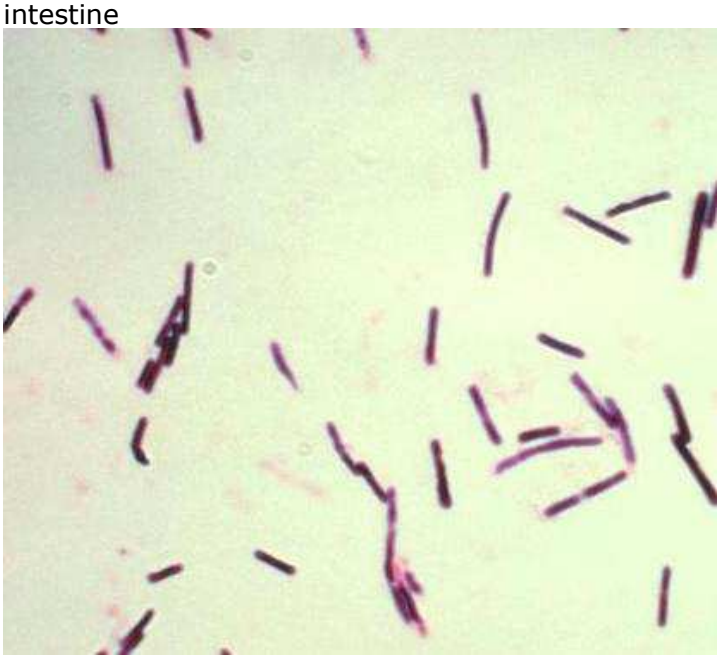

Figure 2. Gram stain of *C. perfringens*

Growth – The optimum temperature for growth is between 43-47^{o}C, however growth has been recorded between 12- 50^{o}C. However optimum temperature for enterotoxin production is between 35-40^{o}C.
C. perfringens prefers a neutral pH, where any major extremes of this result in sporulation.

Stress – When stressed *C. perfringens* forms spores that can survive high temperatures of over 100^{o}C. They can also survive freezing temperatures below -15^{o}C. Unlike the vegetative cells, the spores can survive desiccation

Toxin Stress – The toxin produced by *C. perfringens* cannot survive high temperatures for long. Therefore it is highly advisable to reheat food thoroughly before eating.

Transmission

- Transmission – is generally found when eating meat that has been kept warm for a long time, or when insufficient cooling has taken place. The organism is able to survive cooking temperatures through sporulation, and then is activated to its vegetative form. Where if at an optimum temperature a generation time of around eight minutes can occur (New Zealand Ministry of Health, 2001).
- The toxins are then made if the conditions are ideal in the warming meat, or if after refrigeration the food is not reheated appropriately infection can occur very quickly.
- As well as making preformed toxin, spores that get eaten and survive the digestive process are able to settle in the intestines and then activate and begin making toxins.

Clinical Disease

- The food poisoning normally originates from *C. perfringens* strain A, which produces the pathogenic toxin. This then causes abdominal pains and diarrhoea, and sometimes nausea and vomiting.

- There is a fairly fast onset of disease from when contaminated foods are consumed, between 10 to 15 hours. However if the toxins are produced in the food before eating, then the onset can be as low as six hours.

- People normally recover from the gastroenteritis within 24 hours; however mild symptoms have been known to last for a couple weeks after infection.

- This organism can infect anyone at any age; however the elderly or very young are more susceptible. There are very few fatalities from this organism, but the few that do die are mainly the elderly.

Pathogenicity

- Members of the species *C. perfringens* can be sub typed into five toxin types (A, B, C, D and E) based on the production of four major toxins: alpha, beta, epsilon and iota (Songer, 1996). Type A and C are only pathogenic to humans.
- *Clostridium perfringens* enterotoxin (CPE) is the main toxin produced from type A strains of *C. perfringens.* It is produced in varying amounts by almost all *C. perfringens* isolates and causes hydrolysis of membrane phospholipids in different cells, resulting in lysis (Titball, 1993).
- CPE is the major cause of food poisoning and non food borne GI diseases as this strain is found naturally in the gut. Symptoms can occur after antibiotic treatment that alters the balance of flora in the intestine. *C. perfringens* would then proliferate and induce symptoms of these CPE-associated non-foodborne GI illnesses, that include diarrhea and cramping, which tend to be more severe and long-lasting than typically

seen with most cases of *C. perfringens* type A food poisoning (Carmen, 1997).

- CPE is the main toxin involved in food poisoning. However the other toxins are thought to play a role in synergistically working with CPE to cause more serious conditions.
    - The beta toxin (*β* toxin) is a highly trypsin-sensitive protein. It causes mucosal necrosis resulting in central nervous symptoms observed in domestic animals (Hunter, *et al.,* 1993).
    - Epsilon toxin (*ε* toxin) is a potent toxin responsible for potentially lethal enterotoxaemia in livestock (Songer, 1996).
    - The iota toxin (*ι* toxin) consists of two proteins, one active component (Ia) and a binding protein (Ib). The iota toxin is known to increase vascular permeability and has been known to cause necrosis of the skin (Gibert and Popoff, 1999).
- Necrotizing enteritis (or Pigbel) is caused by toxin produced by type C strains of *C*. *perfringens.* This is a very serious condition and accounts for the majority of deaths associated with *C. perfringens.*

Epidemiology

- *C. perfringens* can infect anyone at any age. The most severe cases being found in the elderly or in people that may have debilitating conditions.
- Generally speaking the pathogen is found in meat dishes, as *C. perfringens* is demanding for its nutrition (several amino-acids and growth factors), and therefore prefers protein-rich foods (Louisiana Department of Health and Hospitals, 2008).
    - Poisoning normally occurs when large amounts of food is prepared (schools, hospitals) and not stored appropriately. It may be left warm for an extended period of time or not cooled sufficiently. Heat resistance spores survive the cooking process, and when left out begin to activate and proliferate.
    - The cells in high number manage to survive the acid environment or when reheating, the cells form spores that can survive stomach acid. Then in the intestine, toxins are made and this then causes the abdominal cramps and diarrhoea.
    - Food poisoning occurs only when heavily contaminated food is ingested.
- *C. perfringens* is ubiquitous in nature and can be found in many soil samples, water, many different foods (mainly meat) and in the gastrointestinal tracts and of many animals, including humans. Transmission occurs from improper treatment of these foods as mentioned above.
- It is not generally thought to be transmissible from person to person, unless unsanitary conditions are observed. The incubation period is normally between eight to 12 hours, but can be as long as 24 hours.
- There are few fatalities in general associated with *C. perfringens,* with the majority being related to elderly patients or people with underlying health problems, and the majority of these fatalities are related to type C of the organism not type A.

Table 2: Reported Cases of *C. perfringens* in England and Wales from Health Protection Agency

Year	Number of outbreaks	Total number Ill
1992	32	748
1993	33	529
1994	22	467
1995	25	352
1996	28	498
1997	38	661
1998	22	523
1999	11	257
2000	7	144
2001	20	439
2002	9	385
2003	2	23
2004	6	486
2005	11	509
2006	8	147

- The number of outbreaks has steadily declined over the years, which may show an increased trend of better cooking and education related to the storage of food.

Laboratory Diagnosis

- The ISO method to detect *C. perfringens* involves the use of tryptose sulfite cycloserine (TSC) agar. The organism should then form round black colonies on the agar which can then be put through confirmatory tests.
    - Definitive results are normally gained based on the following tests: sulphite reduction and lactose fermentation in lactose sulfite broth.
    - Alternative tests such as the reverse CAMP test could be carried out in conjunction with morphology tests, such as gram stain and motility experiments would yield definitive results.
- The ISO methods and subsequent confirmatory tests can be quite slow, and time consuming. Therefore other genotypic, and immune assays have been designed to be more specific and sensitive, as wells as being faster than the standard tests.
    - Genotypic methods have been developed, which uses a reliable species-specific multiplex PCR for the detection of the *cpa*, *cpb*, *cpb*2, *cpe*, *etx* and *iap* genes of *C. perfringens* isolates in a single reaction. The benefits of this means that DNA purification is not required.
    - Enzyme Linked ImmunoSorbent Assays (ELISA) can also be carried out on the toxins. Although the ELISAs allow reliable typing of *C. perfringens* isolates, the options for subtyping are limited. For example, so far no ELISA is available to detect the β_2-toxin. However the main toxin CPE is able to be tested very readily and so ELISA would still be applicable in the vast majority of suspected *C. perfringens* cases. ELISA kits for CPE are generally less expensive compared to the kits that detect the other toxins, and the results are seen within one day so this a very useful method

- The Reverse Passive Latex Agglutination Test (RPLA) is commonly used. Polystyrene latex particles are coated with rabbit anti-entero toxin A antibodies. In presence of stools containing *C. perfringens* entero-toxin A, agglutination is observed (Louisiana Department of Health and Hospitals, 2008). This method is extremely rapid and sensitive although expensive, due to application of antibodies onto the beads (monoclonal are dearer than polyclonal).

Prevention/Control

- *C. perfringens* is reportable, as it can be a serious disease, especially in hospitals and schools where large amount of food is served, and so investigations would be required to determine the food safety regulations in the establishments.
- In hospitals only standard precautions are required as it cannot be transferred person to person.
- Good cooking times and storage is recommended, as well as avoiding long periods of time when the food is held at a constant temperature. Reheating food properly is also beneficial to kill any vegetative cells that may have grown.

Treatment

- No antibiotic treatment is recommended, as the symptoms usually are gone within 24 hours. However fluid replacement is essential during diarrhoea to prevent dehydration.

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Disclaimer

The information contained in the Education Series text was obtained through collaboration with laboratories identifying and diagnosing *C. difficile* as well as scientific publications and relevant text books. We believe the information specified in this text to be correct at the time of writing although it is the responsibility of the participant to confirm information validity. Cosmos Biomedical Ltd claim no responsibility for misinterpretation of information.

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Appendix 1.3: Web text for *L. monocytogenes* for CPD scheme

Background/History

Organism name – *Listeria monocytogenes* is a gram positive, motile (under 30°C), facultative anaerobic, rod shaped bacterium. This organism is responsible for causing Listeriosis.

Outbreaks – Listeriosis is a serious condition formed from *L. monocytogenes.* A fairly high proportion of fatalities are linked to people contracting this disease (20-25%). The infection is found mostly in new born babies, pregnant women and immunocompromised patients. However it has been known to infect the general population as well.

U.K Outbreaks – In May 2008, two cancer patients contracted listeriosis in a hospital ward. Neither of the patients died, but there was an increased risk as they were going through chemotherapy, which can severely weaken the immune system. The *Listeria monocytogenes* was isolated from sandwiches they had eaten. The other major risk in hospitals is that there is a real possibility of cross-infection between people infected in different wards and neonates in delivery suites (Shetty, *et al.,* 2009).

Worldwide Outbreaks – An epidemic outbreak of food borne listeriosis occurred in 1992 in France. It was responsible for 279 cases including 22 abortions and 63 death cases (Goulet, *et al.,* 1993). The responsibility was put down to two separate delicatessen plants that served supermarkets had not been applied standard cleaning an disinfection protocols, therefore contaminating food.

Where found – *Listeria monocytogenes* is widespread in the environment and can be found in raw food, soil, vegetation, sewage and in the faeces of many mammals, birds, and fish. It is thought that up to or maybe more than 5% of the population may be carriers of the disease. This organism is also found in ready-to-eat foods, pre-prepared cooked and chilled meals, soft cheeses, and meat products.

Table 1: Classification of *C. perfringens*

Classification	
Kingdom	*Prokaryotae, (Bacteria)*
Phylum	*Firmicutes*
Class	*Bacilli*
Order	*Bacillales*
Family	*Listeriaceae*
Genus	*Listeria*
Species	*monocytogenes*

Genome Size

Figure 1. *Listeria monocytogenes* complete genome (BacMap,

The size of the *L. monocytogenes* genome is approximately 2,905kbp with approximately 2,995 genes found on the genome.

Morphology

Organism characteristics:

- Short blunt end rods
- Gram positive
- About 0.4-0.5 by 1-2μm
- Facultative anaerobe
- Motile with use of flagella (only at room temperature, not body temperature) (see Figure 2)
- Catalase positive
- Oxidase negative
- Produces beta-haemolysin

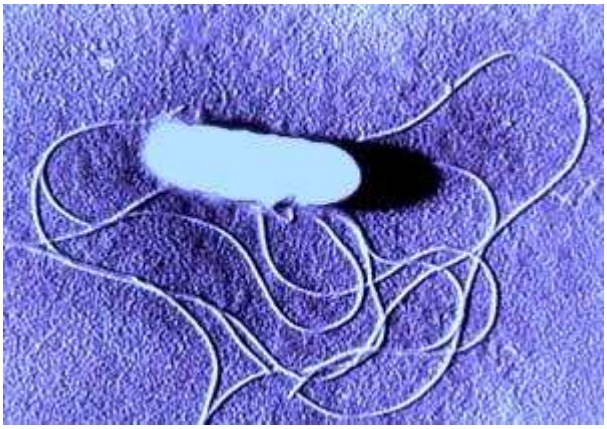

Figure 2. *Listeria monocytogenes* under electron microscope with flagella (sourced from NHS wales)

Growth

L. monocytogenes optimum growth temperature is 37°C, however it can grow between -1.5°C and 45°C.

- Its optimum is pH 7; however studies have shown limited growth from between pH 4.5 and 9.5.
- This organism is also able in aerobic and anaerobic conditions, and so is not affected by atmosphere. It can survive the freezing process quite well, and is able to grow in refrigerated conditions.

Transmission

- Transmission – The main route of transmission for humans is thought to be through food. Ready-to-eat foods have been shown to cause outbreaks of listeriosis, especially in hospitals.
- Most foodstuffs are considered to contain small numbers of *L. monocytogenes,* therefore undercooked food is problematic.
- Also food that has been cooked may be contaminated after, via improper hygiene standards. Because, even if the food has been food is properly stored and refrigerated, *L. monocytogenes* can still grow and proliferate at low temperatures. As well as this is can survive freezing.
- Human to human transmission is possible as the cells can survive in high numbers in faeces. As well as occupational transmission especially for veterinarians or other people who work with animals, as *L. monocytogenes* can be cause from them. Which can be a result from infection through the skin.

Clinical Disease

- Listeriosis is quite rare, but when it does infect, can have a very high mortality rate associated with it. However in animals it is a fairly common disease and there has been records of outbreaks found on farms.
- Listeriosis can infect anyone of any age; however immunocompromised, elderly, pregnant women and new born babies are particularly susceptible.
    - It can cause gastroenteritis in anyone, which is non invasive, symptoms include vomiting, nausea, stomach cramps, diarrhea
    - If pregnant, the organism can induce fevers and flu like symptoms as well as premature labour or cause stillbirths.
    - For new born babies, they can contract listeriosis from their mothers in the womb, can cause sepsis or meningitis.
    - For all of the above and immunocompromised people, and the elderly another invasive form can infect the nervous system causing 'flu like' symptoms, meningitis and it has been known to cause convulsions.

- The incubation time of this infection can be very lengthy. The invasive form can take up 90 days to cause any problems, with an average time of 30 days, where as the non invasive, gastroenteritis form is relatively shorter with an incubation time of 11 hours to seven days.

- People suffering from the non invasive form of listeriosis should suffer no long term effects, however people who suffer meningitis from *L. monocytogenes* are likely to have possibly neurological problems, as well as new born babies possibly having long term side effects from premature birth (New Zealand Ministry of Health, 2001).

Pathogenicity

- Listeriosis is mainly caught from contaminated food products. Its severity is dependent on several factors:
    - The infectious dose found in food is anything over 10^6 CFU (Buncic, *et al.,* 1996).
    - The virulence of the *L. monocytogenes* strain is also important. As out of the 13 strains identified, only four a thought to be pathogenic to humans. These are strains; 1/2a, 1/2b, 1/2c and 4b (Liu, 2008).
    - The hosts involved, where elderly or new born babies may have weaker or underdeveloped immune systems respectively. Or immunocompromised people that may have had a transplant, cancer patients or HIV/AIDS sufferers. As well as pregnant women who may be more susceptible to disease that may pass it on to their unborn children.
    - It should be noted that healthy individuals that ingest large amounts of *L. monocytogenes* would more than likely become infected and possibly depending on the strain, may cause the invasive disease.
- *L. monocytogenes* has an ingenious method of infecting cells. The organism induces its own internalisation into cells in the intestine using zipper-like phagocytosis (Liu, 2008). Once in the cell a combination of haemolysin, listeriolysin and two phospholipases enable the bacteria to enter the cytoplasm of the host cell for replication.
- *L. monocytogenes* is not motile at body temperature and so the organism has developed a method of moving between cells without having to leave them and possibly exposing them to any immune response. The cells

Population Group	No. of cases (%)	Common Symptoms
Pregnant women and infants	141/603 (23%)	Bacteraemia (fever, headache, malaise
		Amnionitis
		Abortion and stillbirth
		Septicaemia and meningitis in infants
Elderly and immunocompromised	414/603 (65%)	Bacteraemia
		Septicaemia
Health individuals	78/603 (12%)	Asymptomatic faecal carriers or bactera
		Gastroenteritis (nausea, vomiting or di

initialise actin tails to be produced that create a propulsive force , propelling the pathogen to neighbouring cells. These then get taken up in 'listeriopods' (a double membrane pod, formed from moving into a new cell) which then are lysed and replication begins again in a new cell (Giardini and Theriot, 2001).

- This method of moving between cells enables the *L. monocytogenes* to be protected by their host cells from the immune system.
- It is not fully understood how the cells move from the blood stream into brain cells. As well as how the pathogens move across the feto-placental barriers.

Epidemiology

- *L. monocytogenes* can be found widely in the environment, such as water sources, soils, plants and vegetation. A main concern is the ability for *L. monocytogenes* to survive on processing plant machinery, where several strains have been known to survive disinfection processes (Samelis and Metaxopoulos, 1999).

- The organism is also very adept at surviving harsh environmental stresses, especially as it does not form spores. *L. monocytogenes* can grow in very low temperatures as well as surviving the freezing process. It can also survive relatively high temperatures (up to 45^{o}C). It can also survive drying processes, and so this organism is very robust.

- *L. monocytogenes* is generally found in ready-to-eat foods, meat and dairy products. Although the organism is not able to survive the cooking process, it can readily contaminate packaging or equipment and then grow on foods that have been stored at low temperatures.
- *L. monocytogenes* is also wide spread in animals, with more cases of listeriosis being reported on farms compared to the human population. Therefore cases of listeriosis may be higher in people working with animals, such as veterinarians.
- Anyone can contract listeriosis; however it is more prevalent, as discussed above, in pregnant woman, the elderly or in immunocompromised people. The range of disorders that they may contract is varying (see Table 2).

		CNS listeriosis (meningitis, meningoenc

Based on a survey of 603 human listeriosis cases that occurred in France during 2001-2003 (Goulet, *et al.,* 2006).

Table 3: Reported Cases of *L. monocytogenes* in England and Wales from Health Protection Agency

Year	Non pregnancy-associated	Pregnancy-associated*	Total
1995	80	10	90
1996	99	17	116
1997	100	24	124
1998	84	22	106
1999	87	18	106
2000	88	13	101
2001	127	18	146
2002	128	10	138
2003	199	35	234
2004	190	21	211
2005	164	25	189
2006	160	25	185
2007	199	28	227
2008	161	19	180
Total	**3035**	**919**	**3956**

- The number of cases of listeriosis has slowly seen an increase since 1995, which may be because of the wide range of ready-to-eat food products increasing on the market.

Laboratory Diagnosis

- The ISO standard method to test for *L. monocytogenes* is a very lengthy one. Normally it takes two cycles of enrichments using Fraser broth, one taking 24 hours, the other 48 hours. After enrichment, the solution containing the suspected organism is plated out onto Listeria agar for a further 24 hours. After this a range of morphological or biochemical tests such as; cAMP test, gram stains, haemolysis and motility.
- A new very simple and much quicker kit has been developed by Bio-Rad Laboratories. It involves a chromogenic reaction based on the specific beta-glucosidase activity of *Listeria*. The selectivity of the medium inhibits the interfering flora and the nutrients of the medium, providing rapid growth and identification of *Listeria* spp. in 48 hours (Bio-Rad Laboratories…).
- The use of immunomagnetic separation could be used as a rapid method that can detect low numbers of *L. monocytogenes* in a sample. However Hudson, *et al* (2001) described how the immunomagnetic separation and concentration procedures reduce the detection time to about 1 day, but was limited in terms of sensitivity, since the recovery of cells on the beads was only about 20%. Therefore further improvements in the method would be required before use in food industry.
- Another method that involves genotyping the organisms has been developed which uses random amplification of polymorphic DNA (RAPD) together with multiplex-PCR serotyping allowed rapid discrimination of *L. monocytogenes* (Aurora, *et al.,* 2009). However it should be noted that although cheaper than using an immune assay, the use of the RAPD assay is difficult to perform and requires specialist training.

Prevention/Control

- As discussed above, *L. monocytogenes* is a very hardy organism that can survive in a wide range of environments. To control the numbers of this organism foods should be cooked properly at temperatures at least above 60°C for ten minutes or longer.
- However drying foods will not kill *L. monocytogenes* as they have been known to survive weeks in dried powdered milk. This organism can also survive cooling or freezing temperatures as *L. monocytogenes* is pschrophilic. Therefore eating cooked foods that have been refrigerated for a long time may be dangerous.
- Rigorous cleaning and disinfection of food processing plants is also encouraged as this organism can form biofilms on a wide range of equipment that could contaminate foodstuffs.
- Person to person transmission does not seem to occur, and so standard protocols in hospitals would be sufficient.

Treatment

- Antibiotic treatment has been proven to be very efficient, as only low numbers of resistances have been documented. A mix of penicillin and ampicillin. Some more serious cases may require longer term use of these antibiotics and can sometimes be combined with gentamicin (New Zealand Ministry of Health, 2001).

Recent Developments

- In July 2009, a new report was published by the Health Protection Agency, on the levels of contamination found in ready-to-eat foods. The majority (99%) of the foods tested were shown to be fine to eat. However a small proportion had dangerously high numbers of pathogens including *L. monocytogenes* in it. It concludes that the main worries are with foods with long shelf lives as this organism is able to grow in standard storage conditions.

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Disclaimer

The information contained in the Education Series text was obtained through collaboration with laboratories identifying and diagnosing *C. difficile* as well as scientific publications and relevant text books. We believe the information specified in this text to be correct at the time of writing although it is the responsibility of the participant to confirm information validity. Cosmos Biomedical Ltd claim no responsibility for misinterpretation of information.

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Appendix 1.4: Web text for *S. aureus* for CPD scheme

Background/History

Organism name – *Staphylococcus aureus* is a facultative anaerobic, Gram positive coccus, that is non-motile, and when viewed under a microscope normally forms golden, grape like bunches. The name is organism is derived from this appearance; *staphylo* (bunch of grapes) and *aureus* (gold).

Outbreaks – The organism is found naturally on the skin, but mainly colonised in the nostrils, throat, hair and hands (Moroni *et al.,* 2009). Foodborne outbreaks however a mostly associated with foods that are handled. This would be seen as a primary route of infection. The exception may be milk from mastitic cows, as *Staphylococcus aureus* is a cause of mastitis (EcoLab, 2010).

U.K Outbreak –In 1986 there was an outbreak of Staphylococcus aureus sourced from dried pasta. Forty-seven cases were reported in the UK. Outbreaks of staphylococcal food poisoning attributed to mishandling during the food processing stage are uncommon and pasta as the food vehicle is rare (Woolaway, *et al.,* 1986).

Worldwide Outbreaks –1,364 children became ill out of a total of 5,824 who had eaten a chicken salad school lunch served at 16 elementary schools in Texas. The lunches were prepared in a central kitchen and transported to the schools by truck. It was thought that the transmission from handlers as the chicken was deboned and that improper refrigeration and storage conditions contributed to the growth of the organism (adapted from FDA website).

Where found – *Staphylococcus aureus* can be found in many places in the environment. It is a very hardy organism that survives in the air, in sewage, in water and on a wide range of surfaces. The organism is associated with foodborne illnesses mainly due to contamination via food handlers. It is thought that a high proportion of the human population carry the organism on their skin. The human body can be seen as a natural reservoir for this bacterium.

Table 1: Classification of *B. cereus*

Classification	
Domain:	Bacteria
Kingdom:	Eubacteria
Phylum:	Firmicutes
Class:	Bacilli
Order:	Bacillales
Family:	Staphylococcaceae
Genus:	*Staphylococcus*
Species:	*aureus*

Genome Size

Figure 1. *Staphylococcus aureus* complete genome (BacMap, 2007).
The size of the genome is approximately 2,809Kbp, with 2,688 genes.

Morphology

Organism characteristics:

- Coccoid shaped
- Gram positive
- Facultative anaerobe
- Heat stable enterotoxin former, causes human illness
- Form grape like clusters, or can be found in pairs
- Non-motile

INSERT OF MY PICTURE HERE

Figure 2 and 3. *Staphylococcus aureus* colony formation on Baird Parker.

Growth – *Staphylococcus aureus* can grow between 7°C and 45°C. With its optimal range being around 35-39°C. Generally grows best at a neutral pH, however can grow between a range of 4-9. Can grow in both aerobic and anaerobic conditions (better in the presence of oxygen) and can survive in highly desiccated environments.

Survival – *Staphylococcus aureus* is readily killed by cooking, however its toxin is very heat resistant for example the D time of enterotoxin B at 149°C is 100min (NZFSA, 2001).

Transmission

- Transmission – The organism is found on many different surfaces, but is naturally found on the human body. As the organism is sensitive to heat through cooking, normal transmission and then subsequent growth occurs after cooking if the food is not properly stored. The enterotoxin is normally synthesised during this period and relates to the rapid onset of infection.
- The organism is also thought to be able to survive for hours on different surfaces which would contribute to the transmission rates especially in hospitals and in the food industry
- Human to human transfer of the bacteria is also a very important problem. As an opportunistic pathogen, this organism can cause a lot of problems in hospitals, especially for those patients with open wounds or who are immunocompromised.

Clinical Disease

- Certain strains of *Staphylococcus aureus* produce an enterotoxin that is involved in food poisoning, this can be called staphyloenterotoxicosis.
- Symptoms usually develop within one to six hours after eating contaminated food. The illness usually lasts for one to three days and is normally self limiting.
- Symptoms usually consist of abdominal pains, vomiting and diarrhoea. In severe cases extreme muscular cramps and nausea have been attributed to the disease.

- Anyone of any age can be infected, and it is widely considered that the majority of the population has been infected by *Staphylococcus aureus* at some time. Very few deaths are related to the food poisoning infection from *Staphylococcus aureus*.

Pathogenicity

- There are up to 15 different enterotoxins that this organism can produce. These are all able to cause a wide range of illnesses in humans. Food poisoning is attributed to the around eight of these toxins; SEs SEA to SEE and only SEH, SEG and SEI (McLauchlin, *et al.,* 2000, and Omoe, *et al.,* 2002), which have been proven to induce gastroenteric syndrome. However the most prevent toxin associated with food poisoning is SEA (Klotz, *et al.,* 2003).
    - The majority of the toxins produced by this organism have similar three dimensional structures, however some differ slightly in that they have small additional loops. The most notable of these is a cysteine loop structure present in many of the Staphylococcal enterotoxins (SE). This loop is thought to be important for emetic activity in SEs. This is because some of the SE's identified without these loops have a severely lower emetic activity (Orwin, *et al.,* 2003).
    - The structure of the SE is also very similar to those that cause Toxic Shock Syndrome, which is a very serious condition caused by *Staphylococcus aureus.*
- The enterotoxins are produced when the food contaminated with *Staphylococcus aureus* is not stored at low or high enough temperatures allowing it to proliferate. Toxins produced are heat stable. They can survive a reheating process. They are also highly stable and resist most proteolytic enzymes, such as pepsin or trypsin, and thus keep their activity in the digestive tract after ingestion (Loir, *et al.,* 2003).
- The infective dose required to induce staphylococcal food poisoning in humans is estimated to be around 0.1 µg and it may vary with patient sensitivity (Evenson, *et al*., 1988)

Epidemiology

- Anyone of any age can be infected by *Staphylococcus aureus,* it is thought that the majority of people have had a food poisoning case related to this organism. The most severe cases are found in those that are immunocompromised, such as neonates or in the elderly population.
- The food borne infection normally is the result of eating contaminated meat. However many different foods have been implicated in the food poisoning, such as dairy products, salads and sandwiches.

    - Trends can change geographically. In the UK the majority of food poisoning cases were attributed to meat products, especially ham, whereas in France, cheese and dairy products were the main culprits involved in food poisoning (Adapted from Loir, *et al.,* 2003).
- Transmission – *Staphylococcus aureus* is ubiquitous in nature and can be found in a wide variety of hard surfaces and is part of the natural skin flora of the human body, where up to 50% of the population is thought to

be carriers. Transmission occurs from improper treatment of these foods through handling, storing, or insufficient heating and cooling methods.

- It is difficult to get definitive numbers for the number of cases of food poisoning attributed to *Staphylococcus aureus* as it is generally as it is self limiting and does not last long. Therefore it is not widely reported.
- There are very few recorded fatalities related to being infected with *Staphylococcus aureus* food poisoning.
-

Year	Number of Outbreaks	Number Affected
1992	7	107
1993	1	15
1994	2	17
1995	3	46
1996	5	146
1997	2	18
1998	0	0
1999	4	81
2000	0	0
2001	6	25
2002	2	14
2003	0	0
2004	2	31
2005	0	0
2006	1	5

Figure 4. Reported Cases of *S. aureus* in the UK from Health Protection Agency

Laboratory Diagnosis

- ISO method 6888 is the standard method to detect *Staphylococcus aureus.* It involves preparing a solution of 25g of my food sample into 225ml of Buffered peptone water. This is them plated onto Baird Parker agar. These should form shiny round black colonies with a halo.
    - Basic microscopy and Gram stains should be carried out to determine it is a non motile coccus and is Gram positive.
    - Postive catalase and subsequent coagulase tests should also be carried out to presumptively identify the organism as *Staphylococcus aureus* (Not all *Staphylococcus aureus'* are coagulase positive).
- The ISO method above is considered fairly slow and labour intensive and due to the possibility of coagulase negative *Staphylococcus aureus* false negatives could be reached. Therefore PCR reactions can be carried out.
    - Multiplex polymerase chain reactions (PCR's) have also been designed to allow positive identification of *Staphylococcus aureus,* with primers designed around a conserved 16s sequence found in *Staph. aureus* DNA, as well as determining whether the organism is toxigenic or not. These PCR's can be carried within a day with high specificity and sensitivity and act as a good identification step.

Prevention/Control

- In food care should be taken to thoroughly cook all raw food stuffs. As the organism is sensitive to a prolonged period of cooking. However after

cooking food should be stored at an appropriate temperature to prevent cell proliferation and toxin synthesis.

- Cleaning equipment thoroughly is also good practice as this organism can survive on food processing equipment for a long period of time and therefore contaminate the food sources and proliferate rapidly if at an ambient temperature.
- The main route of contamination would be through food handlers. They must be sure to wash their hands thoroughly before handling food as *Staphylococcus aureus* is part of the natural flora on humans.

Treatment

- There is not a wide range of treatment for food poisoning caused by *Staphylococcus aureus,* as it is a very short lived infection period and will normally last no longer than a couple of days. However replacing lost fluids is vital especially after diarrhoea.

Acknowledgements

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Appendix 3
Appendix 3.1: APC of organisms from IFM

	Aerobic Plate Count (PCA)		
Product/Dilution	**10^{-1}**	**10^{-2}**	**10^{-3}**
B-AF	ND	ND	ND
B-AD	ND	ND	ND
B-AH	ND	ND	ND
B-CC	ND	ND	ND
B-CF	ND	ND	ND
B-CH	ND	ND	ND
B-CI	ND	ND	ND
B-E	*	*	ND
B-HH	ND	ND	ND
B-HN	33	ND	ND
B-HPF	5	ND	ND
B-HPS	4	ND	ND
B-SH	4	ND	ND
B-SF	2	ND	ND
B-SLF	ND	ND	ND
B-S	ND	ND	ND
B-W	1	ND	ND

Aerobic plate counts for infant formula milk. The (*) represents irregularities on the plate, such as contaminants or foreign bodies on the plate (fluff) and ND stands for None Detected.

Appendix 3.2: APC from EBU's

	Aerobic Plate Count (PCA)		
Product/Dilution	**10^{-1}**	**10^{-2}**	**10^{-3}**
E-BC	ND	ND	ND
E-CO	*	*	ND
E-CC	ND*	ND	ND
E-COC	2*	ND	ND
E-COF	2	ND	ND
E-C	ND*	2	ND
E-CS	1	1	ND
E-CV	ND*	ND	ND
E-NC	7	1	ND
E-NV	TMTC *[5]	TMTC *5	TMTC *5
E-NS	4	ND	ND
E-SAN	ND	ND	ND

Aerobic plate counts for elderly build up foods. The (*) represents irregularities on the plate, such as contaminants or foreign bodies on the plate and ND stands for None Detected. TMTC stands for too many to count (above 300)

Appendix 3.3: APC from SD

	Aerobic Plate Count (PCA)		
Product/Dilution	**10^{-1}**	**10^{-2}**	**10^{-3}**
S-BFW	*	*	ND
S-MEP	*1	*1	*1
S-BSN	ND	ND	ND
S-C90	ND	ND	ND
S-GE	ND	ND	ND
S-HPC	ND	ND	ND
S-SPP	*	*	*
S-IEO	ND	ND	ND
S-LSP	*	*	ND
S-LSR	ND	ND	ND
S-N1	ND	ND	ND
S-PV	*	ND	ND
S-PESF	ND	ND	ND
S-PSP	*	*	ND
S-RRSF	1 (*1)	ND	ND
S-SMN	ND	ND	ND
S-WP	ND	ND	ND
S-MMP	2	ND	ND
S-MMX	ND	ND	ND

Aerobic plate counts for sports drinks. The (*) represents irregularities on the plate, such as contaminants or foreign bodies on the plate (fluff) and ND stands for None Detected.

Appendix 3.4: Colonies that formed on TSC agar for SD and EBU's

	Clostridium perfringens											
Product	**10^0**			**10^1**			**10^2**			**10^3**		
S-LSP	19	4	12	0	1	0	0	0	0	0	0	0
S-BSN*	2	7	0	0	0	0	0	0	0	0	2	0
S-WP*	42	32	32	6	12	17	0	5	2	0	0	0
S-MMX	>300	>300	>300	>300	>300	>300	97	107	82	29	19	22
S-MMP	>300	>300	>300	>300	>300	>300	66	122	121	11	3	5

	Clostridium perfringens											
Product	**10^0**			**10^1**			**10^2**			**10^3**		
E-NC	116	107	114	0	0	0	0	0	0	0	0	0
E-NS	>300	>300	>300	255	212	199	42	16	0	0	0	0
E-BC	1	0	1	0	0	0	0	0	0	0	0	0

A table (panel 1) showing the number of colonies formed from sports powders on TSC agar. The (*) means that the colonies formed here were not characteristic black colonies that are presumed to be *Clostridium perfringens*
A table (panel 2) showing the number of colonies formed on TSC agar from elderly build-up powders. Each of these products formed characteristic black with or without a zone of precipitation.

Appendix 3.5: List of ingredients of all the products that were contaminated

B-HPF:
Organic skimmed milk, organic demineralised whey powder, organic vegetable oils, prebiotic fibres (galacto-oligosaccharides from milk), organic lactose, calcium carbonate, LCP oils (vegetable, fish), potassium chloride, emulsifier (soya lecithin), vitamin mix (vitamin C, vitamin E, niacin, pantothenic acid, vitamin A, thiamin (vitamin B1), vitamin B6, riboflavin (vitamin B2), folic acid, vitamin K, biotin, vitamin D, vitamin B12), L-tyrosine, L-phenylalanine, L-tryptophan, magnesium carbonate, zinc sulphate, iron sulphate, stabiliser (L-lactic acid), copper-lysine complex, potassium iodate, manganese sulphate, sodium selenate.

B-HPS:
Organic skimmed milk, organic lactose, organic vegetable oils, emulsifier (soya lecithin) calcium carbonate, L-cystine, vitamin C, L-tryptophan, iron diphosphate, inositol, magnesium carbonate, vitamin E, niacin, zinc, oxide, pantothenic acid, vitamin A, copper sulphate, thiamin (vitamin B1) vitamin B6, riboflavin, potassium iodate, folic acid, manganese sulphate, vitamin K, sodium selenate, biotin, vitamin D, vitamin B12.

B-AH:
Lactose, skimmed milk, vegetable oils, dietary fibres (galacto-oligosaccharide, polyfructose), calcium carbonate, fish oil, L-cysteine hydrochloride, choline chloride, vitamin C, emulsifier (soya lecithin), taurine, ferrous sulphate, inositol, vitamin E, uridine 5'-monophosphate, zinc sulphate, adenosine 5'-monophosphate, inosine 5'-monophosphate, pantothenic acid, folic acid, copper

sulphate, vitamin A, biotin, vitamin B_{12}, thiamine, L-carnitine, vitamin D, vitamin B, manganese sulphate, potassium iodide, vitamin K, sodium selenite.

B-SLF:
Dried glucose syrup, vegetable oils, soya protein isolate, calcium phosphate, sodium citrate, magnesium chloride, potassium chloride, emulsifier (soy lecithin), potassium citrate, L-menthionine, vitamin C, choline chloride, potsssium hydroxide, potassium bicarbonate, taurine, inositol, ferrous sulphate, zinc sulphate, L-carnitine, vitamin E, niacin, pantothenic acid, vitamin A, riboflavin, thiamine, copper suplphate, vitamin B, vitamin D, folic acid, potassium iodide, vitamin K, sodium selenite, biotin, vitamin B.

B-SW:
Dried glucose syrup, vegetable oils, soya protein isolate, calcium phosphate, sodium citrate, magnesium chloride, potassium chloride, emulsifier (soy lecithin), potassium citrate, L-menthionine, vitamin C, choline chloride, potsssium hydroxide, potassium bicarbonate, taurine, inositol, ferrous sulphate, zinc sulphate, L-carnitine, vitamin E, niacin, pantothenic acid, vitamin A, riboflavin, thiamine, copper suplphate, vitamin B, vitamin D, folic acid, potassium iodide, vitamin K, sodium selenite, biotin, vitamin B.

S-PV:
Ingredients: Whey Protein Isolate, Maltodextrin, Dutch Processed Cocoa Powder, Vitamin and Mineral Blend [Dicalcium Phosphate, Dipotassium Phosphate, Potassium Chloride, Sodium Chloride, Dimagnesium Phosphate, Magnesium Oxide, Vitamin C (as Ascorbic Acid and Sodium Ascorbate), Vitamin E (as dl-Alpha Tocopheryl Acetate and dl-Alpha Tocopherol), Ferrous Sulphate, Tricalcium Phosphate, Calcium Sulphate, Zinc Oxide, Magnesium Chloride, Niacin (as Niacinamide), Sodium RNA, Cupric Gluconate, Sodium Borate, Manganese Sulphate, Pantothenic Acid (as d-Calcium Pantothenate), Beta Carotene, Thiamin Hydrochloride (Vitamin B1), Riboflavin (Vitamin B2), Vitamin B6 (as Pyridoxine Hydrochloride), Biotin (as d-Biotin), Folic Acid, Vitamin A (as Retinyl Palmitate), Chromium Chloride, Potassium Iodide, Sodium Molybdate, Sodium Selenate, Vitamin D3, Vitamin B12 (as Cyanocobalamin)], Flavourings, Bulking Agent (Xanthan Gum), Emulsifiers (Soya Lecithin, Acacia Gum), Dextrose, Starch, L-Glutamine, Taurine, Sucrose, Citric Acid, Sweetener (Sucralose), Gelatine, Coconut Oil, Glycine, Anti-Caking Agent (Silicon Dioxide), Sorbic Acid, Mono & Diglycerides, Preservative (Sodium Benzoate), Rice Protein Hydrolysates, Medium Chain Triglycerides, Antioxidant (Butylated Hydroxytoluene).

S-HPC:
Dextrose, HPLC Pure Creatine Monohydrate, Taurine, Disodium Phosphate, Trimagnesium Phosphate, Monopotassium Phosphate, Citric Acid, Colour (Grapeskin Extract), Sweetener (Aspartame), Starch, Flavouring, Maltodextrin, Antioxidant (Butylated Hydroxytoluene).

S-PSP:
Soya Protein Isolate, Cocoa Powder, Flavourings, Emulsifier (Soya Lecithin), Sweetener (Acesulfame K), Sodium Chloride.

S-BSN:
Whey protein concentrate (Milk), dextrose, maltodextrin, fat reduced cocoa powder, D-Glucosamine sulphate 2 potassium chloride, fructo-oligosaccharide, creatine monohydrate, conjugated linoleic acid (with antioxidant: tocopherols), L-carnitine, l-glutamine, taurine, tri-calcium phosphate, magnesium phosphate,

vitamin blend (dextrose monohydrate, ascorbic acid, vitamin e-acetate, niacinamide, biotin, vitamin a-acetate, calcium-d-pantothenate, pyridoxine hydrochloride, folic acid, cholecalciferol, thiamine hydrochloride, riboflavin, vitamin B12), ferri pyrophosphate, niacimide, zinc oxide, chromium chloride, potassium iodide.

S-MMX:
Biomax, (90%, a Maximuscle proprietary high quality blend of whey protein isolates, hydrolosates and whey peptides), cocoa powder in Promax chocolate, Flavours: either vanilla/cocoa/strawberry/banana depending on the flavour displayed on each tub, Colours: riboflavin in vanilla and banana, beta carotene in vanilla, chocineal in strawberry; betain hydrochloride, patented in zinc stabilised purified prolase preparation derived from carcia papaya, Glutamine peptides, N-acetyl-cysteine (NAC), L-ornithine-keto-glutarate (OKG), Sweetener: Acesulfame-K, chromium picolinate.

S-MMP:
Biomax, (90%, a Maximuscle proprietary high quality blend of whey protein isolates, hydrolosates and whey peptides), strawberry powder in Promagain strawberry, Flavours: either vanilla/cocoa/strawberry/banana depending on the flavour displayed on each tub, Colours: riboflavin in vanilla and banana, beta carotene in vanilla, chocineal in strawberry; betain hydrochloride, patented in zinc stabilised purified prolase preparation derived from carcia papaya, Glutamine peptides, N-acetyl-cysteine (NAC), L-ornithine-keto-glutarate (OKG), Sweetener: Acesulfame-K, chromium picolinate.

S-LSP:
Sugar (38%), Whey Protein Isolate (from Milk) (28%), Dried Skimmed Milk, Fat-reduced Cocoa Powder, Creatine Monohydrate (7%), Flavourings, Stabiliser (Sodium Carboxy Methyl Cellulose), Soya Lecithin, Anti-caking Agent (Tri-calcium Phosphate), Sweeteners (Aspartame, Acesulfame K). Contains a source of Phenylalanine. May contain traces of Celery.

S-BFW:
Protein Blend (Whey Protein Concentrate, Hydrolysed Whey Protein, Whey Protein Isolate), Dextrose, Maltodextrin, Flavourings, Emulsifiers (Acacia Gum, Soya Lecithin), Triacetin, Starch, Sweetener (Sucralose), Cinnamon Powder, Anti-Caking Agent (Silicon Dioxide), Coconut Oil, Sucrose, Lactoperoxidase, Colour (Beta Carotene), Vitamin C (as Sodium Ascorbate), Vitamin E (as dl-Alpha Tocopherol).

S-MEP:
Milk & Egg Protein Complex (Calcium Caseinate, Egg White Solids, Whey Protein Isolate), Vitamin and Mineral Blend [Dicalcium Phosphate, Magnesium Oxide, Sodium Chloride, Potassium Citrate, Potassium Chloride, Tricalcium Phosphate, Vitamin C (as Ascorbic Acid), Ferrous Sulphate, Vitamin E (as dl-Alpha Tocopheryl Acetate and Mixed Tocopherols), Niacin (as Niacinamide), Calcium Sulphate, Zinc Oxide, Cupric Gluconate, Pantothenic Acid (as d-Calcium Pantothenate), Magnesium Chloride, Vitamin A (as Retinyl Palmitate), Thiamin Hydrochloride (Vitamin B1), Riboflavin (Vitamin B2), Vitamin B6 (as Pyridoxine Hydrochloride), Biotin (as d-Biotin), Folic Acid, Potassium Iodide, Vitamin D2, Vitamin B12 (as Cyanocobalamin)], Maltodextrin, Corn Syrup, Flavourings, Beet Juice Powder, Sweetener (Acesulfame K), Emulsifier (Soya Lecithin), Bromelain, Papain, Anti-Caking Agent (Silicon Dioxide), Gelatine, Lactic Acid, Citric Acid, Sucrose, Starch, Mono & Diglycerides, Sorbic Acid, Preservative (Sodium Benzoate), Antioxidant (Butylated Hydroxytoluene), Corn Oil.

S-WP:
Milk Protein, Dextrose, Soya Protein Isolate, Flavouring, Extract of Molasses, Vegetable Oil, Salt, Sweetener, Aspartame, Iron (III) Diphosphate, Vit C, Niacin, Zinc Oxide, Vit E, Pantothenic Acid, Vit B6, Copper Carbonate, Vit B2, Vit B1, Vit A, Sodium Iodide, Folacin, Sodium Selenite, Biotin, Vit D, Vit B12.

E-CV:
Skimmed milk, lactose, vegetable oil, maltodextrin, glucose syrup, sugar, flavourings, magnesium sulphate, thickener (xanthan gum), vitamin C, iron sulphate, niacin, vitamin E, zinc sulphate, pantothenic acid, vitamin B6, manganese sulphate, riboflavin, thiamine, copper sulphate, vitamin A, folic acid, vitamin K, potassium iodate, sodium selenite, biotin, sodium molybdate, chromium chloride, vitamin D, vitamin B12.

E-CS:
Skimmed milk, lactose, vegetable oil, maltodextrin, glucose syrup, beetroot powder, flavourings, magnesium sulphate, thickener (xanthan gum), vitamin C, iron sulphate, niacin, vitamin E, zinc sulphate, pantothenic acid, vitamin B6, manganese sulphate, riboflavin, thiamine, copper sulphate, vitamin A, folic acid, vitamin K, potassium iodate, sodium selenite, biotin, sodium molybdate, chromium chloride, vitamin D, vitamin B12.

E-CO:
Maltodextrin, milk proteins, vegetable oil, flavourings, salt, emulsifiers (di-potassium phosphate, lactoglyceride, glyceryl monostearate), magnesium sulphate, tricalcium phosphate, spinach flakes, thickener (xanthan gum), citamin C, iron sulphate, niacin, zinc sulphate, vitamin E, pantotenic acid, vitamin B6, manganese sulphate, riboflavin, copper sulphate, thiamine, vitamin A, folic acid, biotin, vitamin K, potassium iodate, sodium selenite, sodium molybdate, chromium chloride, vitamin D, vitamin B12.

E-CC:
Skimmed milk, lactose, vegetable oil, maltodextrin, glucose syrup, beetroot powder, flavourings, magnesium sulphate, thickener (xanthan gum), vitamin C, iron sulphate, niacin, vitamin E, zinc sulphate, pantothenic acid, vitamin B6, manganese sulphate, riboflavin, thiamine, copper sulphate, vitamin A, folic acid, vitamin K, potassium iodate, sodium selenite, biotin, sodium molybdate, chromium chloride, vitamin D, vitamin B12.

E-NV
Skimmed milk powder, dextrose monohydrate, sugar, fibre, colour, mineral mix (magnesium hydroxide, iron orthophosphate, zinc oxide, potassium iodide), lactose, flavouring, citric acid, vitamin mix (C, E, A ,D, B6, thiamine, riboflavin, niacin, calcium pantothenate, folacin, biotin).

E-NS
Skimmed milk powder, dextrose monohydrate, sugar, fibre (fructo-oligosaccharide), colour: beetroot red,, mineral mix (magnesium hydroxide, iron orthophosphate, zinc oxide, potassium iodide), lactose, flavouring, citric acid, vitamin mix (C, E, A ,D, B6, thiamine, riboflavin, niacin, calcium pantothenate, folacin, biotin).

E-NC:
Skimmed milk powder, dextrose monohydrate, fat-reduced cocoa, fibre, flavouring, lactose, mineral mix (magnesium hydroxide, iron orthophosphate,

zinc oxide, potassium iodide), vitamin mix (C, E, A, D, B6, thiamine, riboflavin, niacin, calcium pantothenate, folacin, biotin).

Appendix 3.6: Milk and non-milk based products contamination

How many contam by (milk):			
Bacilus	**clos**	**lis**	**staph**
13.00	7.00	7.00	7.00
36.11	19.44	19.44	19.44

How many contam by (non-milk):			
Bacilus	**clos**	**lis**	**staph**
3.00	1.00	0.00	2.00
25.00	8.33	0.00	16.67

overall milk	**overall other**
36.00	12.00
8.33	0.00

Tables to show how many mlk or non-milk products were contaminated and by what in per cent.

Appendix 4.1: Restriction digest of ARDRA analysis

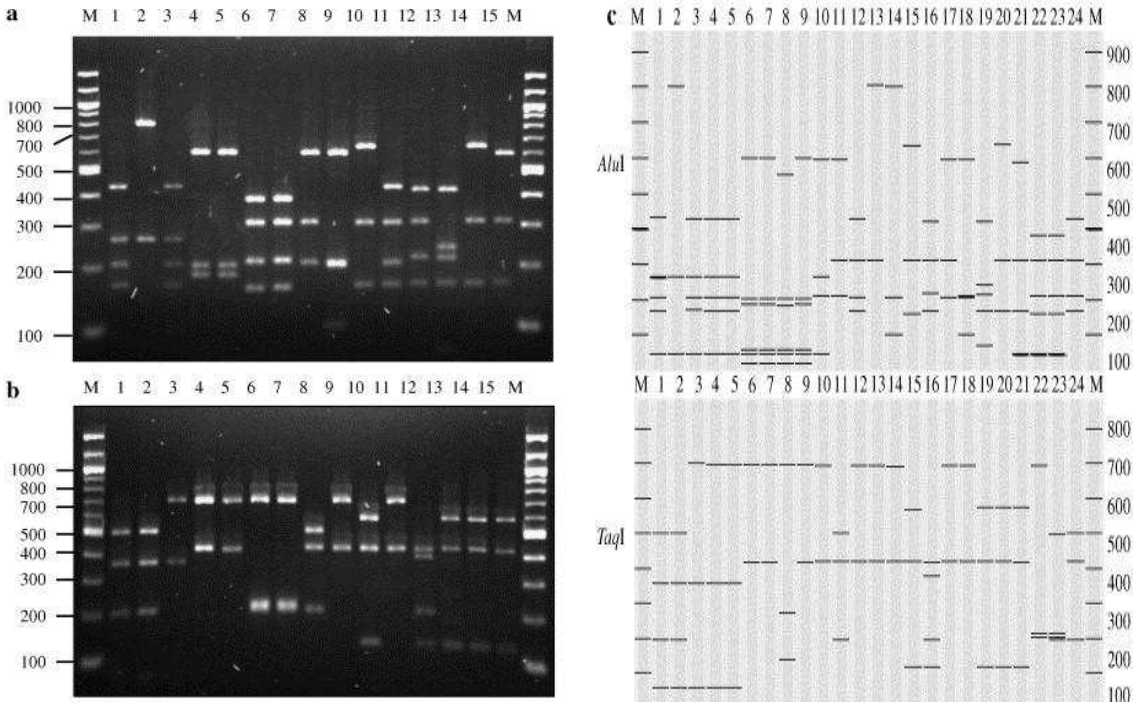


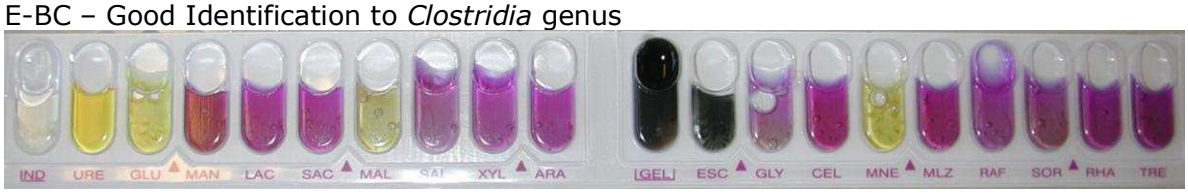
Fig. 2. (a) *Alu*I restriction profiles of amplified regions of the 16S rRNA genes of *Bacillus* reference strains. Lane M, 100 bp+ DNA ladder (size are indicated on the left in bp); lane 1, *B. subtilis* ATCC6633; lane 2, *B. licheniformis* ATCC25972; lane 3, *B. pumilus* ATCC21356; lane 4, *B. cereus* ATCC14579; lane 5, *B. thuringiensis* ATCC10792; lane 6, *B. laterosporus* ATCC64; lane 7, *B. laterosporus* ACM5117; lane 8, *B. coagulans* ATCC7050; lane 9, *B. sphaericus* ATCC14577; lane 10, *B. circulans* ATCC15518; lane 11, *B. badius* ATCC14574; lane 12, *B. clausii* ATCC700160; lane 13, *P. polymyxa* ATCC842; lane 14, *P. larvae* ATCC9545; lane 15, *P. lentimorbus* ATCC 14707. (b) *Taq*I restriction profiles of *Bacillus* reference strains. Lane M, 100 bp+ DNA ladder (size are indicated on the left in bp); lane 1, *B. subtilis* ATCC6633; lane 2, *B. licheniformis* ATCC25972; lane 3, *B. pumilus*ATCC21356; lane 4, *B. cereus*ATCC14579; lane 5, *B. thuringiensis* ATCC10792; lane 6, *B. laterosporus* ATCC64; lane 7, *B. laterosporus* ACM5117; lane 8, *B. coagulans* ATCC7050; lane 9, *B. sphaericus* ATCC14577; lane 10, *B. circulans* ATCC15518; lane 11, *B. badius* ATCC14574; lane 12, *B. clausii* ATCC700160; lane 13, *P. polymyxa* ATCC842; lane 14, *P. larvae* ATCC9545; lane 15, *P. lentimorbus* ATCC 14707. (c) Theoretical prediction of ARDRA profiles generated with restriction enzymes *Alu*I and *Taq*I based on published 16S rDNA sequences from GenBank. Lane M, DNA ladder (size are indicated on the right in bp); lane 1, *B. subtilis* (accession no. AF198249); lane 2, *B. licheniformis* (AF234841); lane 3, *B. pumilus* (AF234856); lane 4, *B. atrophaeus* (AB021181); lane 5, *B. amyloliquefaciens* (AY055223); lane 6, *B. cereus* (AF206326); lane 7, *B. thuringiensis* (Z84584); lane 8, *B. mycoides* (AB021192); lane 9, *B. anthracis* (AF176321); lane 10, *B. megaterium* (AB022310); lane 11, *B. coagulans* (AF466695); lane 12, *B. badius* (D78310); lane 13, *B. firmus* (D16268); lane 14, *B. lentus* (AB021189); lane 15, *B. circulans* (Y13062); lane 16, *B. clausii* (AJ297492); lane 17, *B. simplex* (AJ439078); lane 18, *B. sphaericus* (AF169495); lane 19, *P. polymyxa* (AJ320493); lane 20, *P. larvae* (X60619); lane 21, *P. lentimorbus* (X60622); lane 22, *B. laterosporus* (D16271); lane 23, *B. brevis* (AF424048); lane 24, *G. stearothermophilus* (AY491497) **(Taken from Wu, *et al.,* 2006).**

Appendix 4.2: API 20A read outs from each trip, showing which organisms identified or not.

S-LSP - Very good Identification: *Clostridium difficile*

B-CC – Not Valid

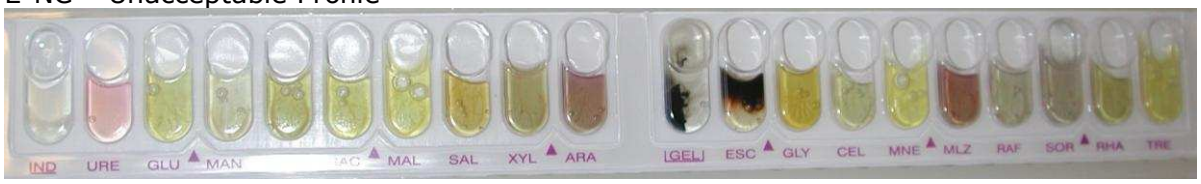
E-BC – Good Identification to *Clostridia* genus



S-MMP – Unacceptable Profile

S-MMX – Good Identification: *Clostridium beijerinckii/butryricum*

E-NC – Unacceptable Profile

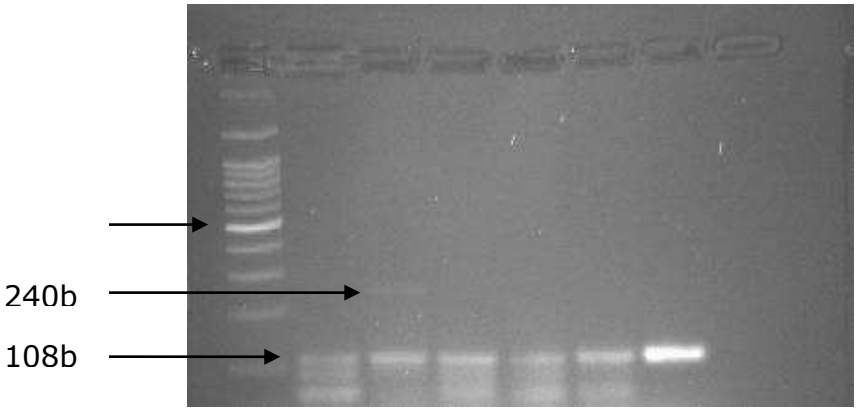


E-NS – Very Good Identification: *Clostridium perfringens*

S-WP – Very Good Identification: *Clostridium beijerinckii/butryricum*

Appendix 4.3: *S. aureus* toxin PCR with too faint bands

Lanes: 1 2 3 4 5 6 7 8



2% agarose gel separated for 1.5 h with PCR amplification products specific for *S. aureus* and its toxins. Lane 1 is marker (Promega 100bp ladder). Lanes 2-6 were; B-SW, S-LSP, E-CC, S-BFW and S-MMX. Lane 7 was a positive control and lane 8 was a negative control. With lane 3 possibly having toxigenic genes however it is too faint and not accurate and the positive control did not show it.

Appendix 4.4: Microscopic analysis of presumptive *B. cereus*

MYP Agar				
Product	**Cell Morphology**	**Motility**	**Gram Stain**	**Indicative of Organism?**
B-AH	Rods	-ve	+ve	No
B-HPS	Rods	+ve	+ve	Yes
B-HPF	Large Rods	-ve	+ve	No
S-MEP	Rods	+ve	+ve	Yes
S-WP	Rods	+ve	+ve	Yes
S-LSP	Short Rods	+ve	+ve	Yes*
S-SPP	Rods	+ve	+ve	Yes
S-PSP	Rods	+ve	+ve	Yes
S-HPC	Rods	+ve	+ve	Yes
S-PV	Rods	+ve	+ve	Yes
S-MMX	Rods	+ve	+ve	Yes
S-MMP	Long Rods	+ve	+ve	Yes*
E-NS	Long Rods	+ve	+ve	Yes*
E-CV	Rods	+ve	+ve	Yes
E-CS	Rods	+ve	+ve	Yes
E-CO	Rods	+ve	+ve	Yes
E-NV	Rods	+ve	+ve	Yes
E-NC	Rods	+ve	+ve	Yes

Table showing the microscopic analysis of cells that have come from presumptive positive *Bacillus cereus* colonies grown on MYP Agar. * means that the length of the rods may be indicative of a different organism but are considered presumptive.

Appendix 4.5: Microscopic analysis of presumptive *C. perfringens*

TSC Agar				
Product	**Cell Morphology**	**Motility**	**Gram Stain**	**Indicative of Organism?**
B-CF	Cocci	-ve	+ve	No
S-MMX	Rods	-ve	+ve	Yes
S-MMP	Rods	+ve	+ve	No
S-LSP	Rods (in Chains)	-ve	+ve	Yes
E-BC	Rods	-ve	+ve	Yes
E-NS	Rods (in Chains)	-ve	+ve	Yes
E-NC	Rods	-ve	+ve	Yes

Table showing the microscopic analysis of cells that have come from presumptive positive *Clostridium perfringens* colonies grown on TSC Agar.

Appendix 4.6: Microscopic analysis of presumptive *L. monocytogenes*

PALCAM Agar				
Product	**Cell Morphology**	**Motility (25oC)***	**Gram Stain**	**Indicative of Organism?**
B-HPF	Rods	+ve	+ve	Yes
B-SF	Small Rods	-ve	-ve	No
B-CI	Small Rods	-ve	+ve	Yes*
S-MMP	Rods	-ve	+ve	Yes*
S-MMX	Rods	+ve	+ve	Yes
S-BSN	Rods	+ve	+ve	Yes
S-MEP	Small Rods	-ve	+ve	Yes*
E-CS	Rods	-ve	+ve	Yes*
E-NC	Long Rods	-ve	+ve	Yes*
E-NV	Small Rods	-ve	+ve	Yes*

Appendix 9. Table showing the microscopic analysis of cells that have come from presumptive positive *Listeria monocytogenes* colonies grown on PALCAM Agar. * means that the motility at 25^{o}C was not considered definitive due to problems.

Appendix 4.7: Microscopic analysis of presumptive *S. aureus*

BP Agar				
Product	**Cell Morphology**	**Motility**	**Gram Stain**	**Indicative of Organism?**
B-E	Cocci (Clustered)	-ve	+ve	Yes
B-SLF	Cocci	-ve	+ve	Yes
B-CC	Negligible	Neg	Neg	No
B-HPS	Rods	-ve	-ve	No
B-SW	Cocci (Clustered)	-ve	+ve	Yes
S-WP	Cocci	-ve	+ve	Yes
S-PV	Cocci	-ve	+ve	Yes
S-BFW	Cocci (Clustered)	-ve	+ve	Yes
S-PSP	Cocci (Clustered)	-ve	+ve	Yes
S-LSP	Cocci	-ve	+ve	Yes
S-MMX	Cocci (Clustered)	-ve	+ve	Yes
S-MMP	Cocci	-ve	+ve	Yes
E-NV	Cocci	-ve	-ve	Yes
E-CO	Rods	+ve	-ve	No
E-CC	Cocci (Clustered)	-ve	+ve	Yes
E-NC	Rods	-ve	+ve	No
E-COF	Rods	-ve	+ve	No
E-CV	Rods	-ve	+ve	No

Table showing the microscopic analysis of cells that have come from presumptive positive *Staphylococcus aureus* colonies grown on BP Agar.

Appendix 5.1: Temperature curve of uninoculated powdered food sample

Time (s)	Temp (°C)
0	25
9	30
13	40
19	50
28	60
39	65
51	68
74	70
92	71
132	72
142	72
143	70
151	65
154	60
156	55
158	50
160	45
163	40
167	35
170	30
176	25
193	20
198	15
207	10
229	5
239	4
254	3

Table showing the time taken for a powdered food product to reach 72oC and then drop down below 5oC.

Appendix 5.2:

	Agar (log10)		
Time	**BHI**	**TSAYE**	**PALCAM**
1	5.146128	6.69897	0
2	5.423246	7.113943	1
3	5.845098	6.924279	0
4	6.130334	6.69897	1.954243
5	5.69897	6.662758	1.69897
6	5.845098	6.102605	1.69897
7	5.322219	5.939519	1.477121
8	5.255273	5.863323	2.079181
9	6.021189	-	1.477121
10	5.778151	5.845098	-

Table showing the log10 cfu/ml of *Listeria monocytogenes* after 10s of heating from BHI, TSAYE and PALCAM Agar.

Appendix 5.3:

	Agar(log10)		
Time	**BHI**	**BP**	**TSAYE**
1	5.176091	5.662758	5.919078
2	4.845098	5.267172	5.623249
3	4.041393	4.477121	5.342423
4	3.217484		4.778151
5	4.176091	4.544068	5.322219
6	4.681241	4.778151	5.045323
7	4.30103	4.954243	5.190332
8	4.255273	4.653213	4.968483
9		4.477121	
10		4.39794	

Table showing the log10 cfu/ml of *Staphylococcus aureus* after 10s of heating from BHI, TSAYE and BP Agar.

Appendix 5.4:

	Agar (log10)		
Time	**BHI**	**TSAYE**	**PALCAM**
1	7.845098	7.225309	7.361728
2	7.69897	6.892095	6.845098
3	6.556303	5.788875	5.977724
4	6.812913	8.102091	7.352183
5	7.332438	8.021189	7.041393
6	6.977724	7.079181	7.021189
7	6.079181	6.146128	7.060698
8	6.255273	7.176091	6.90309
9	7.643453	5.342423	6.778151
10	6.477121	4.90309	6.954243

Table showing the log10 cfu/ml of *Listeria monocytogenes* after 3d of refrigeration from BHI, TSAYE and PALCAM Agar.

Appendix 5.5:

	Agar (log10)		
Time (s)	**BHI**	**TSAYE**	**BP**
1	6.113943	5.236789	2.30103
2	5.20412	5.238046	0
3	5.041393	5.047275	2.60206
4	4.812913	4.740363	2.30103
5	3.477121	4.662758	0
6	4.322219	4.591065	0
7	4.875061	4	2
8	4.690196	4.30103	0
9	4.672098	4.30103	0
10		4.690196	0

Table showing the log10 cfu/ml of *Staphylococcus aureus* after 3d of refrigeration from BHI, TSAYE and BP Agar.

Appendix 5.6: Statistical analysis between *S. aureus* growth before and after cold storage

	Staphylococcus after 72°C	**Staphylococcus after refrigeration**
	5.66	2.30
	5.27	0.00
	4.48	2.60
	0.00	2.30
	4.54	0.00
	4.78	0.00
	4.95	2.00
	4.65	0.00
	4.48	0.00
	4.40	0.00
Mean	4.32	0.92
Median	4.60	0.00
SD	1.570257322	1.19669043

Table showing the comparisons between *Staphylococcus aureus* before and after refrigeration. Using an unpaired t-test as the data is parametric, shows how there is a significant difference ($p>0.05$) between the growth before and after refrigeration.

Appendix 5.7: Statistical analysis between *L. monocytogenes* growth before and after cold storage

	***Listeria after* 72°C**	***Listeria* after refrigeration**
	0.00	7.36
	1.00	6.85
	0.00	5.98
	1.95	7.35
	1.70	7.04
	1.70	7.02
	1.48	7.06
	2.08	6.90
	1.48	6.78
	-	6.95
Mean	1.27	6.93
Median	1.48	6.99
SD	0.780646533	0.38598205

Table showing the comparisons between *Listeria monocytogenes* before and after refrigeration. Using an unpaired t-test as the data is parametric, shows how there is a significant difference ($p>0.05$) between the growth before and after refrigeration.

Appendix 5.8: Positive and negative control for *S. aureus* after cold storage

Panel 1

	Postive Control (post-refrigeration)		
Time	**BP**	**BHI**	**TSAYE**
0	1.56	5.1203	3.45
5	0.45	3.89	3.456
10	0.215	2.115	1.264

Panel 2

	Negative Control (post-refrigeration)		
Time	**BP**	**BHI**	**TSAYE**
0	0	1.9985	0.85
5	0	1.02	0
10	0	0	0

Tables showing the log10 of the viable counts from *S. aureus* (R3293/62) (Panel 1) and the viable count from the uninoculated samples (Panel 2).

Appendix 5.9: Effect of cold storage on recovery of heat treated *L. monocytogenes* control samples

Panel 1

Postive Control (post-refrigeration)		
PALCAM	**BHI**	**TSAYE**
2.62	4.65	4.32345
0.264	1.56	3.32
1.6	1.236	1.023

Panel 2

Negative Control (post-refrigeration)		
PALCAM	**BHI**	**TSAYE**
0	1.9	1.2
0	0	0
0	0	0.16

Tables showing the log10 of the viable counts from *L. monocytgoenes* (NLTL 23074) (Panel 1) and the viable count from the uninoculated samples (Panel 2).