ANA LUÍSA COSTA RAMOS

STUDIES ON THE BIOCHEMICAL AND MICROBIOLOGICAL QUALITY OF INFANT FORMULA POST EXPOSURE TO NOVEL PROCESSING TECHNOLOGIES

UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologias

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Mestrado em Biotecnologia

Trabalho efetuado sob orientação de:

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Ever tried. Ever failed. No matter. Try again. Fail again. Fail better.

- Samuel Beckett

Abstract

Infant milk formula (IMF) is produced by adding all individual components to dehydrated milk, creating a non-sterile produce. Even though IMF undergoes a pasteurisation step during its manufacturing process, recontamination may occur during handling and reconstitution. Therefore, such produce carries microbiological load to an extent that may cause enteric disease in newborns and infants. However, public acceptance of irradiated food varies depending on access to information. Far East markets, as China and the Republic of Korea, are critical IMF markets. A joint FAO/IAEA work showed that Asian consumers would accept irradiated foods and trade benefits would ensue from the application of the technology. Also, commercial food irradiation is increasing significantly in Asia, but decreasing in EU.

This multidisciplinary project investigates the microbiological quality of two brands of powdered IMF commercially available in Ireland (Cow&Gate and SMA), and different sterilisation methods (pulsed UV light, e-beam and gamma irradiation) with potential to be applied to industrial manufacturing of IMF and other infant and enteral feeds, without compromising their nutritional value. It was found that both brands are contaminated with unacceptable levels of microorganisms, and those contaminating bacteria are reduced when the powder is irradiated. These intrinsic microorganisms belong to the genus *Bacillus*. It was not possible to demonstrate if these were enterotoxin producers because BCET-RPLA's test kits are not available in Ireland at the time. PBS buffer and rehydrated IMF were supplemented with four *Bacillus* strains, *Cronobacter sakazakii* and *Listeria monocytogenes*, and individually irradiated with PUV, e-beam and gamma rays. PUV treatment was ineffective in killing all bacteria; however both e-beam and gamma irradiation successfully decontaminated samples to acceptable levels of microorganisms. Further studies must be conducted to assess the effectiveness of gamma irradiation in IMF, since these samples could not be treated due to spoilage risk. After e-beam irradiation, IMF powder was subjected to nutritional testing to assess potential nutritional losses. Tests to both treated and untreated samples revealed none of the samples were altered during irradiation, and European Commission standards regarding IMF composition were respected.

Keywords: Infant milk formulae, sterilisation, pulsed UV light, e-beam, gamma radiation.

Resumo

As fórmulas para lactentes são produzidas pela adição de todos os seus componentes individuais a leite em pó, originando um produto não estéril. Embora as fórmulas para lactentes sofram pasteurização durante o processamento, pode ocorrer recontaminação durante o empacotamento e reconstituição. Assim, tais produtos apresentam um teor microbiológico tal que poderá causar doença em recém-nascidos e crianças.

Os principais microorganismos encontrados em fórmula para lactentes em pó são *Salmonella*, *Cronobacter* spp. (*Cronobacter sakazakii*) e *Bacillus cereus*. *Listeria monocytogenes* é uma bactéria a ter também em atenção, pois não é normalmente encontrada no leite em pó mas pode estar presente na água com que se reidrata a fórmula. Neste trabalho deu-se ênfase às bactérias *Bacillus cereus*, *Listeria monocytogenes* e *Cronobacter sakazakii*.

Bacillus cereus é uma bactéria Gram-positiva e anaeróbia facultativa, comummente encontrada no solo e na vegetação. É um espécie formadora de endósporos, que são estruturas resistentes a elevados calor e acidez e raios ultravioleta, sendo capaz de originar dois tipos de doença: os síndromes emético e diarreico. A sua patogenicidade deve-se à produção de fatores extracelulares tais como fosfolipase, cereulida, enterotoxina Hbl, toxina não-hemolítica (Nhe), hemolisina IV e está associada à indução da citotoxina de enterocolite necrótica (CytK).

Listeria monocytogenes é um bacilo Gram-positivo, não formador de endósporos dotado de motilidade que tem sido isolado de diferentes espécies animais. Possui grande capacidade de crescer em ambientes hostis. A maior parte dos surtos de listeriose devem-se à pasteurização inadequada de laticínios, carnes de aves mal cozinhadas e alimentos prontos a comer. Até ao momento, 13 serotipos foram identificados e todos têm potencial para causar doença em seres humanos, contudo os mais prováveis de causar listeriose são os serotipos 4b, 1/2a e 1/2b.

Relativamente a *Cronobacter sakazakii*, trata-se de um novo género onde se inclui a espécie antes designada como *Enterobacter sakazakii*. Conhecido pela sua resistência a *stress* osmótico, este bastonete Gram-negativo é o agente causador de uma rara infeção invasiva com taxa de mortalidade de 40% a 80% dos infetados infantis – bacterémia, meningite e enterocolite necrotizante, associadas com a ingestão de fórmula para lactentes. Pertence à família *Enterobacteriaceae*, a mesma de *Escherichia coli*.

A composição nutricional de fórmulas para lactentes e fórmulas de transição segue, na Europa, critérios rígidos definidos pela Comissão Europeia, que por sua vez se baseou no artigo *Global Standard for the Composition of Infant Formula: Recommendations of an ESPGHAN*. Tal como esperado, a composição da fórmula para lactentes é baseado na composição do leite

humano: 0,45g/100 mL a 0,7 g/100 mL de proteínas; um conteúdo energético de 60 a 70 kcal/100 mL; um total de hidratos de carbono entre 9 e 14 g/100 kcal, dos quais 4,5 g/100 kcal de lactose; sem esquecer a suplementação com, entre outros, cobre, ferro e vitamina C, que possibilita o aumento da absorção intestinal de ferro. Em termos de critérios microbiológicos, e de acordo com o Regulamento da Comissão Europeia 1441/2007, a bactéria *Salmonella* deverá estar ausente em 25 g, as bactérias *Cronobacter* spp. e *Enterobacteriaceae* ausentes em 10 g e *Bacillus cereus* deverá apresentar uma concentração entre 50 e 500 UFC/g de amostra.

A esterilização é necessária para a destruição completa ou remoção de todos os microrganismos em alimentos, de acordo com a Organização Mundial de Saúde. No grupo dos métodos fisicos de esterilização incluem-se as radiações ionizantes (e-beam, radiação gama) e não ionizantes (luz ultravioleta).

A desinfeção por luz UV é um processo de transferência de energia eletromagnética da fonte para o organismo. Nesta transferência, os efeitos desta energia atingem o material genético dos organismos, afetando a sua capacidade de replicação. Geralmente, as bactérias Gram-negativas são as menos resistentes à radiação UV, seguidas das Gram-positivas, leveduras e esporos bacterianos. A luz UV pulsada funciona recorrendo a uma lâmpada de xénon capaz de produzir flashes várias vezes por segundo. Esta luz é rica em radiação UVC (UV germicida, com um comprimento de onda de 200 a 280 nm).

A radiação ionizante tem como fonte eletrões de elevada energia, suficiente para penetrar uma grande variedade de materiais. Os eletrões interagem com as moléculas do material, gerando radicais livres. Assim são capazes de alterar polímeros, mas estes também destroem microrganismos pois atingem o DNA cromossomal e exercem um efeito secundário na membrana citoplasmática. A quantidade de radiação necessária para alcançar a completa e irreversível inativação de enzimas microbianos é demasiado alta e pode provocar efeitos indesejáveis, como por exemplo perdas nutricionais. Segundo a Organização Mundial de Saúde, a irradiação de alimentos com uma intensidade até 10 kGy é considerada segura para consumo humano.

O estudo de novos métodos de esterilização de fórmula para lactentes é de vital importância. Em primeiro lugar, a República da Irlanda é um dos maiores produtores mundiais deste produto, cujas exportações atingem aproximadamente 10% do total mundial. O mercado mundial de comida infantil alcançou cerca de 55 mil milhões de dólares em 2015, com ênfase nos chamados mercados em desenvolvimento – América Latina, Ásia-Pacífico e Médio Oriente. Considerando a importância da indústria para o país, desenvolver um produto nutricionalmente relevante e seguro para consumo humano é o próximo passo a ser tomado.

Este projeto multidisciplinar investiga a qualidade microbiológica de duas marcas de fórmula para lactentes disponíveis para venda na República da Irlanda, e métodos de esterilização (luz UV pulsada, e-beam e irradiação gama) potencialmente aplicáveis na indústria de processamento de fórmulas e outro tipo de alimentação infantil e entérica, sem comprometer o seu valor nutricional.

Neste estudo, foram utilizadas as seguintes estirpes: *Cronobacter sakazakii* NCTC 8155, *Bacillus cereus NCTC 11145, Bacillus cereus* NR3, *Bacillus coagulans* NB11, *Listeria monocytogenes* 7071 e uma estirpe de *Bacillus* isolada de uma preparação de formula para lactentes, à qual foi dado o nome de *Bacillus cereus* ALCR. Todas elas foram sujeitas a testes para confirmar a sua identidade. 10 g de fórmula para lactentes foram reconstituídos em 100 mL de água desionizada esterilizada. Para a preparação de amostras, as bactérias supracitadas foram diluídas em tampão PBS para uma concentração final de 10^9 UFC/mL (OD~2.0) a 560 nm. De modo a utilizar apenas os endósporos, as estirpes de *Bacillus* foram colocadas num banho a 63°C durante 30 minutos, com o objetivo de eliminar as células vegetativas. Para obter as amostras a tratar, 1 mL da suspensão bacteriana foi adicionada a 9 mL de fórmula ou PBS, conforme o meio desejado. As condições de tratamento das amostras com luz UV pulsada estão especificadas na Secção 4. Relativamente à inativação com e-beam e radiação gama, *Cronobacter sakazakii* e *Listeria monocytogenes* foram irradiadas com 0, 1,5 e 2 kGy e as estirpes de *Bacillus* com intensidades de 0, 2, 5 e 10 kGy.

Testes realizados sugerem que ambas as marcas estão contaminadas com níveis inaceitáveis de microrganismos, e tais estirpes contaminantes são reduzidas quando a fórmula é irradiada. Estes microrganismos intrínsecos pertencem ao género *Bacillus*. Não foi possível demonstrar se tais estirpes são produtoras de enterotoxinas, uma vez que os kits de deteção de enterotoxinas BCET-RPLA não estavam disponíveis para venda na República da Irlanda aquando do estudo.

Amostras de PBS e fórmula para lactentes reconstituída foram suplementados com quatro estirpes do género *Bacillus, Cronobacter sakazakii* e *Listeria monocytogenes*, e individualmente irradiadas com luz UV pulsada, e-beam e radiação gama. O tratamento com luz UV pulsada foi ineficaz contra todas as estirpes; contudo tanto o e-beam como a radiação gama descontaminaram com sucesso as amostras a níveis aceitáveis de carga microbiana. Serão necessários estudos futuros para avaliar a eficácia da radiação gama na descontaminação de fórmula para lactentes, uma vez que estas amostras não puderam ser irradiadas devido a risco de degradação.

Após a irradiação, amostras de fórmula para lactentes foram sujeitas a avaliação nutricional para testar potenciais perdas nutricionais. Tal avaliação revelou que nenhuma das amostras sofreu alterações na sua composição devidas à irradiação, e que os padrões da Comissão Europeia no que diz respeito à composição da fórmula para lactentes foram respeitados.

Palavras-chave: Fórmula para lactentes; esterilização; luz UV pulsada; e-beam; radiação gama.

Abbreviations

AGE: Advanced glycation end products
API: Analytical Profile Index
Bp : Base pairs
CML: N^{ε}- (carboxymethyl) lysine
CFU: Colony-forming unit
CG: Cow&Gate
EC: European Commission
FAO: Food and Agriculture Organisation
FUR: Furosine
g: grams
$g.L^{-1}$: grams per litre
Gy: Gray
HMF: hydroxymethylfurfural
IAEA: International Atomic Energy Agency
IF: Infant formula
IMF: Infant milk formula
kb: kilobase
kGy: kiloGray
$kJ.mol^{-1}$: kilojoule per mole
LAB: Lactic Acid Bacteria
LCT: Lactulose
LL: Lactulosyllysine
MeV: Million electron volts
mg: milligrams
$mg.kg^{-1}$: milligrams per kilogram
$mg.L^{-1}$: milligrams per litre
MR: Maillard reaction
MRP: Maillard reaction products
OD: Optical density
PBS: Phosphate-buffered saline
PCR: Polymerase chain reaction
PIF: Powdered infant formula
PL: Pulsed light
PUV: Pulsed ultraviolet
UFC: Unidades formadoras de colónia
UHT: Ultra-high temperature
UV: Ultraviolet
VRBG agar: Violet red bile glucose agar
WHO: World Health Organisation

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1. Introduction

Biotechnology can be defined in several ways. The most common definition is the usage of biological processes, organisms or systems to genetically modify living cells, for them to originate new products and/or perform new functions. The first biotechnologists were farmers who developed improved plant and animal species through crossbreeding. Currently, biotechnology is divided in four main domains: red (medicine), white (industrial processes), green (agriculture) and blue (marine). According to the New Zealand Technology Curriculum (1995): "Biotechnology involves the use of living systems, organisms, or parts of organisms to manipulate natural processes in order to develop products, systems, or environments to benefit people. These may be products, such as foods, pharmaceuticals, or compost; systems, such as waste management or water purification; or environments, such as hydroponics. Biotechnology also includes genetic or biomedical engineering." The Organisation for Economic Cooperation and Development (OECD) defines biotechnology as "the application of science and technology to living organisms, as well as parts, products and models thereof, to alter living or non-living materials for the production of knowledge, goods and services." In its strict sense, Biotechnology is Biology-based technology, using both biomolecular and cellular processes for the development of technologies and products that may improve the quality of life of human beings, and also Earth's health itself.

The theme of this dissertation implies carrying out microbiological and biochemical tests to assess the quality of novel sterilisation methods (e-beam and gamma irradiation) applied in infant formula, comparing it with pulsed UV light. Nowadays, disease related to the ingestion of reconstituted infant formula is mainly caused by improper storage, which can lead to the growth of microorganisms within formula. If one can minimize the impact of these intrinsic microorganisms through sterilisation, the number of children affected by this issue in their early months of life might decrease and provide a better quality of life for themselves and their parents. Despite most frequently cited definitions of Biotechnology revolve around the applicability of microorganisms to solve problems, as mentioned before, Biotechnology is an interdisciplinary area of studies that is related to food engineering, agriculture, biochemistry and microbiology. Thereby, the chosen topic is obviously included in the broadest concept of Biotechnology, hence its achievement is coherent with the goals of a Master's Degree in Biotechnology. All research work was conducted at the Athlone Institute of Technology (Athlone, Ireland), supervised by Dr. Neil Rowan.

1.1. Infant Food Industry around the world: The figures

The global baby food market is a very competitive industry and retail sales in this segment were projected to grow from 36.7 billion U.S. dollars in 2010 to about 55 billion U.S. dollars by 2015. Key market companies, such as Abbott Nutrition and Mead Johnson Nutrition, dominated 80% of the ready-to-drink baby formula market in the United States of America. Abbott Nutrition was the major market leader, with sales amounting to 149.5 million U.S. dollars in 2013. *Similac Advance*, owned by Abbott Nutrition, was the top powder baby formula brand in the United States of America, with about 791 million U.S. dollars worth of sales in 2014. According to The Nielsen Company report *Trends in the global baby food and diaper market* (dated August 2015), 87% of global baby food and 66% of baby formula sales come from North America and Europe, but developing markets are growing.

In 2013 the infant formula market grew rapidly with the development of markets like Asia, particularly China with a growth rate close to 20% per year, Eastern Europe, and in a lesser extend Middle East and Latin America. This market development is linked with the economic growth of those countries and the growing number of working women. Japan's market is characterised by innovative use of ingredients such as prebiotics and specific milk protein fractions (UBIC Consulting 2014). In 2014 sales grew 4% over the previous year in Africa/Middle East, 3.4% in Asia-Pacific and 2.6% in Latin America. Value sales in Europe and North America were flat over the same period. Nielsen Company estimated global baby food and formula sales reached nearly 35 billion U.S. dollars in 2015.

Ireland currently produces approximately 10% of the entire global exports of infant milk formula. Three of the world's biggest infant nutrition companies – Abbott, Wyeth and Danone – operate manufacturing facilities in Ireland.

1.2. Infant Milk Formula's Manufacture and Composition

First, it is important to specify the differences between infant formula and follow-on formula. European Commission Directive 2006/141/CE defines:

- Infants: children under the age of 12 months;
- Young children: children aged between one and three years;
- Infant formula: foodstuffs intended for particular nutritional use by infants during the first months of life and satisfying by themselves the nutritional requirements of such infants until the introduction of appropriate complementary feeding;

- Follow-on formula: foodstuffs intended for particular nutritional use by infants when appropriate complementary feeding is introduced and constituting the principal liquid element in a progressively diversified diet of such infants.

In Portugal, "infant formula" and "follow-on formula" are, respectively, "fórmula para lactentes" and "fórmula de transição".

Powdered infant milk formula is manufactured through dry-mix process, wet-mix process or a combination of both. The dry-mix process involves the mixture of individually processed components, in a dry environment, reducing the chance of contamination. Wet-mix process involves adding both unprocessed and processed ingredients together, followed by a pasteurisation step and drying. The pasteurisation step destroys harmful microorganisms (Food and Drug Administration 2008).

According to the Food and Drug Administration, there are three types of infant formula:

- Cow milk formula;
- Soy-based formula, which is useful if one wishes to exclude animal proteins from the child's diet or for a case of intolerance/allergy to lactose;
- Protein hydrolysate formula, which contains protein that has been hydrolysed, partially or extensively.

In terms of commercial offer, there are powdered, liquid and ready-to-use formulae.

Human milk contains 8–13 $g.L^{-1}$ of a complex mixture of oligosaccharides, which is 20 times higher than cow milk, and infant formula's composition is based on it (Sabater et al. 2016). Proteins (at a twice higher content), carbohydrates (essentially lactose, at a concentration of 60-70 $g.L^{-1}$), lipids, minerals and vitamins have to be adjusted to emulate its levels in human milk. Infant formula is also typically enriched in iron and vitamin C, to increase iron intestinal absorption (Birlouez-Aragon 2004). Some formulae substitute lactose with maltodextrin, in order to decrease the glycation potential (Birlouez-Aragon 2004). The common copper levels fluctuate between 0.4 $mg.L^{-1}$ and 0.6 $mg.L^{-1}$ (Sarriá & Vaquero 2006).

The detailed specifications for infant formula, according to European Commission's Directives, are described in **Annex I**. The indispensable amino acids in human breast milk, and that should be emulated in formulae, are specified in **Annex II**. In **Annex III** it is described the specification for the protein content and source, and the processing of protein used in the manufacture of infant formulae manufactured from hydrolysates of whey proteins derived from cows' milk protein.

1.3. Infant food quality standards in the European Union

According to Boekel (2008), "Quality in a very broad sense means satisfying the expectation of the consumer; in other words, quality experience delivered by a food should match quality expectations of a consumer." However, the concept of "quality" must be categorised using standards that have to be followed by food industry, monitoring some indicators as "colour, presence or absence of some microorganisms, texture, vitamins, protein composition, and so on" (Boekel 2008).

Infant and follow-on formulae are specifically covered by Commission Directive 2006/141/EC. The Directive lays down the requirements for the composition and labelling of infant formula and follow-on formula. The annexes of the Directive give criteria for the composition (protein, carbohydrate, fat, mineral substances, vitamins and certain other ingredients) of infant formulae and follow-on formulae including minimum and maximum levels. It requires that infant formulae and follow-on formulae contain no detectable levels of pesticide residues, meaning no more than 0.01 mg of pesticide residues per kilogram. It also prohibits the use of certain very toxic pesticides in the production of infant and follow-on formulae and establishes levels lower than the general maximum level of 0.01 $mg.kg^{-1}$ for a few other very toxic pesticides (European Comission 2016).

Commission Regulation EC No 1609/2006 authorised the placing on the market of infant formulae based on hydrolysates of cow's milk in accordance with specifications for the protein content source, processing and quality set out in the Annex there to for a period of two years. Further to the expiry of such Regulation, these specifications concerning protein quality were added to Directive 2006/141/EC by adopting Commission Regulation No. 1243/2008. Commission Directive 2013/46/EU extended these specifications to follow-on formulae based on hydrolysates of cow's milk and also authorised the placing on the market in the EU of infant formulae and follow-on formulae manufactured from goats' milk protein. Commission Regulation No. 1258/2011 states that the maximum nitrate content in processed cereal-based foods and baby foods for infants and young children is 200 mg $NO_3.kg^{-1}$.

The microbiological criteria for infant and follow-on formulae, established by the European Commission's Regulations, are specified in **Annex IV**. These criteria follow the *Global Standard for the Composition of Infant Formula*: *Recommendations of an ESPGHAN coordinated International Expert Group* (Koletzko et al. 2005).

1.4. Microorganisms most commonly found in food. Related health issues.

According to Cahill et al. (2008), "powdered infant formula is not a sterile product and may be intrinsically contaminated with pathogens that can cause serious illness in infants".

Outbreaks associated with *Salmonella*-contaminated milk products were recognised in the 1950's in the United Kingdom and Bulgaria. In the 1960's and 1970's there were also a number of outbreaks related to *Salmonella* in various powdered milk products. Between 1985 and 2005 at least six outbreaks of salmonellosis have been associated with PIF. In 2005, in France, an outbreak affecting more than 100 infants was associated with PIF contaminated with *Salmonella* Agona. Experts from two FAO/WHO Expert Consultations, held in 2004 and 2006, concluded that intrinsic contamination of PIF with *Cronobacter sakazakii* and *Salmonella* has been a cause of infection and illness in infants (Toyofuku et al. 2006). The microbiological criteria for infant and follow-on formulae are described in **Annex IV**. In this study, focus will be on microorganisms commonly associated with PIF, milk and other dairy products.

1.4.1. *Bacillus cereus*

Bacillus cereus is a facultative anaerobic Gram-positive bacterium commonly found in soil and vegetation, notorious due to the formation of highly heat, acid, UV, and desiccation-resistant endospores (Ehling-Schulz et al. 2015; Gallo et al. 2016). It is a major contaminant of raw or processed foods of plant or animal origin, since it can proliferate in the lower tract of the gastrointestinal system. Certain strains of *B. cereus* are used as probiotics but others cause food poisoning in humans, being responsible for two types of disease: emetic syndrome and diarrhoeal syndrome. The pathogenicity of *B. cereus* is attributed to the species' production of extracellular factors such as phospholipase, cereulide, enterotoxin Hbl, non-haemolytic toxin (Nhe), haemolysin IV, and is associated with the induction of necrotic enterocolitis cytotoxin (CytK) (Kotiranta et al. 2000; Logan 2012; Hwang & Park 2015; Berthold-Pluta et al. 2015).

The diarrhoeal syndrome is attributed to the presence of enterotoxins Nhe and Hbl (da Silva Fernandes et al. 2014). The estimated dose required for causing the diarrhoeal syndrome is about 10^5 *B. cereus*'s vegetative cells or spores (Hwang & Park 2015).

The emetic syndrome emerged in the United Kingdom in the 1970s and is characterised by nausea and vomiting 0.5h to 6 h after eating contaminated food – predominantly oriental

rice dishes, pasteurised cream, milk pudding, pasta dishes and reconstituted infant formulas, as shown by Hwang & Park (2015) in their research for the identification of enterotoxins through PCR in powdered infant formula, sushi, instant cereals and steamed rice. Production of the emetic *B. cereus* toxin cereulide (**Figure 1.1**) is restricted to strains with specific genotypic traits, associated with distinct environmental habitats, and linked to severe clinical manifestations including acute liver failures and encephalopathy (Kotiranta et al. 2000; Logan 2012).

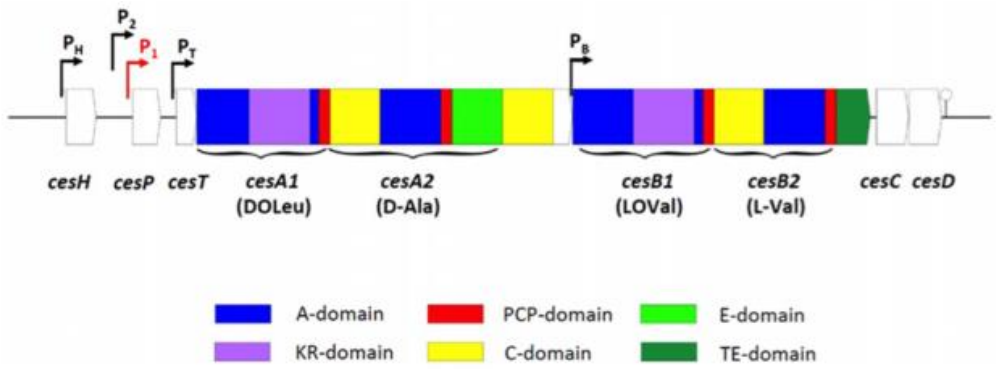


Figure 1.1. Cereulide synthetase gene cluster (Ehling-Schulz et al. 2015). NRPS (non-ribosomal peptide synthetase) are large modular multienzyme complexes that act to produce natural products, such as antibiotics and peptide toxins, following the thiotemplate model instead of the ribosomal route. A NRPS contains an adenylation (A) domain that specifically recognises and activates a certain substrate, its cognate peptidyl carrier protein (PCP) and a condensation (C) domain that catalyses the chain growth. NRPS also contains auxiliary domains, such as epimerization (E) domain. The genetic locus ces encodes the cereulide synthetase. The A-domains in the structural genes *cesA* and *cesB* are interrupted by unusual insertions showing homologies to ketoreductases. These A-domains recognize and select α-ketocarboxylic acids, the precursors in the toxin sequence. The KR-domain carries out chiral reduction on the toxin assembly.

Among the factors that most significantly inhibit the survival of *B. cereus* in the human gastrointestinal tract are low pH and presence of pepsin in the stomach, oxygen deficiency and presence of bile in the small intestine, and the indigenous microbiota in the lower part of the gastrointestinal tract. However, about 10% of vegetative cells can survive at low pH. Acid resistance is achieved through a mechanism of cross-protection between the different stresses microorganisms are exposed to. For example, exposure of the microorganism to various stresses they encounter in the course of food production, e.g. heat processing, dehydration, or acidification, can elicit a higher tolerance to stresses encountered passing through the stomach (Berthold-Pluta et al. 2015).

The acid tolerance response (ATR) involves F_0F_1 ATPase and/or glutamate decarboxylase, modifications of metabolism, and synthesis of proteins which act as protect

and/or repair factors (Thomassin et al. 2006). Bile salts at high concentrations can dissolve membrane lipids and cause the dissociation of integral membrane proteins, damage to RNA, DNA, and the enzymes involved in DNA repair. Generally, Gram-positive bacteria seem to be more sensitive to the activity of bile than Gram-negative bacteria. The absence or deficiency of oxygen affected the growth of diarrhoeal enterotoxin-producing strains *B. cereus* F4430/73, ATCC 14579, and ATCC 10987 (Rosenfeld et al. 2005; Mols et al. 2009). In the anaerobic conditions that prevail in the small intestine, *B. cereus* produces more Hbl and Nhe toxins than during aerobic growth. The toxicity of *B. cereus* depends on *plcR* gene expression, which encodes most of the Hbl, Nhe, CytK toxins (Duport et al. 2004; Zigha et al. 2006). The indigenous intestinal LAB prevent growth of pathogens and spore germination through the production of lactic acid and bacteriocins, again due to the environment's high pH (Røssland et al. 2005).

Combined with its ability to produce toxins, it has been reported that some strains of *B. cereus* are resistant to antibiotics due to β-lactamases, which are a clinically important cause of β-lactam antibiotic resistance, making *B. cereus* a greater problem when infecting infants through formula (Kotiranta et al. 2000; Ankolekar & Labbé 2010; Chaves et al. 2011).

1.4.1.1. Toxin detection kits applied to *Bacillus cereus* enterotoxin detection

Reversed passive latex agglutination (RPLA) methods have been developed commercially for use with food and culture filtrates, with the purpose of detecting the *B. cereus*' diarrhoeal enterotoxin, as well as to demonstrate enterotoxin production by isolates grown in culture. BCET-RPLA Toxin Detection Kit, developed by Oxoid, requires only a simple extraction procedure and provides a result in 24 hours. In a reversed agglutination assay the antibody, which is attached to particles, reacts with the soluble antigen. The particles, in this case latex, do not play a part in the reaction. The cross-linking of the latex particles by the specific antigen/antibody reaction results in the visible latex agglutination reaction (Thermo Scientific 2016).

1.4.2. *Listeria monocytogenes*

Listeria monocytogenes is a Gram-positive, motile, non-spore-forming bacillus that has been isolated from a variety of animal species (Bortolussi 1999). This organism has a great ability to grow and persist in hostile environments, even in food with high salt or acidic content

due to the formation of biofilms, or through cross-contamination, representing a major concern for the food industry (Jadhav et al. 2012; Lomonaco et al. 2015).

L. monocytogenes is the major bacterial pathogen for newborn infants in North America. Their high susceptibility is attributed to delayed activation of macrophages, since tumour necrosis factor-α (TNF-α) and interleukin-12 (IL-12), which are essential mediators of host defense, are produced in lower concentrations at such a young age (Bortolussi 1999; Day & Basavanna 2015a).

Foodborne outbreaks of listeriosis (one of the most virulent and life threatening bacterial foodborne diseases known) are due to inadequately pasteurised dairy products (e.g. soft cheeses), undercooked chicken, and ready-to-eat foods. The severity of infection is dependent on several factors including the virulence of the infecting strain and susceptibility of the host. There are 13 serotypes that have been identified so far and each has the potential to cause disease in human hosts (Day & Basavanna 2015a). Serotypes 1/2a, 1/2b and 4b have been mainly isolated from both sporadic and outbreak cases of human listeriosis. Serotypes 1/2a, 1/2b, 1/2c, 3a, 3c and 4b are commonly found in food and appear widely distributed in natural and farm environments (Kathariou 2002). However, serotypes 4b, 1/2a and 1/2b are the most likely to cause listeriosis in humans (Kathariou et al. 2006).

Day & Basavanna (2015b) developed a rapid detection procedure for *L. monocytogenes* in Infamil^{R} powdered infant formula resuspended in distilled water, using a macrophage-based enrichment protocol and real-time PCR. Macrophage monolayers were exposed to IF contaminated with serial dilutions of *L. monocytogenes*. Approximately 10 CFU mL^{-1} (or g^{-1}) of *L. monocytogenes* were detected in infant formula 16h after infection by real-time PCR, concluding that the detection method is highly sensitive and specific for *L. monocytogenes* in infant formula, since co-inoculation with *L. innocua* did not reduce the *L. monocytogenes* detection sensitivity. Macrophage enrichment of *L. monocytogenes* eliminates PCR-inhibitory food elements and contaminating food microbiota.

The specific detection of the genus *Listeria* by PCR was evaluated with six sets of primers against 16S rRNA present in all *Listeria* spp. and *iap, hly* and *prf* virulence genes. The primer pair against 16S rRNA resulted in a 1.2 kb amplification product. Primers targeted against different regions of *iap* produced 371 and 660 bp products, respectively. The primer pair targeted against *prf* gene could produce 508 bp product. Three primer pairs targeted against *hly, hly A* and *hly K9* were able to amplify 713, 276 and 384 bp products, respectively. While optimizing the parameters, the authors observed that only two of the amplified products, 1.2 kb

and 713 bp, could be invariably detected. The conditions were optimized for application in dairy foods for detection of *Listeria monocytogenes* (Kumar et al. 2015).

1.4.3. *Cronobacter* spp.

Cronobacter is a newly described genus which includes opportunistic pathogens formerly known as *Enterobacter sakazakii*, well known for its resistance to osmotic and dry stresses (Osaili & Forsythe 2009). The original name of *E. sakazakii* was reclassified as *Cronobacter* spp., which includes *Cronobacter sakazakii* (Iversen et al. 2004; Kim et al. 2010), using partial 16S ribosomal DNA and *hsp60* sequencing. Iversen et al. (2004) divided 126 *Cronobacter* isolates into four clusters, suggesting that the genus might require re-classification. Following further extensive polyphasic analysis, Iversen et al. (2007) and Iversen et al. (2008) proposed the reclassification of these bacteria into a new genus called *Cronobacter*.

Cronobacter sakazakii is a Gram-negative, facultative anaerobic, rod-shaped bacterium, belonging to the *Enterobacteriaceae* family. The species differentiate based on biochemical reactions, and serological and molecular techniques. It is considered an opportunistic pathogen causing severe form of colitis and meningitis, and severe neurological sequelae such as hydrocephalus, quadriplegia and retarded neural development can occur (Nazarowec-White & Farber 1997; Shaker et al. 2007). Apart from C. *sakazakii, C. cloacae* and C. *agglomerans* are also a severe hazard for populations, especially neonates, with a mortality rate of 40%-80% (Muytjens et al. 1988).

The first sequenced *Cronobacter* genome, *C. sakazakii* ATCC®BAA-894's, was published by Kucerova et al. (2010). It revealed a single chromosome of 4.4 Mb (57% GC) along with two plasmids, designated as pESA2 and pESA3 (31-kb, 51% GC and 131-kb, 56% GC, respectively). The source of this isolate was a contaminated PIF used in a neonatal intensive care unit and which gave rise to an outbreak in 2001 in Tennessee, United States of America. *Cronobacter sakazakii* ATCC®BAA-894 contained approximately 4392 genes as part of its core genome. However, using the Comparative Genome Hybridization approach, 4382 unique, annotated genes from both the chromosome and plasmids were noted, and only 54.9% of genes were common to all *C. sakazakii* with 43.3% being common across all *Cronobacter* species. 21 genes were found to be unique in 5 of the *C. sakazakii* tested, and these proteins were involved in pilus assembly, a phosphotransferase system, an acid transporter, N-acetylneuraminate lyase and a toxin/antitoxin system.

Franco et al. (2011) reported the *in silico* analysis of *C. sakazakii* ATCC®BAA-894 plasmid pESA3, which contained a RepFIB replicon. Plasmid pESA3 also contains two iron acquisition systems (eitCBAD and iucABCD/iutA) essential for survival and successful pathogenesis. The presence of a *Cronobacter* plasminogen activator-encoding gene (*cpa*), encoded on pESA3, a type 6 secretion system (T6SS) also encoded on pESA3, and a filamentous haemagglutinin/adhesin (FHA) gene locus (located on pCTU1) suggested the existence of unique virulence determinants in these species. The *cpa*-encoding gene encodes an outer membrane protease implicated in serum resistance, which may facilitate *Cronobacter* in crossing the blood–brain barrier and causing meningitis (Franco et al. 2011). The T6SS acts to translocate putative effector proteins aiding in bacterial pathogenesis, while FHA pCTU1 contains *fhaB, fhaC* genes (which encode proteins with similarly identity to a transported and transporter protein as part of a two-partner secretion system) and five associate putative adhesins.

The *Cronobacter* species have been isolated from soil, insects, milk powder and chocolate factories, and common households as well as a wide range of foods, e.g. UHT milk, cheese, meat, vegetables, spices, tofu and powdered infant formula (Muytjens et al. 1988; Leclercq et al. 2002; Iversen & Forsythe 2004). In order to decrease the risk of disease in young children, some measures must be taken, e.g. controlling the microbiological load in infant foods, knowing the optimal growth conditions and understanding the epidemiology (Yan et al. 2012).

The first time *Cronobacter* spp. was reported in powdered infant formula was by Muytjens et al. (1988), using 141 samples from 35 countries. The level of contamination ranged from 0.35 to 66.0 CFU/100 g, and this low level of contamination has been reported in many studies. Despite the fact PIF is subjected to heat treatment during processing, *C. sakazakii* was still found in it, possibly due to post-processing contamination from the factory environment (Shaker et al. 2007). *C. sakazakii* is more thermotolerant than other *Enterobacteriaceae* in dairy products, but it is more heat-sensitive than other pathogens like *L. monocytogenes* (Nazarowec-White & Farber 1997). Lin & Beuchat (2007) and Gurtler & Beuchat (2007) studied the survival of *Cronobacter* spp. in infant formula under different storage conditions for 12 months. The survival was greater at low water activities and a low storage temperature (4°C). The persistence of the bacterium was lower with higher water activity, and it decreased with increasing storage temperature (4, 21 and 30°C).

In terms of virulence, transmembrane signal proteins of two-component regulatory system in Gram-negative pathogens contributed to immune escape or biofilm formation (Kim et al. 2010). Outer membrane proteins A (OmpA) and X (OmpX) in *C. sakazakii* play critical

roles in adhesion to and invading human cells (Mohan Nair & Venkitanarayanan 2007; Nair et al. 2009; Kim et al. 2010). Ye et al. (2015) studied the different expression of genes of membrane proteins between virulent *C. sakazakii* isolate G362 and attenuated L3101: *ompA* and *ompX*, and some novel factors (*envZ*, *LptE*, *MdtD*, and *OsmY*) were successfully identified by 2-DE gel electrophoresis by potentially being involved in virulence differences. However, the detailed roles and regulation of these potential virulence factors of *C. sakazakii*, and study on interaction between host and bacteria have not been determined.

1.4.3.1. Chemical inactivation of *C. sakazakii*

Shi et al. (2016) studied the inhibitory effect of syringic acid against *C. sakazakii*, using field emission scanning electron microscope to assess the morphological changes of bacterial cells. Syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid, $C_9H_{10}O_5$) is a hydroxybenzoic acid of phenolic compound that has been found in *Lentinula edodes* (shiitake mushrooms), cereal grains, *Herba dendrobii*, *Radix isatidis* and the leaves of *Alpinia calcarata Roscoe*. It was shown that syringic acid retarded bacterial growth, and caused cell membrane dysfunction, which was evidenced by decreasing intracellular ATP concentration, reduction of intracellular pH, cell membrane hyperpolarization and changes in cellular morphology, indicating that syringic acid could be developed as a natural preservative to control *C. sakazakii* in foods.

Various natural substances have been proved to be bacteriostatic or bactericidal against *C. sakazakii* in reconstituted powdered infant formula: caprylic acid (Mohan Nair et al. 2004), trans-cinnamaldehyde (Amalaradjou et al. 2009), polyphenol-rich cocoa powder (Pina-Pérez et al. 2012), bovine lactoferrin and nisin (Al-Nabulsi & Holley 2007), and thymoquinone combined with mild heat (Shi et al. 2015).

1.4.4. ***Clostridium botulinum***

The *Clostridium* genera represents ubiquitous bacilli commonly found in soil, water, and gastrointestinal tracts of insects, animals, and humans, growing in low-oxygen environments; however, it is better adapted for anaerobic life. Pathogenic *Clostridium* species have developed mechanisms for survival within and outside different host types, as evidenced by the various diseases frequently linked to their protein toxins and spores that include gas gangrene, food poisoning, antibiotic-associated diarrhoea, pseudomembranous colitis, and enterotoxaemia (Stiles et al. 2014).

Clostridium botulinum is a Gram-positive, rod-shaped, anaerobic, spore-forming, motile bacterium with the ability to produce the neurotoxin botulinum. It was first described in 1895 following a food poisoning incident in Belgium (Devriese 2010). *C. botulinum* is divided into four distinct phenotypic groups (I-IV) and is also classified into seven serotypes (A-G) based on the antigenicity of the botulinum toxin produced. In contrast to the classic botulinum neurotoxins, the C2 toxin produced by types C and D lacks neurotoxicity (Ohishi et al. 1980; Iwasaki et al. 1980). C2 toxin is synthesized by *C. botulinum* during sporulation (Ohishi & Sakaguchi 1982). This species can occur in dried milk products and infant milk formula but it is not a common problem (Doyle & Glass 2013).

1.4.5. *Salmonella* spp.

Salmonella spp. are Gram-negative, facultative anaerobic and motile bacteria, which are transmitted by infected animals, contaminated food, or water via the fecal–oral route (Erhardt & Dersch 2015). *Salmonella*-contaminated dried milk products caused outbreaks of salmonellosis in the 1950s in the United Kingdom and Bulgaria. Salmonellosis still represents an important foodborne disease that constitutes a major threat to human public health in both developed and developing countries, since *Salmonella* serotypes are widespread in nature and can be found in the intestinal tract of both domestic and wild animals species (Carrasco et al. 2012). In the European Union, *Salmonella* Enteritidis and *Salmonella* Typhimurium are the two major agents of salmonellosis in humans.

The preparation of raw food (such as fresh chicken) increases the dissemination of *Salmonella* to hands and food contact surfaces. A significant part of cross-contamination scenarios occurs in domestic kitchens. In fact, more than 50% of reported foodborne outbreaks were observed at home (Scott 1996). *Salmonella* is able to contaminate food preparation areas because of their ability to form biofilms in different surfaces (Carrasco et al. 2012). De Boer & Hahné (1990) showed that *Salmonella* can easily be transferred from chicken to utensils, a variety of kitchen surfaces, hands and other foods; *Salmonella* cells were recovered from surfaces up to 6 h after contamination. *Salmonella* Enteritidis is linked to consumption of poultry, eggs, and egg-derived products (Schmid et al. 1996; Hayes et al. 1999). Slinko et al. (2009) described an outbreak in a series of restaurants across Brisbane, Australia, in a two-month period. Investigation of these restaurants identified potential cross-contamination issues, related to cracked eggs contaminated with *Salmonella* Typhimurium Phage type 197. *Salmonella* spp. is able to survive for weeks in water and for years in soil if environmental

conditions are favourable. Processed foods such as powdered milk, chocolate, peanut butter, infant food, and bakery products are characteristically products with low water activity, which represents a barrier to growth for *Salmonella* spp. (Dimakopoulou-Papazoglou et al. 2016). Epidemiological and environmental data investigations have indicated that cross-contamination plays a major role in the contamination of these products (Smith et al. 2004).

Salmonella spp.'s virulence arises from intrinsic mechanisms which allows them to survive inside the host's organism, with relevance to metabolic adaptation (e.g. catabolic or biosynthetic metabolism, or selection of the best energy source), signalling strategies, and conserved regulatory proteins (Erhardt & Dersch 2015).

Bacteria sense external stimuli through two component systems (TCSs) and convert it into an adaptive cellular response. Among the most virulence-relevant TCS are the PhoP/PhoQ, EnvZ/OmpR, and BarA/SirA(UvrY) systems (Groisman 2001; Groisman & Mouslim 2006), composed of the membrane-bound sensor kinases PhoQ, EnvZ, and BarA. These kinases sense the signals and phosphorylate the cytoplasmic response regulator PhoP, OmpR, and UvrY(SirA).

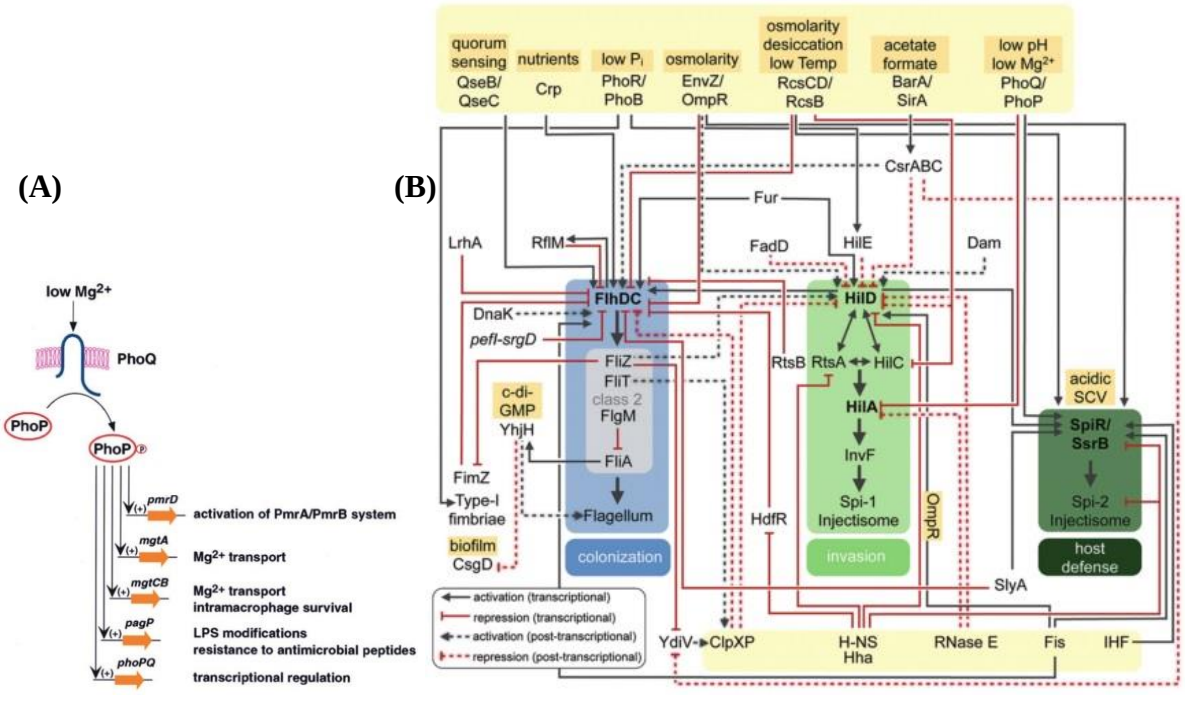


Figure 1.2. (A) PhoP/PhoQ signals control the regulation of virulence-related genes (Groisman 2001). The TCS PhoP/PhoQ responds to low Mg^{2+}, low pH environments, and host-secreted cationic antimicrobial peptides. It is essential for virulence and survival within macrophages (Miller et al. 1989). PhoP represses *hilA* (an invasor gene activator) and the PhoP-repressed genes (Pegues et al. 1995; Bajaj et al. 1996), while the transcription of PhoP-activated genes (required for survival within macrophages) is activated (Miller et al. 1989; Belden & Miller 1994). MgtC also plays a major role in *Salmonella*'s virulence. It is an inner membrane protein with five transmembrane helices at the N-terminus and one cytoplasmic domain at the

C-terminus (Rang et al. 2007). *mgtC* genes from all these pathogens are required both for survival inside macrophages and for growth in low Mg^{2+} media (Blanc-Potard & Groisman 1997). MgtC has the ability to promote *Salmonella* pathogenicity depending on maintaining ATP homeostasis by inhibiting F1Fo ATP synthase (Lee et al. 2013). **(B) Regulation model of all *Salmonella* virulence factors (Erhardt & Dersch 2015).** The TCS EnvZ/OmpR regulates the flagellar and virulence genes, but OmpR represses *flhDC*, a flagellar operon, in response to extracellular osmolarity (Ellermeier et al. 2005; Golubeva et al. 2012). The BarA/UvrY (SirA) system is activated by metabolic end products (e.g., short-chain fatty acids such as formate or acetate) and an imbalance of the TCA cycle. It controls the expression of cell invasion genes (Takeuchi et al. 2009; Gonzalez Chavez et al. 2010). The cAMP receptor protein Crp helps the pathogens to analyse available carbon sources in order to optimise their metabolism (Görke & Stülke 2008; Poncet et al. 2009). Bacteria check for the availability of readily digestible sugars such as glucose and fructose. In the absence of these, cAMP is produced by adenylate cyclase, which then binds to Crp to form an active cAMP-Crp complex (Hanamura & Aiba 1991; Ishizuka et al. 1994).

1.5. Microbial Identification

1.5.1. Gram staining method

An initial step in identifying a bacterial species is determining whether the cells are Gram-positive or Gram-negative. The Gram stain is one of the most important and widely used tools in the identification of unknown bacteria. The Gram stain reaction is dependent on the cell wall structure.

Table 1.1. Bacterial structure and staining procedures according to the its structure, and respective colouring stain and colour on microscope observation.

	Cell wall	**Inner membrane**	**Outer membrane**	**Colour on microscope**	**Colouring stain**
Gram-negative bacteria	Yes	Yes	Yes	Pink	Safranin
Gram-positive bacteria	Yes	Yes	No	Purple	Crystal violet
Endospores	-	-	-	-Green (Schaeffer-Fulton stain) -Red (Moeller stain)	-Schaeffer-Fulton stain (malachite green + safranin as counterstain) -Moeller stain (carbol fuchsin + methylene blue as counterstain)

Flagella	-	-	-	-Purple (Ryu stain) -Red (Leifson Flagella stain)	-Flagella stain solution (Ryu stain) -Leifson's stain made of tannic acid and a basic fuchsin prepared in alcohol base + methylene blue (Leifson Flagella stain)
Capsules	-	-	-	- Negative staining: translucid capsule; black or red background - Positive staining: Capsules appear coulourless with stained cells against a dark background	-Negative staining (unstained capsule; background stained with india ink, negrosin or congo red) -Positive staining (mordant to precipitate the capsule + crystal violet or methylene blue as counterstain for vegetative cells)

The procedure requires four solutions: a basic dye, crystal violet; a mordant, Gram's iodine; a decolorizing agent, ethanol; a counterstain, safranin (**Figure 1.3**).

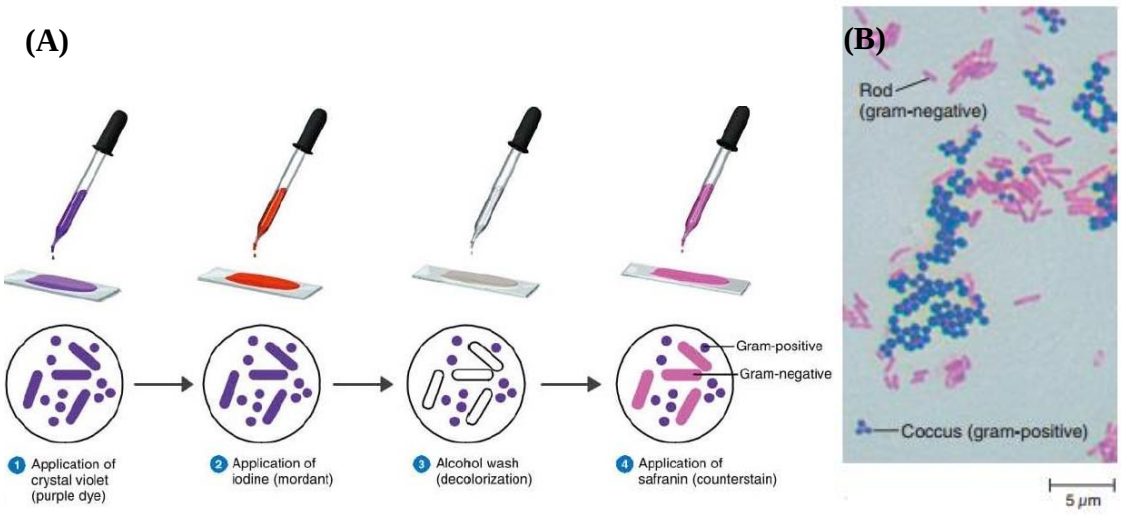


Figure 1.3. (A) Gram staining procedure (retrieved from medicinehack.com). A cell preparation is immersed into the crystal violet stain. The purple dye, crystal violet, is taken by the cell wall of both Gram-positive and Gram-negative bacteria (Step 1).

The cells are then dipped into an iodine solution (Step 2). Iodine forms large complexes with crystal violet and these complexes combine with the peptidoglycan in the cell wall. When ethanol is applied (Step 3), the ethanol dissolves the lipids in the outer membrane of the cell wall of Gram-negative bacteria causing the crystal violet-iodine complex to leave the cells. Thus Gram-negative cells appear colourless. Gram-positive cells therefore stain purple, which is the colour of the crystal violet dye. The decolourising step in the Gram stain is the critical step in distinguishing Gram-positive from Gram-negative bacteria. If Gram-positive cells are exposed to ethanol for too long, they appear as Gram-negative cells. As Gram-negative cells are colourless after the ethanol wash, they are counterstained with safranin (Step 4). Therefore, Gram-negative cells appear pink after the Gram stain procedure (Rao 1997; Beveridge 2001; Coico 2009; Suguna Sivakumar & Dhanalakshmi 2015). **(B)** Exemplification of Gram staining for the identification of Gram-negative and Gram-positive bacteria (Tortora et al. 2016).

1.5.2. Microbial growth conditions

Microorganisms are also classified according to colony morphology and cell morphology. Bacterial colonies grow from a single cell and are composed of millions of cells. Each colony has a characteristic size, form or shape, edge, texture, degree of opacity, and colour. These characteristics describe the morphology of a single colony and may be useful in the preliminary identification of a bacterial species. Colonies with a singular appearance (when grown on the same medium) can be assumed to contain different bacterial species. However, since many species have a similar colony morphology, the reverse (that colonies that look alike are the same species) is not always true.

The three most commonly recognised cell morphologies are *cocci*, *bacilli*, and *spirilla*. C*occi* have a spherical shape. *Bacilli* are rod-shaped. There is often great variation in the length of *bacilli*. Many are long and slender, while others are so short that they may resemble *cocci*. The *spirilla* form spirals, although its number varies considerably from species to species.

In addition to cell morphology, the specific arrangement of cells is an important identifying characteristic of many bacteria. Bacterial cells are found singly, in pairs, in clusters, or in chains when viewed under the microscope.

Motility is observed through the general look of the semi-solid medium in a tube. If the bacteria are motile, the medium will appear turbid. Otherwise, the bacterial growth will only be observed in the inoculation site (Rao 1997; Madigan et al. 2012; Tortora et al. 2016).

1.5.3. Biochemical methods

Biochemical tests that investigate the enzymatic activities of cells are powerful tests in the identification of bacteria. As many as 50-100 tests may have to be performed for a positive

identification. However, the general tests recognise the ability of the bacterial species to use glucose as a carbon source, the specific oxygen requirements for growth of each species, and enzymes. In **Table 1.2** there are some examples of these tests.

Table 1.2. Biochemical characteristics of microorganisms exploited for their identification, and respective tests and description.

Characteristics	Test	Description
Usage of glucose as a carbon source Bacteria are capable of using different carbon sources to obtain the energy needed to sustain life. One main carbon source is glucose, but not all bacteria can metabolise glucose to harness energy. There are several pathways that bacteria can use to metabolise glucose. The end products and side products of each pathway provide an indication of the specific pathway the bacterium uses to metabolise glucose (Tortora et al. 2016).	Malonate	Determines the ability of a strain to use malonate as the only carbon source, because malonate binds competitively to succinate dehydrogenase preventing its catalytic activity over succinic acid. The medium is alkaline, and the indicator bromothymol blue turns the solution blue (Coico 2009).
	Methyl red	Evaluates whether the bacteria produce acids (formic, lactic, or acetic acids) through fermentation. A red colour indicates a pH lower than 4.4 while yellow indicates a pH > 6. It allows the identification of Enterobacteria (Coico 2009).
Usage of lactose or sucrose as carbon source	Phenol red broth	Differentiates Gram-negative enteric bacteria. It contains peptone, phenol red, a Durham tube, and one carbohydrate. Three different kinds of phenol red broths are used: one contains glucose; one contains lactose, and the last contains sucrose. Phenol red turns yellow below a pH of 6.8 and fuchsia above a pH of 7.4. If the organism is able to utilise the carbohydrate, an acid by-product is created, which turns the media yellow. If the organism is unable to utilize the carbohydrate but does use the peptone, the by-product is ammonia, which raises the pH of the media and turns it fuchsia. When the organism is able to use the carbohydrate, a gas by-product may be produced, and an air bubble will be trapped inside the Durham tube.
Usage of citrate as carbon source	Citrate	Simmons citrate agar contains sodium citrate as carbon source, ammonium dihydrogen phosphate as the sole source of nitrogen, other nutrients, and the pH indicator bromthymol blue. This test differentiates *Enterobacteriaceae*. Organisms which can utilize citrate as their sole carbon source

		use the enzyme citrase or citrate-permease to transport the citrate into the cell. These organisms also convert the ammonium dihydrogen phosphate to ammonia and ammonium hydroxide, which creates an alkaline environment in the medium. At pH 7.5 or above, bromthymol blue turns royal blue (citrate positive). At a neutral pH, bromthymol blue is green (citrate negative).
Oxygen needs Bacteria are capable of growing in a variety of environmental conditions. The oxygen requirements for bacterial growth are a factor useful in identification. Bacteria are grouped into several categories based on their oxygen needs.	Obligate anaerobe	Does not tolerate oxygen and growth ceases in the presence of oxygen (Madigan et al. 2012).
	Obligate aerobe	Requires oxygen for survival and growth (Madigan et al. 2012).
	Facultative anaerobe	Survives in aerobic and anaerobic conditions but prefer to grow in the presence of oxygen (Madigan et al. 2012).
Enzymes Some bacterial strains possess specific enzymes making them able to be identified through particular enzyme-catalysed reactions.	Catalase	Decomposes hydrogen peroxide to water and oxygen which forms bubbles visible *in loco*. This reaction allows the distinction between *Staphylococcus* and *Streptococcus* (Rao 1997; Madigan et al. 2012).
	Oxidase	Informs that there is an electron transport system named cytochrome oxidase, meaning natural electron acceptors can be replaced by artificial acceptors. In the presence of atmospheric oxygen, the acceptors are oxidized by cytochrome oxidase, originating a colourful compound. This test differentiates Gram-negative from Gram-positive bacteria (Rao 1997).
	Phenylalanine deaminase	Removes the amino group from the amino acid phenylalanine, originating phenylpiruvic acid. This acid reacts with the ferric chloride (10%) added to the medium, turning it into green; it allows the differentiation of different *Enterobacteria* genera and species (Rao 1997).
	Lipase	One of the lipase tests is tributyrin agar test, a differential medium. Lipase hydrolyzes tributyrin oil, a triglyceride. Triglycerides' glycerol and fatty acids are broken apart and may be converted into a variety of end-products that can be used by the cell in energy production.
	Casease	Skim milk agar is a differential medium that tests the ability of an organism to produce casease, an exoenzyme that hydrolyzes casein. Casease breaks down casein into smaller polypeptides, peptides,

		and amino acids that can cross the cell membrane and be utilized by the organism.
API® test kits (**A**nalytical **P**rofile **I**ndex)	See Annexes V and VI	Biochemical test kits available through BioMerieux, Inc. allowing the identification of bacteria and yeast. The design is identical in all cases: plastic wells are pre-filled with the necessary dehydrated reagents. The operator inoculates wells with the bacterial saline suspension, followed by incubation according to the specifications of the manufacturer (Holmes et al. 1978). After incubation, results are compared to the manufacturer's instructions and confirmed at apiweb™, a BioMerieux *website*. The API® 20E kit allows the 18 to 24-hour identification of *Enterobacteriaceae* and other non-fastidious Gram-negative bacteria, while the API® 50 CHB kit is used in the identification of *Bacillus*.

1.5.4. Real-Time PCR and Microarrays

An accurate microorganism identification is essential for correct disease diagnosis and treatment of infection. Bacterial identification is used in a wide variety of applications including food science, microbial forensics, criminal investigations, bioterrorism threats and environmental studies. Traditional methods of bacterial identification rely on phenotypic identification of the causative organism using Gram staining, culture and biochemical methods. However, these methods have two major disadvantages. First, they can be used only for organisms that can be cultivated *in vitro*. Second, some strains exhibit unique biochemical characteristics that do not fit into patterns.

Real-time PCR and microarrays are currently the most commonly employed molecular techniques. It is highly sensitive and allows quantitation of bacteria at a species level. Using a DNA-based assay, one can easily detect bacterial strains directly from clinical samples or from small amounts of cultured bacterial cells.

PCR has been useful in this regard, which relies on primer sequences designed to facilitate bacterial identification at any level of specificity: strain, species or genus. Real-time PCR is a promising tool for distinguishing specific sequences from a complex mixture of DNA and therefore is useful for determining the presence and quantity of pathogen-specific or other unique sequences within a sample (Mackay 2004). The development of real-time or quantitative PCR (qPCR) provides high-throughput analysis and low risk of cross-contamination since post-PCR processing for the detection of PCR products is not required (Fricker et al. 2007).

Repetitive element PCR (repPCR), amplified fragment length polymorphism (AFLP), and random amplification of polymorphic DNA all utilize PCR to amplify multiple copies of short DNA fragments using defined sets of primers (Versalovic et al. 1994; Vos et al. 1995; Lin et al. 1996). These methods are designed to take advantage of DNA polymorphisms in related organisms (Spigaglia & Mastrantonio 2003; Emerson et al. 2008).

Microarray-based bacterial identification relies on the hybridisation of pre-amplified bacterial DNA sequences to arrayed species-specific oligonucleotides. Each probe is tagged with a different coloured dye which fluoresces upon hybridisation. A typical microarray experiment involves the hybridisation of an mRNA molecule to the DNA template from which it is originated. Thousands of spotted samples known as probes (with known identity) are immobilised on a solid support. The spots can be DNA, cDNA, or oligonucleotides. These are used to determine complementary binding of the unknown sequences. The amount of mRNA bound to each site on the array indicates the expression level of the various genes (Raich & Powell 2015; Rothbauer et al. 2016).

1.6. Sterilisation Methods

According to the World Health Organization, sterilisation is necessary for the complete destruction or removal of all microorganisms, including spore-forming and non-spore-forming bacteria, viruses or fungi). The efficacy of any sterilisation process will depend on the nature of the product, the extent and type of any contamination, and the conditions under which the final product has been prepared. The most important factors for food preservation are microbial growth at high or low temperature, water activity, pH, antimicrobials and competitive microbiota (López-Malo & Palou 2005).

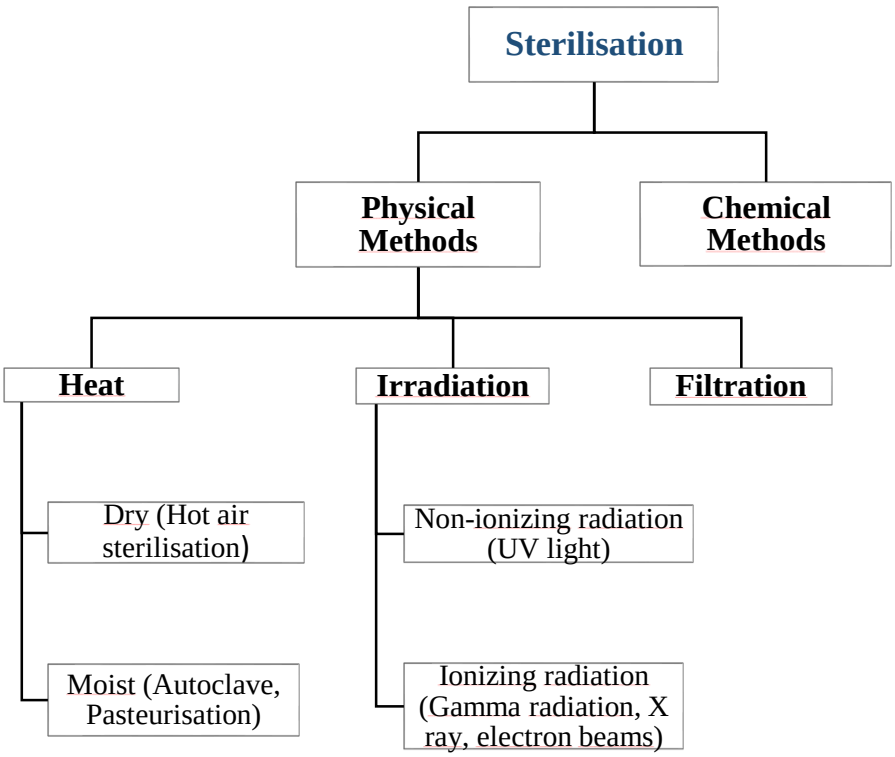


Figure 1.4. Representation of the most utilised sterilisation methods (adapted from World Health Organisation (2004)**).** These are divided between physical and chemical methods, according with the properties involved in said technique. In the physical methods, heat-related techniques rely on dry heat or moist, this last including the autoclave which is used daily in research laboratories. Irradiation techniques are divided into two types of radiation: non-ionizing (e.g. UV light), and ionizing (e.g. gamma radiation and e-beam). Chemical sterilisation is not compatible with food safety standards.

There is another classification system to sterilisation methods: thermal and non-thermal. In **Table 1.3**, there is a description of thermal and non-thermal sterilisation methods.

Table 1.3. Thermal and non-thermal sterilisation methods (Särkka-Tirkkonen 2010).

Method	Industrial relevance	Advantage	Disadvantage
THERMAL			
Conventional heat/autoclave/retort	Covers almost all food products groups	- Direct energy transport to the product (steam) - Well established technology - High food safety	-High heat load for the product, causing structural and nutritional defects -Energy consuming
Ohmic heating	Covers almost all food products groups	- Generates the heat in the food itself, delivering thermal energy where it is needed - Particulate temperatures similar or higher than liquid temperatures	-Lack of temperature monitoring techniques in continuous systems -Differences on electrical conductivity between solids and liquid

		- Faster than conventional heat processing - Minimal mechanical damage to the product and better nutrients and vitamin retention - High energy efficiency because 90% of the electrical energy is converted into heat - Ease of process control with instant switch-on shut-down	-Lack of data concerning the critical factors affecting heating
High frequency/Radio frequency/Microwave		- Direct energy transfer into the food - No structural damages to food - Improved food quality: more uniform heating - Increased throughput - Shorter processing lines - Improved energy efficiency - Maillard reactions may b reduced	- Technology is in an early stage of the development - High energy consumption - Not compatible with organic standards
NON-THERMAL			
High pressure	High-acid products like juices, jams jellies and yoghurts. Pasteurisation of meats and vegetables.	- Textures, taste and retention of nutrients are better than for conventional retort - Shorter treatment times - Lower maximum temperature - Faster heating and cooling - More uniform temperature rise within the product - Independent of the size, shape and composition of the food product - No evidence of toxicity	- Not yet commercial application for shelf-stable low-acid products - Energy consuming - Expensive equipment - Food should have 40% of free water for anti-microbial effect - Limited packaging options - Regulatory issues to be resolved
Pulse-electric field	Liquid foods (juices, soups, liquid egg and milk). Accelerated thawing.	- Kills vegetative cells. - Colours, flavours and nutrients are preserved - No evidence of toxicity - Relative short treatment time	-Difficult to use with conductive materials -Only suitable with liquids or particles in liquids -Energy efficiency not yet certain -No effect on enzymes and spores
Ultrasonic waves	Any food that is heated	- Effective against vegetative cells, spores and enzymes	- Can denaturate proteins and produce free radicals which

		- Reduction of process times and temperatures	can affect the flavour (high fat foods)
Microbiocide gas (ethylene oxide, ethanol, ozone)			- Not accepted in EU at all for food - Not compatible with organic foods - Only for specific foods - Expensive
Irradiation	Covers almost all food products groups	-Pre-packed products can be processed	- Not compatible with organic food
Ultraviolet light	Dry foods, fresh fruit and vegetables	- Medium cost - Rapid process - Little or no changes in food	- Not proven effective against spores - Only surface effects (problems with complex surfaces)

1.6.1. Conventional heating

Exposure of microorganisms to moist heat under pressure in an autoclave provokes their destruction by the irreversible denaturation of enzymes and structural proteins. For example, BSSL- bile salt stimulated lipase- is a very heat labile enzyme which activity is completely eliminated during sterilisation. It breaks down triacylglycerol into monoacylglycerides and free fatty acids, contributing to an easy digestion. Also, alkaline phosphatase (known as ALP) is inactivated slightly above the pasteurisation conditions needed to eliminate the microorganisms targeted in milk, being used in the dairy industry as a biomarker to detect inadequate pasteurisation of milk (Christen et al. 2013). Sterilisation in saturated steam requires precise control of time, temperature and pressure. This method should be used whenever possible for aqueous preparations, thus for liquid infant formula. Another moist heat sterilisation method is pasteurisation. In dry-heat processes, as hot air sterilisation, the primary lethal process is considered to be oxidation of cell constituents. Dry-heat sterilisation requires a higher temperature than moist heat and a longer exposure time. The method is more convenient for heat-stable, non-aqueous materials that cannot be sterilized by steam, as PIF.

IF constituents suffer blending, pasteurisation, homogenisation, concentration, spray-drying and heat sterilisation in order to ensure microbiological safety and to extend its life span. Sterilisation through UHT or spray-drying leads to enormous modifications in the composition of IF, causing low digestibility (Birlouez-Aragon 2004; Sarriá & Vaquero 2006). The methods required for bacterial death include high temperatures, which cause protein denaturation,

vitamin loss and formation of Maillard reaction products, also known as MRP (Diez et al. 2008). Heat-damaged proteins and MRP affect copper bioavailability, absorption and transport in the child's organism (Sarriá & Vaquero 2006).

Thermodynamically speaking, a high activation energy indicates a strong temperature dependence (it will run slowly at low temperature). In food reactions, as the Maillard reaction, the activation energy is about 100 $kJ.mol^{-1}$, whereas 300 $kJ.mol^{-1}$ is the energy necessary to inactivate microorganisms. This energy difference is explored in UHT (ultra-high-temperature treatment), for instance, achieving microbial inactivation and a minimum quality loss (Boekel 2008; Boekel 2007). Most enzymes related to food reactions and/or microbial contaminations tend to become inactivated between 50-80ºC, and time-temperature combinations for microbial inactivations must be chosen in this temperature gap.

Some malign effects and applicability of food sterilisation methods are described in **Table 1.4**.

Table 1.4. Characteristics and applicability of some sterilisation methods (Särkka-Tirkkonen 2010).

Sterilisation method	Food safety affecting factors	Products	Nutritional influence	Sensory quality
Autoclave	Inactivates microbes and bacterial spores	Almost all food products	Nutritional defects	Structural, taste and colour defects
Ohmic heating	Lack of temperature monitoring techniques in continuous systems	Almost all food products	Better nutrient and vitamin retention than in conventional heating	Minimal mechanical damage
High frequency	Heating can be controlled very precisely	Pre-packed food, both solid and liquid	No data	No data
Microwave	Difficulties for controlling heating uniformity	Pre-packed food, both sold and liquid	No data	No data
High pressure	Bacterial spores difficult to inactivate by high pressure alone. Food should have 40% of free water for antimicrobial effect. No evidence of toxicity.	High acid products like juices, jams, jellies and salad dressings, yoghurt, certain meat products	Better nutrient and vitamin retention than in conventional heating	Better texture and taste than in conventional heating systems. Effect on colour product dependent.
Pulse electric field	No evidence of toxicity. Kills vegetative cells but no effect on enzymes and spores	Liquid products like fruit juices, soups and milk	Nutrients are preserved better than in conventional heating	Better colour and flavour than in conventional heating
Ultrasonic waves	Insufficient to inactivate many bacterial species alone. Causes DNA	Any food that is heated	No data	Flavour defects in high fat food

	damage to bacterial cells.			
Irradiation	Restricted use in EU for food and not compatible with organic food	Prepacked food	No data	Flavour defects, especially in high fat food
UV Light	Not proven effective against spores	Fresh fruit and vegetables, dry food	No data	No data

1.6.2. UV Light

Disinfection by ultraviolet radiation is a physical process defined by the transfer of electromagnetic energy from a light source to an organism's genetic cellular material. The lethal effects of this energy are the cell's inability to replicate (Särkka-Tirkkonen 2010), because most microorganisms absorb radiation at 254 nm, due to its purine and pyrimidine bases of DNA, causing bonds to form between adjacent thymines in DNA chains. The forms of radiation in the electromagnetic spectrum differ in frequency, penetrating power and wavelength. Generally, Gram-negative bacteria are the least resistant to UV radiation, followed by Gram-positives, yeast and bacterial spores. The biggest resistance is found in moulds and viruses (López-Malo & Palou 2005).

Typically, UV light for disinfecting techniques ranges from 100 to 400 nm (López-Malo & Palou 2005). Short-wave ultraviolet light (UVC, or monochromatic/germicidal UV), at a wavelength rage of 200-280 nm, is reported to be an effective method for inactivating bacteria on surfaces (for pharmaceutical and aquaculture industries) and on liquids like fruit and vegetable juices (Bintsis et al. 2000; Yaun et al. 2004; López-Malo & Palou 2005). It has been used to control *Bacillus stearothermophilus* growth in thin layers of sugar (Weiser 1963), minimise the risk of foodborne illness associated with the consumption of fresh fruits and vegetables (Yaun et al. 2004), and as a disinfection technique for drinking water supplies (López-Malo & Palou 2005). Kasahara et al. (2015) effectively PUV-inactivated *E. coli* in goat milk using a 6 $\log_{10}$ reduction in the population. Although some aromatic changes were found, there were no differences in the physical or compositional parameters of the irradiated and control samples. Engin & Karagul Yuceer (2012) investigated the effects of UV application as an alternative to heat treatment of milk. UV light had a major effect on total coliforms, *E. coli* and *Staphylococcus* spp.; no major differences were observed in terms of aroma-active compounds in milk, but some volatiles were generated by UV treatments that were not previously present in the milk, and the coagulation time of milk was not affected. Krishnamurthy (2006) inoculated raw milk with *S. aureus* and treated it with PUV by varying

distance of milk sample from the quartz window, volume of milk, and treatment time. The $\log_{10}$ reduction obtained varied from 0.16 to 8.55 $\log_{10}$ CFU/mL. Complete inactivation of *S. aureus* was obtained at two conditions (1 and 2 second-treatments of 12 and 24-mL samples, respectively) with corresponding reductions of 8.55 $\log_{10}$ CFU/mL. *B. subtilis* spores in water were treated with PUV light in an annular flow chamber, and flow rates up to 14 L/min resulted in complete inactivation of *B. subtilis* spores. Ha & Kang (2014) inoculated a cocktail of *C. sakazakii* strains into PIF, followed by NIR, UV, and combined NIR-UV treatments, resulting in greater reductions in cell numbers of *Cronobacter sakazakii* than did either treatment alone (7 minute-long treatment achieved a 2.76 $\log_{10}$ CFU reduction). Additionally, combined NIR-UV treatments did not change the colour or sensory attributes of PIF significantly.

To overcome the turbidity problem in milk, Reinemann et al. (2006) exposed milk to UV irradiation using the PureUV system for turbid liquids, designed to increase the penetration of UV throughout the liquid being treated and to ensure maximum exposure to individual bacterial cells, inoculating it with two strains of each bacteria types (e.g. *E. coli*, *Lactobacillus casei*, *S. aureus* and *Enterobacter sp.*). This treatment was shown to be capable of reducing plate count in raw milk.

The benefits of UV in comparison to other methods are that no chemicals are used; it is a non-heat-related process; there is no change in colour, flavour, odour or pH; no residuals are left in the fluid stream. Moreover, it has been recently reported that PUV decreased (but did not eliminate) the allergenic potential in cow's milk, peanuts, wheat and soybean extracts, since allergens (glycoproteins) suffered aggregation (Yang et al. 2010; Verhoeckx et al. 2015).

Technically speaking, short-wave, continuous UV lamps are mercury lamps covered with a glass, instead of quartz. Quartz gives high UV transmittance but it is expensive, thus cheaper glasses are produced to increases the transmittance of a regular glass. The UV light is generated by a lamp within the quartz/glass tube, radiation crosses the tube and reaches microorganisms in the surroundings (López-Malo & Palou 2005; Bintsis et al. 2000). Only medium-pressure UV lamps produce the necessary broad range of wavelengths. Low-pressure lamps emit a single wavelength peak which only affects DNA. Polychromatic medium-pressure UV light is so effective because of the lamps exceptionally high UV energy output at specific wavelengths across the UV spectrum (Kalisvaart 2004).

1.6.2.1. Pulsed UV Light

Pulsed light (PL) is a technique used to decontaminate surfaces using pulses of an intense broad spectrum, rich in UVC light (Gómez-López et al. 2007). It has been used in a variety of studies regarding *Listeria monocytogenes* contamination of meat and meat-slicing materials (Rajkovic et al. 2010), 13 food-related microorganisms, 8 of them pathogenic (*Listeria monocytogenes*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Escherichia coli*, *Providencia alcalifaciens*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Salmonella* Enteritidis, *Aeromonas hydrophila*, *Yersinia enterocolitica*, and *Salmonella* Typhimurium) in liquid environment (Ogihara et al. 2013), and *Escherichia coli* and *Salmonella* spp. (Luo et al. 2014).

UVC treatment to preserve food has been applied since the 1930s, even though the classical technique works in a continuous mode, as opposed to the pulsed light here described. However, pulsed UV light is considered to be four times more efficient than continuous UV-light due to the high peak power over a short period of time (Bialka 2006). Some of the food products subjected to pulsed UV light are bulk tank milk (Smith et al. 2002), corn meal (Jun et al. 2003) chicken (Keklik et al. 2010), iceberg lettuce and white cabbage (Gómez-López et al. 2005), alfalfa seeds (Sharma et al. 2002), grated carrots (Gomez-Lopez et al. 2005), raw salmon fillets (Ozer & Demirci 2006), honey (Hillegas & Demirci 2003), and black pepper and wheat flour (Fine & Gervais 2004).

Pulsed UV light works with xenon lamps, which are able to produce flashes several times per second (Gómez-López et al. 2007). According to Gómez-López et al. (2007), these following units are commonly used to characterize a PL treatment:

- Fluence rate: the energy received from the lamp by the sample per unit area per second. It is measured in Watt/meter^2 (W/m^2).
- Fluence: the energy received from the lamp by the sample per unit of area during the treatment. It is measured in Joule/meter^2 (J/m^2).
- Dose: sometimes it is a synonym of fluence.
- Exposure time: length in time (seconds) of the treatment.
- Pulse width: time interval during which energy is delivered.
- Pulse-repetition-rate (prr): number of pulses per second (Hertz [Hz] or commonly expressed as pps- pulses per second).
- Peak power: pulse energy divided by the pulse duration. It is measured in Watt (W).

1.6.2.1.1. PUV advantages

- Fast method, due to the short pulse width and high doses of the pulsed UV source (Wang et al. 2005).
- The lack of residual compounds, and the absence of are an advantage comparing to other methods. Xenon flash lamps are more environment friendly because they do not use mercury (Gómez-López et al. 2007).
- Medium cost process.
- No proven changes in processed food's colour, flavour, or odour (Särkka-Tirkkonen 2010).

1.6.2.1.2. PUV disadvantages

- Sample heating during treatment is perhaps the most important limiting factor. Heat can originate from the absorption of light by the food or by lamp heating (Jun et al. 2003).
- Another disadvantage of PL treatments is the possibility of shadowing occurring when microorganisms readily absorb the rays, and are present one upon another. This makes the organisms in the lower layers very hard to destroy in contrast to those in the upper layer (Hiramoto 1984; Särkka-Tirkkonen 2010; Cudemos et al. 2013), although the use of higher peak powers can overcome the shadowing effect. According to Gómez-López et al. (2007), "this restriction is different when flashing solid foods, and when flashing fluid foods or microorganism suspensions. Regarding fluid foods and microorganism suspensions, the liquid will absorb light depending on its absorption coefficient and depth."

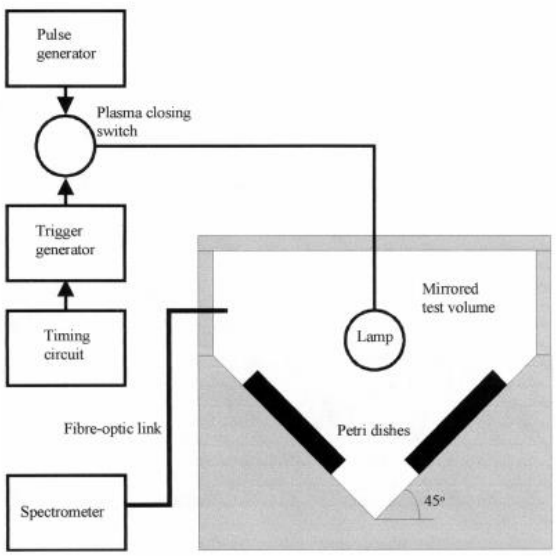


Figure 1.5. Schematic representation of a facility for microbial inactivation through pulsed light (Rowan et al. 1999).

1.6.3. Novel irradiation technologies: Electron Beam (e-beam) and Gamma Irradiation

Irradiation of food (also known as cold pasteurisation) is a process during which foods are subjected to controlled ionizing radiation to either bulk or packaged product for a pre-established time to preserve them by inhibiting enzymatic and microbial activities (Patterson & Loaharanu 2000; Hui et al. 2007). The process utilizes high energy electrons as its radiation source. The electrons, which are produced by normal electrical current, are accelerated to near the speed of light by means of a linear accelerator. The resulting energies, ranging normally from 3 to 10 million electron volts (MeV) and coupled with 1 to 50 kW of power, have sufficient energy to penetrate a range of materials.

1.6.3.1. E-beam

The electron beam process can achieve widely differing effects, such as sterilisation, polymer modification, crosslinking and chain scission, via its action as an ionizing radiation.

The bombardment of a material with high-energy electrons results in a cascade of these electrons moving through the target material. These electrons are free to interact with molecules within the material, ejecting electrons from their orbits and generating free radicals. It is primarily the reactions of these species that are responsible for the ability of the beam to modify polymers. The process takes place behind a thick concrete wall, which prevents radiation from leaving the cell.

The mechanism of microbial inactivation by ionizing radiation has been attributed to direct interaction of the radiation with cell components and food molecules, or indirect action from radiolytic products such as H^+ and OH^- (Wilkinson & Gould 1996; Morris et al. 2007). It targets chromosomal DNA and exerts a secondary effect on the cytoplasmic membrane. Low levels of gamma rays cause a stochastic health risk (meaning an unpredictable event because if its randomness), which for radiation dose assessment is defined as the probability of cancer induction and genetic damage. High doses produce deterministic effects, which is the severity of acute tissue damage that is certain to happen.

The technique is used in meat, seafood, fruits, and vegetables (especially cereal grains) to extend food shelf life (Hui et al. 2007). It does not involve heating, so the food retains most of its organoleptic features, except for high irradiation doses (Patterson & Loaharanu 2000).

The amount of irradiation needed to achieve complete and irreversible inactivation of enzymes may be too high and could provoke undesirable effects, e.g. nutritional loss; therefore has low appeal to consumers and it is not extensively used in food processing (Hui et al. 2007). Required doses for a specific goal depend on the rays (type, quantity, and radiation time) and the irradiated environment (absorption capacity, physical, chemical and biological modifications, and secondary reactions). Irradiation dose is measured in Grays (Gy), and defined as the absorption of 1 J of ionizing radiation by 1 kg of matter (1 Gy = 1 J/kg). High-radiation dose in the range of 10–74 kGy is usually applied for microbial sterilisation (Morris et al. 2007). Milder doses (less than 10 kGy) have been applied for pasteurisation applications (Patterson & Loaharanu 2000).

Table 1.5. Maximum absorbed radiation dose allowed for irradiation of foods (FDA 2005).

Maximum Absorbed Radiation Dose	Food Product
1 kGy	Control of growth and maturation in fresh products
3 kGy	Fresh or frozen, uncooked poultry products
4.5 kGy	Refrigerated, uncooked meat products
7 kGy	Frozen, uncooked meat products
> 30 kGy	Microbial disinfection of dry or dehydrated spices

The beam can be directed towards the food allowing a more controlled application, and treatment times are generally shorter than gamma irradiation. However, a disadvantage of e-beam is a limited penetration depth of 3 to 10 cm, and it has not been yet proven effective against foodborne viruses (Predmore et al. 2015).

According to the World Health Organization, food irradiation up to 10 kGy is considered safe for human consumption. Lee et al. (2006) described the inactivation of *E. sakazakii* in infant milk formula subjected to gamma radiation at 5 kGy. Tesfai et al. (2014) applied e-beam to dehydrated infant milk formula at 25 kGy. Fats, minerals and proteins were shown stable at low temperature and under anaerobic conditions, even though this intensity induced lipid oxidation. Most authors dedicate their research to study meat sterilisation through e-beam (Predmore et al. 2015; Kundu et al. 2014), but Velasco et al. (2015) applied it to eliminate *Listeria monocytogenes* from surface mould cheese.

1.6.3.1.1. Main benefits of electron-beam

E-beam irradiation can deliver the irradiating dose in just a few seconds, meaning that the entire process can take place in minutes. As a result of this short exposure period the following benefits have been found:

- Shorter processing times.
- Improved supply chain efficiencies.
- Polymers in the products are less likely to be subject to embrittlement.
- Considerably less oxidative damage to the product.
- Reduced colour change in any present polymers.
- No chemical residuals remain on processed products – clean, safe and environmentally friendly.

1.6.3.2. Gamma irradiation

According to STERIS AST, "Gamma irradiation is a safe and effective process that can be used for controlling microbial contamination of dry food ingredients. As irradiation is chemical and residue free, it has little or no effect on properties such as appearance, flavour, texture or aroma." A key characteristic of gamma irradiation is the high penetration capability. This enables moderately dense or sealed products to be processed with relative ease and facilitates treatment of palletized product (Steris AST 2016). The commercial use of gamma radiation to sterilise healthcare products began in the late 1950s.

A 1 kGy dose applied in e-beam sterilisation is equivalent to 1 kGy in gamma irradiation; however, there are some differences in recommended treatment doses. Those for gamma radiation are presented in **Table 1.6**.

Table 1.6. Typical dose range applied to different products, according to the International Atomic Energy Agency (2006).

Product	Intended Effect	Typical Dose Range (kGy)
Blood	Preventing TA-GVHD	0.020 – 0.040
Potatoes, onions, garlic	Inhibiting sprouting	0.05 – 0.15
Insects	Reproductive sterilisation for pest management	0.1 – 0.5
Strawberries and some other fruits	Extending shelf life by delaying mould growth and retarding decay	1 – 4
Meat, poultry, fish	Delaying spoilage, killing certain pathogenic bacteria e.g. *Salmonella*	1 – 7
Health care products	Sterilisation	15 – 30
Spices and other seasoning	Killing a variety of microorganisms and insects	1 – 30
Polymers	Crosslinking	1 – 250
	Grafting	0.2 – 30

Cobalt-60 and caesium-137 are the most suitable gamma radiation sources because of the relatively high energy of their gamma rays and fairly long half-life. However, the use of caesium-137 has been limited to small self-contained, dry-storage irradiators, used primarily for the irradiation of blood and for insect sterilisation. Currently, the primary industrial sources of gamma rays is cobalt-60, with a half-life of 5.27 years (International Atomic Energy Agency 2006; Martin 2012).

There are two broad types of irradiators (International Atomic Energy Agency 2006):

- **Self-contained irradiators**, specially designed for research and small-dose applications;
- **Panoramic irradiators**, for pilot-scale and full commercial-scale irradiations.

Within the broad spectrum of panoramic irradiators, different throughputs are required according to its specific application. Therefore, these also include:

- **Product overlap irradiators**, in which the user places the product to be irradiated in metal containers named totes. Totes are moved around and the total height of two totes is bigger than the height of the source rack.
- **Source overlap irradiators**, in which totes are moved in four or more rows but only at one level. In this case, the height of the container is no more than the height of the source rack. However, energy utilisation efficiency is lower than in product overlap irradiators.
- **Pallet irradiators**, designed to irradiate an entire pallet of product which is already in standardised containers. Pallet irradiators are similar to product overlap irradiators. Two

advantages are saving the extra effort of removing the product boxes from the pallet and arranging them in an irradiation container for irradiation and then, after the process, replacing them into the pallets for transportation out of the facility; and avoiding any damage to the product due to handling.

- **Batch irradiators** are simpler and suitable for small-scale irradiations. The sample is placed on a turntable that rotates during irradiation.

1.6.3.3. General overview on decontamination methods

Table 1.7. Advantages/disadvantages comparison between heating, UV light, e-beam and gamma irradiation.

Conventional Heating	Ultraviolet Light	E-Beam	Gamma Irradiation
- Applicable to all food products - Structural, taste and colour defects due to high heat load - Requires a precise control of time, temperature and pressure - More convenient to heat-stable, non-aqueous food (Särkka-Tirkkonen 2010)	- Applicable to fresh fruit and vegetables, and dry food - Not proven effective against spores (Särkka-Tirkkonen 2010) - No chemicals are used - Non-heat related process, but heat can be generated during treatment itself - No proven changes in colour, flavour, odour or pH - No residuals are left in the fluid stream (López-Malo & Palou 2005) - Medium cost process - Fast method - Only affects the surface of the food (Särkka-Tirkkonen 2010) - Possible shadowing effect	- Covers almost all food products groups - Pre-packed products can be processed - Not compatible with organic food - Flavour defects, especially in high fat food - Restricted use in EU (Särkka-Tirkkonen 2010) - E-beam can be directed towards the food allowing a more controlled application - Limited penetration depth (3-10 cm) - Not yet proven effective against foodborne viruses (Tesfai et al. 2014; Predmore et al. 2015; Kundu et al. 2014; Velasco et al. 2015)	- Applicable to all products - High penetration capability (better than e-beam) - No effect on food properties (appearance, flavour, aroma, texture) - No chemicals are used - Non-heat related process

1.6.4. Detection of physical changes due to sterilisation in vegetative cells and endospores

1.6.4.1. Scanning Electron Microscopy (SEM)

Endospores are dormant or metabolically inactive forms of a bacterium that allow it to survive the harsh environmental conditions. As previously reported, *Bacillus cereus* bacteria

produce highly heat, acid, UV, and desiccation-resistant endospores. These resistance properties make them of particular importance because they are not readily killed by many antimicrobial treatments, hence being the main cause of gastrointestinal disease in bottle-fed infants. A variety of different microorganisms form spores, but the endospores of low GC content Gram-positive bacteria are by far the most resistant.

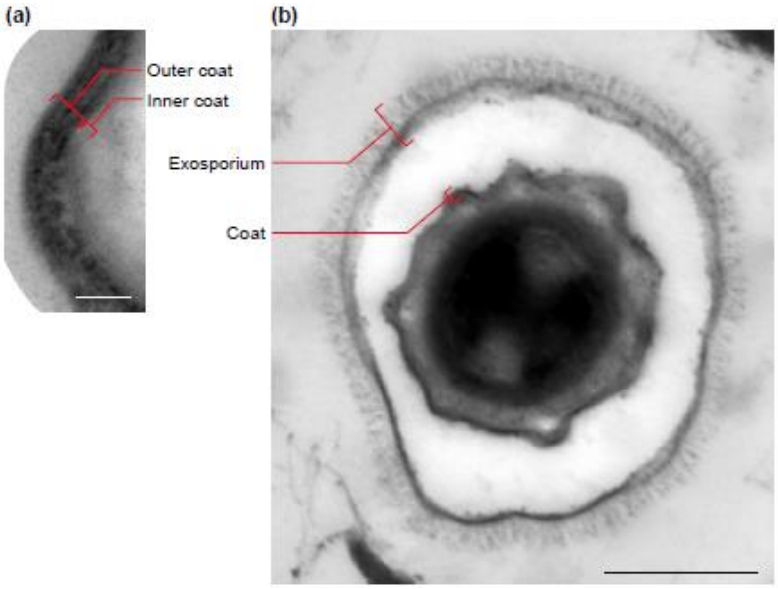


Figure 1.6. Electron micrographs of *Bacillus* spore coats. (a) Arc of *B. subtilis* spore coat (b) *B. anthracis* spore coat. Scale bars: 100 nm for *B. subtilis* and 300 nm for *B. anthracis* (Driks 2002).

The resilience of an endospore is explained by its cellular structure (**Figure 1.6**). The proteinaceous outer coat surrounding the spore provides the chemical and enzymatic resistance. Beneath the coat there is a very thick layer of specialised peptidoglycan- the cortex. Dehydration of the spore core is needed for proper cortex formation, which adds resistance to high temperature; and under the cortex there is a germ cell wall. This layer of peptidoglycan will become the cell wall of the bacterium after the endospore germinates. The inner membrane, under the germ cell wall, is a major permeability barrier against several potentially damaging chemicals. The core exists in a very dehydrated state and houses the cell's DNA, ribosomes and large amounts of dipicolinic acid, an endospore-specific chemical that plays a role in maintaining spore dormancy. Small acid-soluble proteins (SASPs) are also only found in endospores, and they tightly bind and condense the DNA, and are in part responsible for resistance to UV light and DNA-damaging chemicals. Other species-specific structures and chemicals associated with endospores include stalks, toxin crystals, or an additional outer glycoprotein layer called the exosporium (Driks 1999; Chada et al. 2003; Henriques & Moran 2007).

The scanning electron microscopy (SEM) produces images of a sample by scanning it with a focused beam of high-energy electrons. The electrons interact with atoms in the sample, producing various signals (which are secondary electrons that are emitted and collected by an Everhart-Thornley detector) that reveal information about the sample's surface morphology (texture), crystalline structure and chemical composition. SEM can achieve resolution better than 1 nm. Hence, it makes it possible to detect alterations in the endospores/cells topography when subject to heat/UV/e-beam treatment, in order to evaluate the effectiveness of said treatment on spore damage (Schlafer & Meyer 2016; Daemen et al. 2016).

1.6.4.2. Confocal microscopy

Confocal optical microscopy is a technique for increasing the contrast of microscope images. By restricting the observed volume, the technique keeps overlying or nearby scatterers from contributing to the detected signal, providing a three-dimensional imaging of biological cells and tissues as well as topography. It also allows real-time visualization of fully hydrated, living specimens, with a precision in the micrometre range.

Confocal microscopy is a very popular method due to its many advantages: a high variability in usage which results, among other reasons, from the variety of available objective lenses; thus the resolution, measurement range and duration can be adapted.

The main design is common to all confocal microscopes. A laser or halogen lamp is the light source, while the image generation is acquired through an adjustable mirror system. The detectors are photomultiplier tubes or CCD sensors. Most confocal microscopes contain an objective revolver to allow a quick change of the objective and thereby a fast change of measurement parameters. For axial scanning, the objective is mounted on an axial scanning system. However, the most significant for the functionality of a confocal microscope is the pinhole in front of the detector. This pinhole allows light to pass through to the detector, when the specimen is in the focal plane of the objective. When the specimen is not in the focal plane, the pinhole efficiently blocks light and no or very low light intensity is measured by the detector. During a measurement, the distance shift of the objective is carried out by the axial scanning system. The surface topography can be calculated by searching axial position of the objective which has the highest light intensity on the detector. Since the number of axial scanning steps is limited, interpolation techniques are frequently used to precisely determine the position with the highest light intensity (Braga et al. 2004; Mueller et al. 2016).

1.7. Chemical Reactions which alter Infant Formulae's Properties

A large number of products may form due to oxidation and glycation reactions that occur during sterilisation of powdered or liquid infant formulae (Birlouez-Aragon et al. 2005), as explicated in **Table 1.8**.

Table 1.8. Reactions that affect food quality (Boekel 2008).

Example	Type	Consequences
Nonenzymatic browning	Chemical reaction (Maillard reaction)	Colour, taste and aroma, nutritional value, formation of toxicologically suspect compounds.
Fat oxidation	Chemical reaction	Loss of essential fatty acids, rancid flavour, formation of toxicologically suspect compounds.
Fat oxidation	Biochemical reaction (lipoxygenase)	Off-flavours, mainly due to formation of aldehydes and ketones.
Hydrolysis	Chemical reaction	Changes in flavour, vitamin content.
Lipolysis	Biochemical reaction (lipase)	Formation of free fatty acids, rancid taste.
Proteolysis	Biochemical reaction (proteases)	Formation of amino acids and peptides, bitter taste, flavour compounds, changes in texture.
Enzymatic browning	Biochemical reaction of polyphenols	Browning.
Separation	Physical reaction	Sedimentation, creaming.
Gelation	Combination of chemical and physical reaction	Gel formation, texture changes.

1.7.1. Maillard Reaction

In **Table 1.9**, all molecule types and reactions they suffer are described, showing the consequences for food quality.

Table 1.9. Reactions occurring in food molecules/components (Boekel 2008).

Molecule	Reaction	Consequences
Proteins	Denaturation	Gelation, precipitation, solubility, inactivation of antinutritional factors.
	Hydrolysis	Formation of peptides and amino acids, texture changes.
	Deamidation	Loss of charge and change in reactivity.
	Maillard reaction	Crosslinking, browning, loss of nutritional value.
Lipids	Oxidation	Loss of essential fatty acids, rancidity.
	Fat hardening	Formation of trans fatty acids.
	Hydrolysis (usually enzymatically)	Formation of free fatty acids, leading to a soapy off-flavour.
Mono- and dissacharides	**Maillard reaction**	Nonenzymatic browning.
	Caramelisation	Taste and flavour changes.
	Hydrolysis	Sugar inversion.
Polyssacharides	Hydrolysis (enzymatically during ripening, chemically during cooking)	Softening of tissue, texture changes.
	Physical interaction with other components	Gelation, phase separation.
	Gelatinisation and retrogradation of starch	Staling of bread.
Polyphenols	Enzymatic polymerisation	Browning.
	Interaction with proteins	Crosslinking, gelation.
Vitamins	Oxidation	Loss of nutritional value.

Proteins available in IF are prone to suffer the Maillard reaction (MR), known as the non-enzymatic browning, which react with carbonylated substrates derived from vitamin C degradation, lactose or oxidised polyunsaturated fatty acids (Birlouez-Aragon et al. 2005). In fact, it is a cascade of condensation, rearrangement, fragmentation and oxidation reactions (Booth et al. 1997).

The Maillard reaction occurs in lysine's free ε-amino groups (Rabasseda et al. 1988). The first steps lead to the synthesis of Schiff bases, which are rearranged into ketoamines (Amadori compounds) and the following steps arise unsaturated molecules which undergo polymerisation resulting in brown pigments (melanoidins) (Simões & Oliveira 2001; Ferreira et al. 2003). This reaction results in undesirable consequences, like a change in flavour and colour and in a decrease of nutritional quality, especially loss of the amino acid lysine. Other consequence is the formation of fluorescent compounds, which is due to casein and happens at the same time as the browning reaction (Angel Rufián-Henares et al. 2006). However, milk-based IFs are more susceptible to heat damage than soy-based or hydrolysate formulae because whey proteins glycate faster than casein (Cattaneo et al. 2009).

The first consequence of the Maillard reaction is the formation of lactulosyllysine (LL), which means a decrease in lysine bioavailability (Birlouez-Aragon 2004), and lactulose (LCT), derived from lactose isomerisation (Cattaneo et al. 2009). By analysing furosine (FUR), which

concentration depends on the reducing sugar and storage conditions and derives from LL, it is possible to detect LL in an early reaction stage (Damjanovic Desic & Birlouez-Aragon 2011). Further degradation origins N^{ε}-(carboxymethyl)lysine (CML) and erythronic acid through oxidative degradation and hydroxymethylfurfural (HMF) through 1,2-enolisation followed by dehydration. HMF is a genotoxic and mutagenic agent, and CML induces oxidative stress (Damjanovic Desic & Birlouez-Aragon 2011; Xu et al. 2013), as well as immunogenic agents capable of triggering cellular injury responses (Booth et al. 1997). Both CML and HMF are known as advanced glycation end products – AGE- and reactive precursors that are implicated in diabetes, Alzheimer's disease and aging, among others (Booth et al. 1997). AGEs are no more than post-translational protein modifications that result from aforementioned reactions between amino acid residues and carbonyls/carbohydrates (Ferreira et al. 2003).

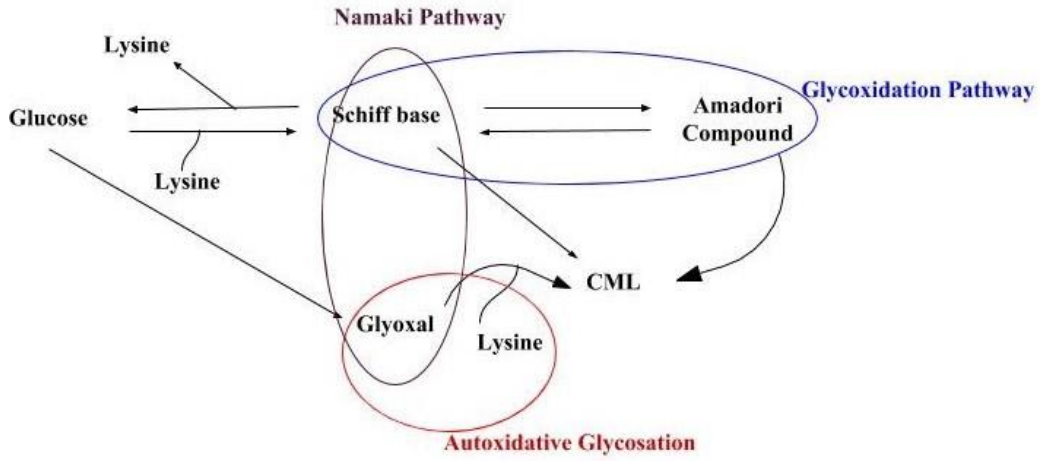


Figure 1.7. Simplified scheme of three possible CML synthesis pathways: Namaki; Glycoxidation and Autoxidative pathways (adapted from Ahmed et al. 1986). Glycoxidation is the most important pathway that leads to the formation of CML. When accumulation of glucose occurs, glycation or non-enzymatic glycosylation is increased proceeding through the formation of a Schiff base between glucose and lysine residues followed by an Amadori rearrangement that origins a stable ketoamine, an Amadori compound. Glycated proteins undergo a Maillard reaction yielding CML. In the autoxidative glycosation, glyoxal, a dialdehyde involved in oxalate synthesis, reacts with lysine residues located in proteins, forming CML. In the Namaki pathway, glyoxal and a Schiff base derived from glycation combine to originate CML.

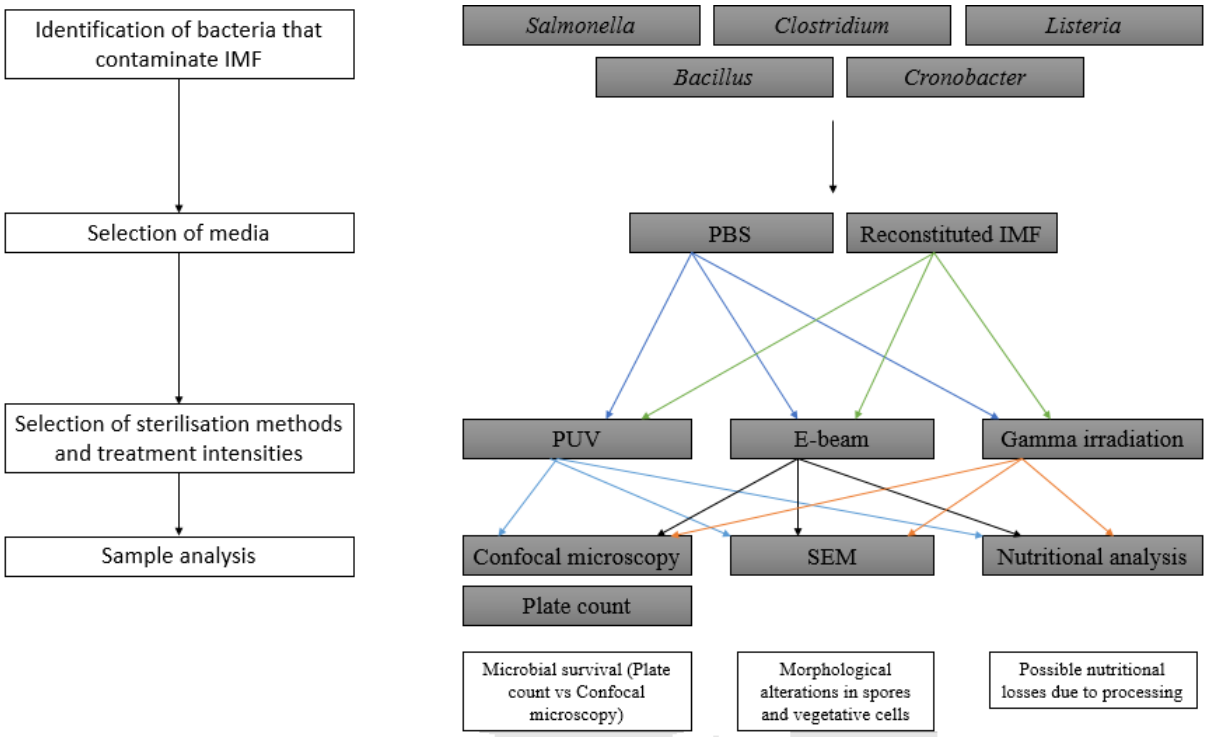
Oxidative conditions, such as the presence of iron or vitamin C, can promote AGE formation and tryptophan degradation, so the loss of tryptophan should also be looked for during the IF analysis (Birlouez-Aragon 2004). Some MRP induce lipid peroxidation *in vivo*, affecting erythrocytes' membrane and altering its functioning (Sarriá & Vaquero 2006).

2. Aims of the study

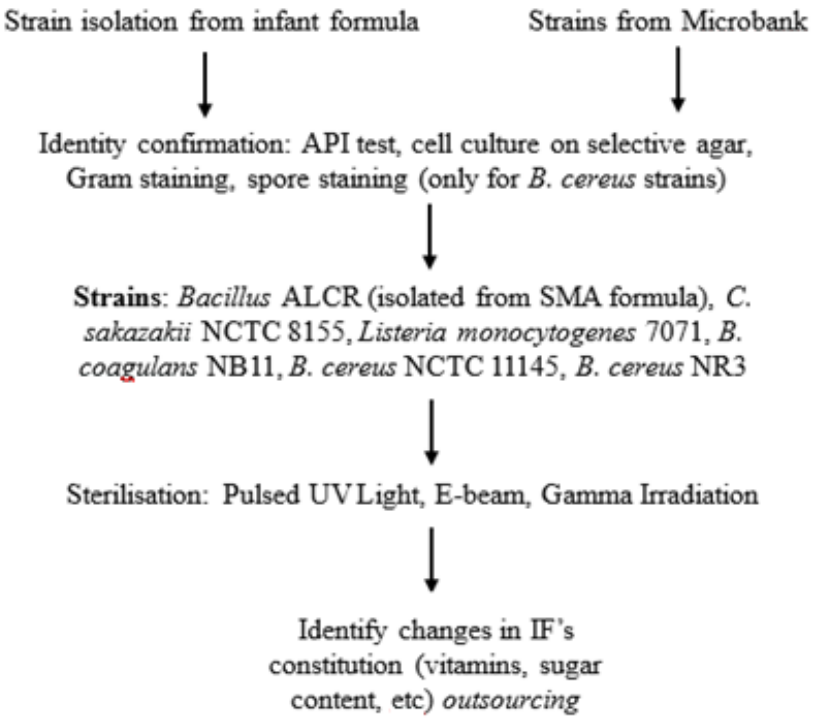
This study primarily aims to investigate inter-related engineering, biochemical and microbiological factors that govern the effectiveness and efficiency of pulsed UV, e-beam and gamma irradiation for decontaminating a limited number of targeted food products (infant formulae in first instance) and water. More specifically:

- Compare the performance of pulsed UV light, e-beam and gamma irradiation regarding microbial death, to determine which of the techniques presents better results;
- Establish a relation between the aforementioned techniques and nutritional losses in infant formulae;
- Optimise sterilisation conditions in order to achieve high microbial death without compromising nutritional requirements, and respecting European Commission's Directives regarding food quality standards.

3. Materials and Methods



3.1. Methodology



3.2. Microorganisms

3.2.1. Bacteria

In this study, single strains of *Cronobacter sakazakii* NCTC 8155, *Bacillus cereus* NCTC 11145, *Listeria monocytogenes* 7071, *Bacillus coagulans* NB11, *Bacillus cereus* NR3, *Bacillus cereus* ATCC 11778 and a *Bacillus* strain isolated from SMA infant formula (named *Bacillus cereus* ALCR) were used. Strains were maintained in Microbank storage vials at -80ºC. Fresh cultures were grown every week.

Strain *Bacillus cereus* ATCC 11778 retrieved from the Microbank was not submitted to further studies since it was confirmed it was contaminated during identification procedures.

3.2.1.1. Bacterial identification

In order to confirm the identity of bacteria retrieved from the Microbank, identification techniques adapted to each species were applied.

All *Bacillus cereus* strains suffered Gram staining, endospore staining with Malachite Green Solution, were tested on an API® 50CHB kit and were grown on Nutrient agar or Plate Count agar (LabM), attaining an identity confirmation in all cases.

Gram staining was performed to identify *Listeria monocytogenes*, resulting in a positive identification.

For *Cronobacter sakazakii*, Gram staining, API® 20E and culture on Violet Red Bile Glucose Agar (VRBG agar) were performed, also reaching a positive identification.

3.2.2. Reconstitution and bacteriological analysis of infant milk formula

The powdered infant milk formulae utilised were SMA (SMA Nutrition, Ireland) and Cow&Gate (Cow&Gate Ireland), purchased from Dunnes. 10 g of powdered IMF were reconstituted in 100 mL of sterile distilled water. The bottles were inverted until a homogenous distribution of the powdered IMF was obtained.

(A)

Nutrition Information Typical values per 100 ml prepared feed		
Energy	280	kJ
	67	kcal
Fat	3.6	g
of which, saturates	1.5	g
of which, unsaturates	2.1	g
Carbohydrate	7.1	g
of which, sugars	7.1	g
Fibre	0.4	g
Protein	1.25	g
Salt* (=Sodium x 2.5)	0.06	g

Vitamins		
Vitamin A	75	µg
Vitamin D	0.9	µg
Vitamin E	1.1	mg
Vitamin K	5.5	µg
Vitamin C	13	mg
Thiamin	0.1	mg
Riboflavin	0.11	mg
Niacin	0.5	mg
Vitamin B_6	0.04	mg
Folic Acid	9.2	µg
Vitamin B_{12}	0.13	µg
Biotin	1.7	µg
Panothenic Acid	0.43	mg

Minerals		
Sodium	24	mg
Potassium	62	mg
Chloride	39	mg
Calcium	43	mg
Phosphorus	24	mg
Magnesium	6.6	mg
Iron	0.7	mg
Zinc	0.7	mg
Copper	0.05	mg
Manganese	0.01	mg
Flouride	<0.01	mg
Selenium	2.2	µg
Iodine	14	µg

Others		
Taurine	4.6	mg
Choline	8.5	mg
Inositol	6.6	mg
L-Carnitine	0.8	mg
Oligosaccharides (GOS/FOS)♦	0.4	g
Omega 3:		
α-linolenic acid (ALA)	67	mg
Docosahexaenoic acid (DHA)†	8.4	mg
Omega 6:		
linoleic acid (ALA)	550	mg
Arachidonic acid (AA)†	8.4	mg

(B)

Nutrition information

Typical values per 100ml

Energy	275kJ 66kcal
Fat	3.4g
of which	
- Saturates	1.5g
- Unsaturates	1.9g
of which LCPs†	0.015g
- Arachidonic acid (AA)	0.006g
- Docosahexaenoic acid (DHA)	0.006g
Carbohydrate	7.3g
of which sugars	7.2g
of which lactose	7.0g
Fibre	0.6g
Protein	1.3g
Whey	0.8g
Casein	0.5g
Salt	0.04g

Vitamins	
Vitamin A	54µg-RE
Vitamin D_3	1.2µg
Vitamin E	1.1mg α-TE
Vitamin K_1	4.4µg
Vitamin C	9.2mg
Thiamin (B_1)	0.05mg
Riboflavin (B_2)	0.12mg
Niacin (B_3)	0.43mg
Pantothenic Acid	0.34mg
Vitamin B_6	0.037mg
Folic Acid	13µg
Vitamin B_{12}	0.19µg
Biotin	1.4µg

Minerals	
Sodium	17mg
Potassium	72mg
Chloride	46mg
Calcium	55mg
Phosphorus	31mg
Magnesium	5.1mg
Iron	0.53mg
Zinc	0.51mg
Copper	0.04mg
Manganese	0.008mg
Fluoride	≤0.003mg
Selenium	1.7µg
Iodine	12µg

Others	
Choline	12mg
Taurine	5.3mg
Inositol	3.8mg
L-carnitine	1.6mg
Nucleotides	3.2mg
GOS/FOS*	0.8g

†Long Chain Polyunsaturated fatty acids
*Galacto-Oligosaccharides & Fructo-Oligosaccharides

Figure 3.1. IMF's nutritional information, as detailed by the manufacturer in the package's label. (A) SMA (B) Cow&Gate.

3.2.3. Bacteria isolation from infant formula

Infant milk formulae's samples were left on the bench to cool down to room temperature (25ºC). A 10 µl sample was plated on Nutrient agar (LabM), followed by incubation overnight at 37ºC. Presumed *Bacillus* colonies were isolated and subject to Gram staining, endospore staining with Malachite Green Solution (BioChemika, Sigma-Aldrich), and API® 50 CHB test (BioMerieux, Inc., France) for identification purposes. All identification methods confirm the SMA isolate colony is a *B. cereus* strain, while the Cow & Gate isolate is a *B. megaterium* strain. After identification, the isolated *B. cereus* strain was stored in agar slopes at 4ºC.

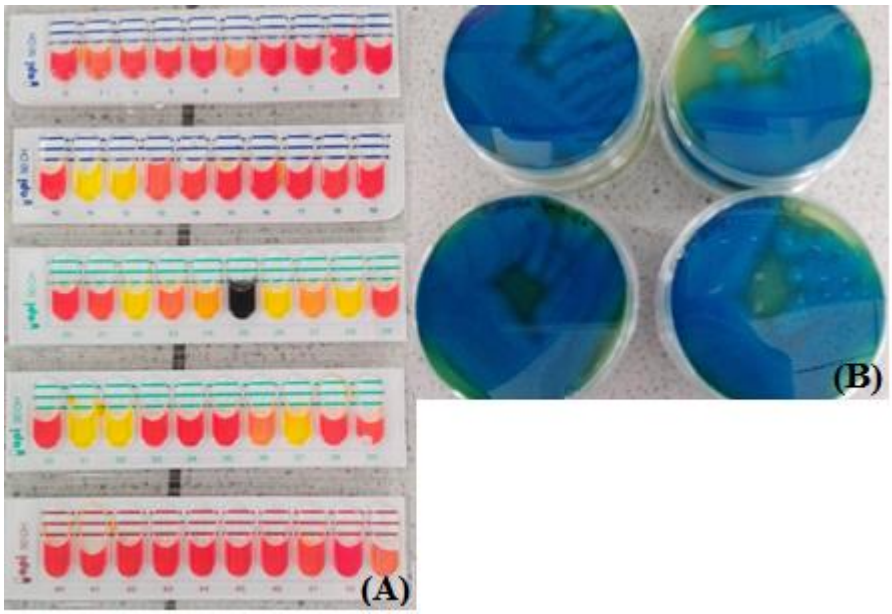


Figure 3.2. (A) API® 50 CHB test kit, confirming SMA's *B. cereus* identity. A detailed description of tests available in each well can be found in Annex VI; **(B)** Growth of *B. cereus* strain isolated from SMA IMF on Nutrient agar supplemented with Malachite Green Solution, showing a positive result (in blue) for endospores.

A 1 mL sample of both IMF's was also plated in two VRBG agar (LabM) plates, a selective agar for *Enterobacteriaceae* bacteria. As observed in **Figure 3.3**, there was no observable growth.

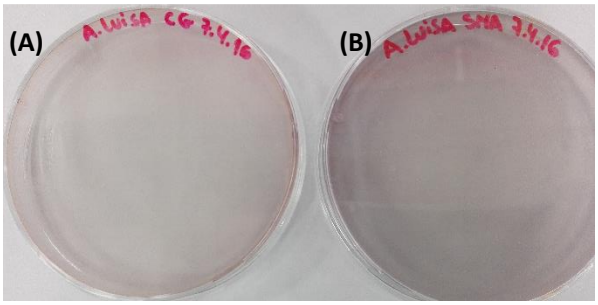


Figure 3.3. VRBG agar plates. 1mL of reconstituted Cow&Gate (A) and SMA (B) IMF were plated on Petri dishes to determine the presence of *Cronobacter* spp.. No visible growth was observed after 24 hours at 37°C.

3.2.4. Bacterial samples preparation

Bacterial cells from the Microbank were diluted in PBS buffer so that a concentrated suspension with an OD of approximately 2.0 (c. 10^9 CFU/mL) at 560 nm was reached. In order to selectively obtain the endospores necessary for the study, *Bacillus cereus* strains were kept in a hot bath at 63ºC for 30 minutes to eliminate vegetative cells. Then, 1 mL was then retrieved from the broth and diluted in 9 mL of PBS buffer.

Regarding infant formula preparations, 9 mL of reconstituted PIF were supplemented with 1 mL of a 10% bacterial suspension in PBS buffer.

3.3. Thermal inactivation

The thermal inactivation studies were not conducted since it was proven, in previous works, that it is capable of killing bacteria and decrease the spore count in peptone water (Haughton 2008).

3.4. PUV inactivation

Bacterial suspensions were prepared as described in **Section 3.2.4**. 10 mL of each sample were transferred into a sterile Petri dish and treated with the desired parameters. Following treatment, serial dilutions were prepared, up to a 10^{-5} concentration, to attain a countable cell culture. 0.1 mL of control and treated samples were plated on Nutrient agar or VRBG agar and incubated for 24 hours at 37ºC. PUV treatment was carried out in triplicate using the same culture to avoid sample variability on a benchtop pulsed power source (Samtech Ltd, Glasgow). Surviving cells were counted and results expressed as $\log_{10}$ CFU/mL versus pulses per second (pps).

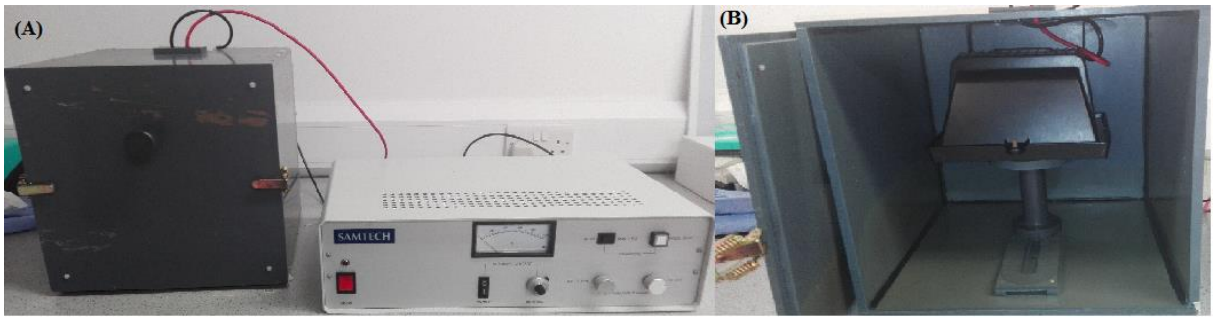


Figure 3.4. Pulsed UV equipment (Samtech Ltd, Glasgow). Outside (A) and pulsing chamber (B) views.

3.5. E-beam inactivation and gamma irradiation

Bacterial suspensions were prepared as described in **Section 3.2.4**. 10 mL of each sample were transferred into a Falcon tube, and couriered to STERIS AST Ireland (Tullamore, Ireland). *Cronobacter sakazakii* and *Listeria monocytogenes* samples were irradiated with an intensity of 0, 1.5 and 2 kGy and *Bacillus* strains with 0, 2, 5 and 10 kGy. Control and treated *Cronobacter* samples were plated in VRBG agar overnight at 37ºC, while *B. cereus* and *L. monocytogenes* samples were plated in Nutrient agar. E-beam inactivation was carried out in a horizontal beam with an operational energy level of 10 MeV, in triplicate using the same culture to avoid sample variability, and surviving cells were counted. Gamma irradiation was performed in triplicate using the same culture to avoid sample variability by STERIS AST Ireland (Westport, Ireland) using a pallet irradiator, and surviving colonies were counted. Results are expressed as $\log_{10}$ CFU/mL versus irradiation intensity in kGy.

4. Results

4.1. Pulsed UV Light

4.1.1. Plate count results

Pulsed UV results were expressed as $\log_{10}$ CFU/mL against pulses per second (pps) and the number of pulses taken to reduce the population of microorganisms by a single $\log_{10}$ was reported as the D-value for that particular microorganism and medium. The D-values for microorganisms was read directly from the plots and are presented in **Table 4.1**.

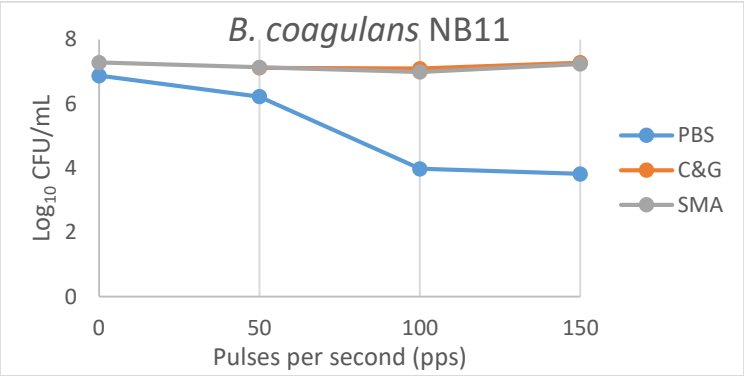


Figure 4.1. Log_{10} reduction of *Bacillus coagulans* NB11 in PBS buffer following treatment with pulsed UV light. Parameters: pulse frequency 1Hz; total pulse number 0, 50, 100 and 150 pulses; 600V. Following PUV treatment, samples were diluted in 10% PBS buffer and plated on Plate Count agar and grown overnight, at 37°C.

Studies involving rehydrated IMF were also conducted in all the other strains (data not shown) to assess the effect of pulsed UV light. However, it was demonstrated that this method causes no alteration in plate count numbers, not being effective for decontamination (see **Section 4.1.2.**).

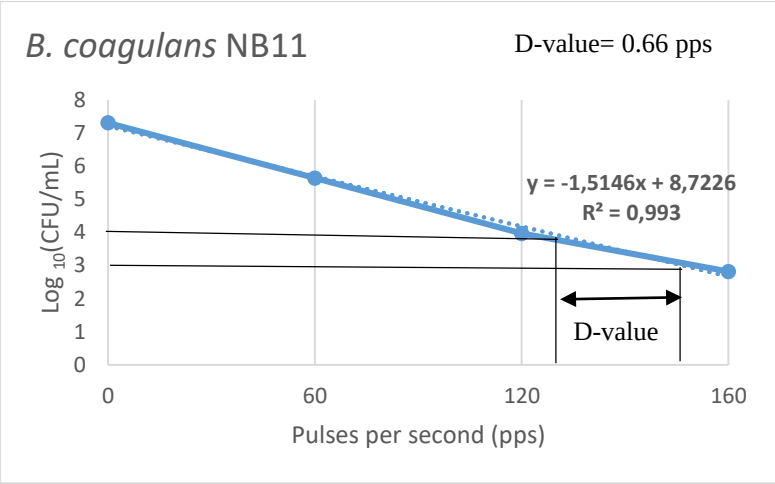


Figure 4.2. Log$_{10}$ reduction of *Bacillus coagulans* NB11 in PBS buffer following treatment with pulsed UV light. Parameters: pulse frequency 1Hz; total pulse number 0, 60, 120 and 160 pulses; 600V. Following PUV treatment, samples were diluted in 10% PBS buffer and plated on Plate Count agar and grown overnight, at 37°C.

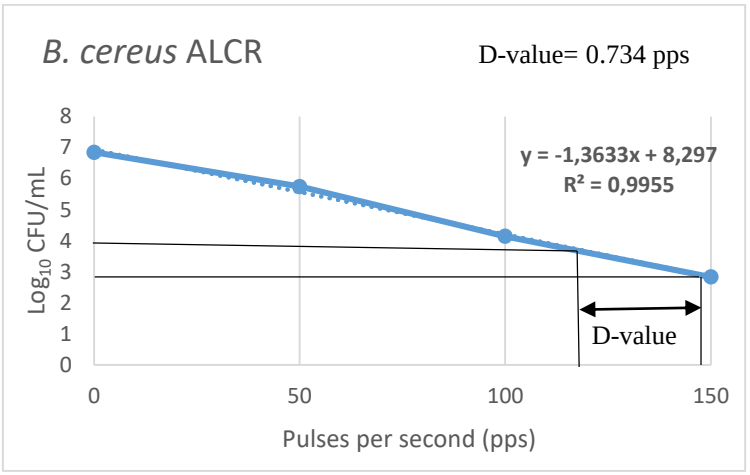


Figure 4.3. Log$_{10}$ reduction of *Bacillus cereus* strain isolated from reconstituted SMA IMF (named *Bacillus cereus* ALCR) in PBS buffer following treatment with pulsed UV light. Parameters: pulse frequency 1Hz; total pulse number 0, 50, 100 and 150 pulses; 600V. Following PUV treatment, samples were diluted in 10% PBS buffer and plated on Plate Count agar and grown overnight, at 37°C.

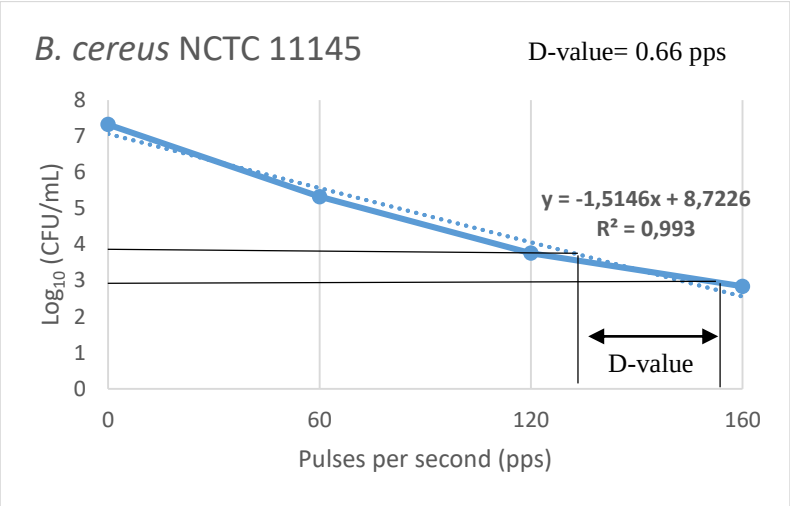


Figure 4.4. Log_{10} reduction of B*acillus cereus* NCTC 11145 in PBS buffer following treatment with pulsed UV light. Parameters: pulse frequency 1Hz; total pulse number 0, 60, 120 and 160 pulses; 600V. Following PUV treatment, samples were diluted in 10% PBS buffer and plated on Plate Count agar and grown overnight, at 37°C.

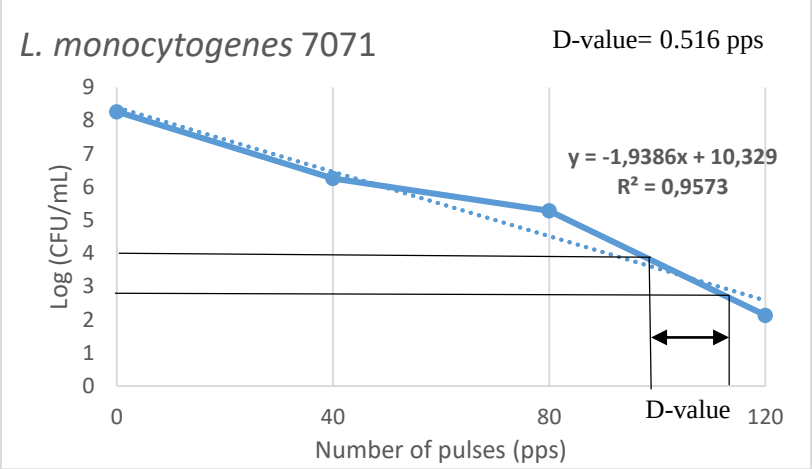


Figure 4.5. Log_{10} reduction of *Listeria monocytogenes* 7071 in PBS buffer following treatment with pulsed UV light. Parameters: pulse frequency 1Hz; total pulse number 0, 40, 80 and 120 pulses; 400V. Following PUV treatment, samples were diluted in 10% PBS buffer and plated on Plate Count agar and grown overnight, at 37°C.

As previously mentioned, *Listeria monocytogenes* is not commonly found in reconstituted IMF; however it might be present in the water used to reconstitute aforementioned IMF, therefore the study of its death curve is of critical importance.

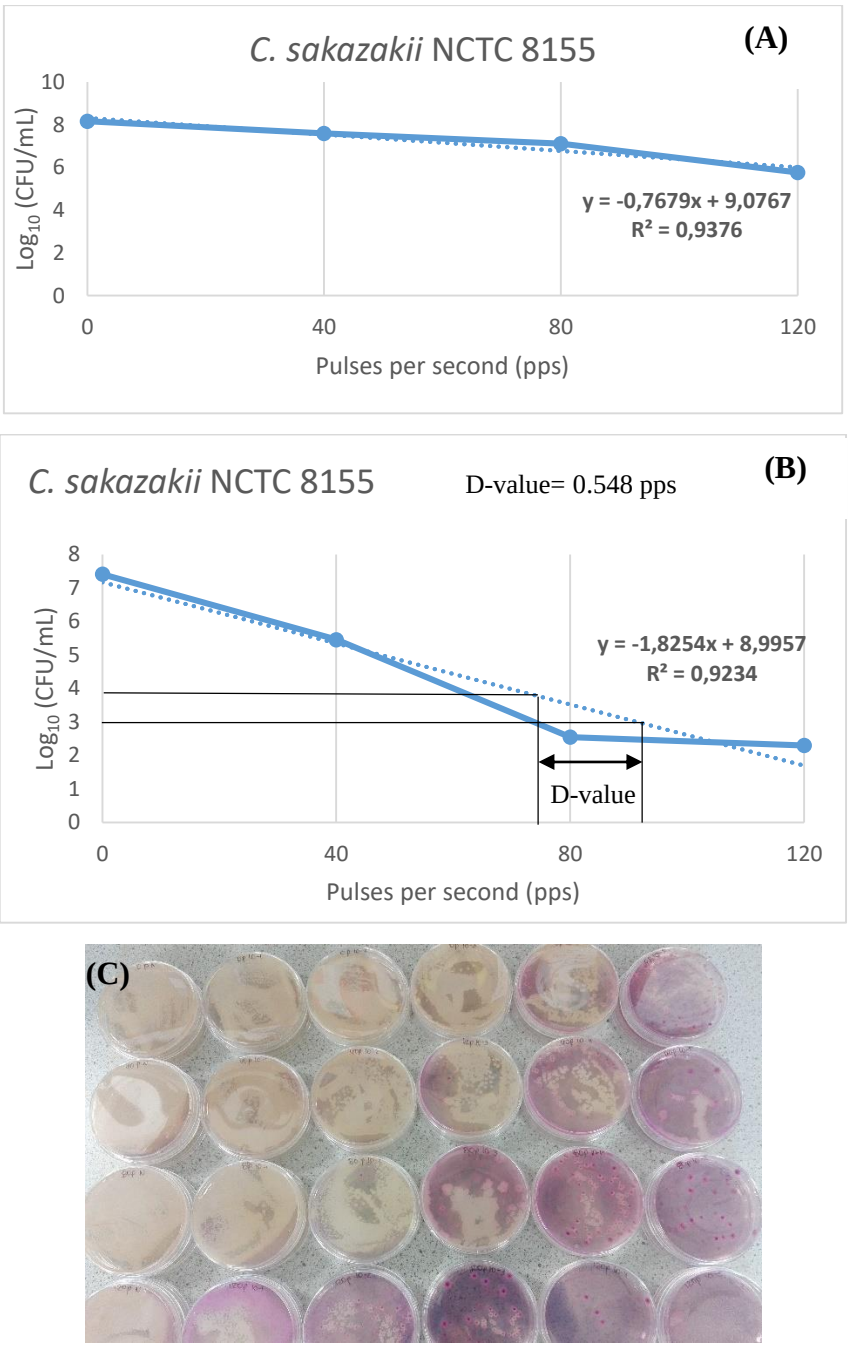


Figure 4.6. Log_{10} reduction of *Cronobacter sakazakii* NCTC 8155 in PBS buffer following treatment with pulsed UV light. Parameters: pulse frequency 1Hz; total pulse number 0, 40, 80 and 120 pulses; **(A)** 400V and **(B)** 600V. Following PUV treatment, samples were diluted in 10% PBS buffer and plated on VRBG agar and grown overnight, at 37°C. **(C)** *Cronobacter sakazakii* NCTC 8155 treated in PBS buffer with pulsed UV light at 400V, plated on VRBG agar. From top to bottom: 0, 40, 80 and 120 pulses; Left to right: serial dilutions (10^0 to 10^{-5} concentrations). Petri dishes located in the bottom row and farther right positions present lower colony counts.

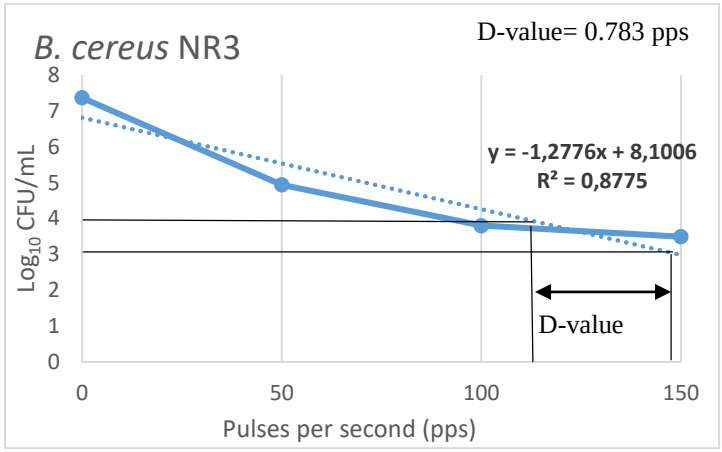


Figure 4.7. Log_{10} reduction of *Bacillus cereus* NR3 in PBS buffer following treatment with pulsed UV light. Parameters: pulse frequency 1Hz; total pulse number 0, 60, 120 and 160 pulses; 600V. Following PUV treatment, samples were diluted in 10% PBS buffer and plated on Plate Count Agar and grown overnight, at 37°C.

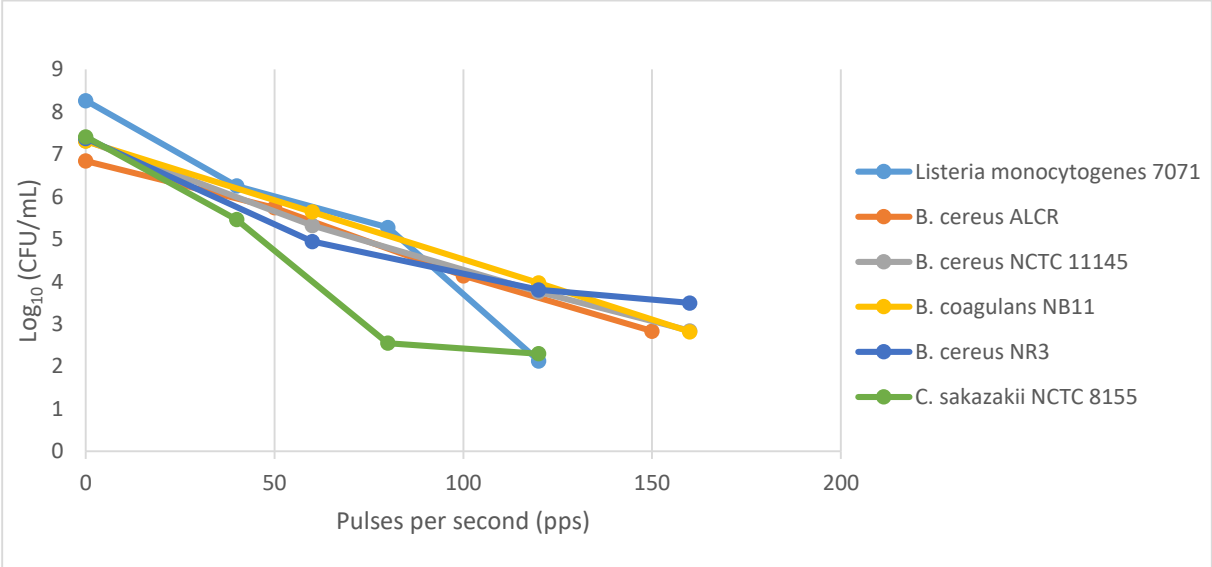


Figure 4.8. Comparison between log_{10} reduction of *B. cereus* ALCR, *B. coagulans* NB11, *B. cereus* NR3, *L. monocytogenes* 7071, *B. cereus* NCTC 11145, and *C. sakazakii* NCTC 8155 in PBS buffer, following treatment with PUV. *C. sakazakii* NCTC 8155 and *L. monocytogenes* 7071 were treated with 0, 40, 80 and 120 pulses, while *B. cereus* NR3, *B. coagulans* NB11 and *B. cereus* NCTC 11145 were treated with 0, 60, 120 and 160 pulses. *B. cereus* ALCR differed from all the other strains since t was treated with 0, 50, 100 and 150 pulses. Irradiated samples were diluted in 10% PBS buffer according to the serial dilution method and plated on VRGB or Plate Count agar, grown overnight at 37ºC.

It then became possible to calculate the D-values for each strain through the trend line's equation. Each D-value represents the intensity that kills one $\log_{10}$ of bacteria (or 90% of a bacterial population) in said media.

Table 4.1. D-values for all six studied strains of bacteria, for PUV treatment.

Strain	D-value (pps) in PBS buffer	R^2
L. monocytogenes 7071	0.516	0.9573
C. sakazakii NCTC 8155	0.548	0.9234
B. cereus NR3	0.783	0.8775
B. coagulans NB11	0.66	0.993
B. cereus NCTC 11145	0.66	0.993
B. cereus ALCR	0.734	0.9955

4.1.2. Turbidity Test

Reconstituted Cow&Gate IMF was pulsed for 100 pulses (1 pps; 600 V), followed by spectrophotometric measurements to evaluate IMF's turbidity.

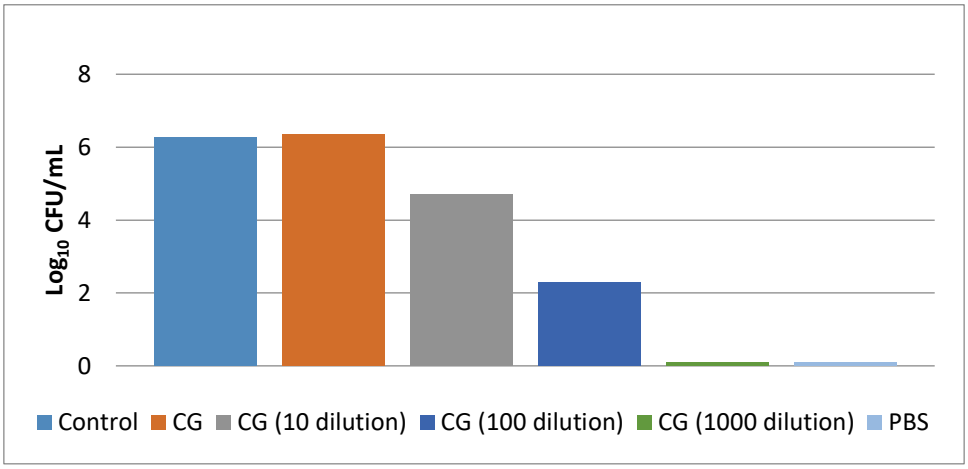


Figure 4.9. Reconstituted Cow&Gate IMF treated with PUV light. Total number of pulses: 100 pulses, Frequency 1 pps, 600V. Control: Non-pulsed CG sample; CG: non-diluted CG sample; CG (10 dilution): CG at 10^{-1} concentration; CG (100 dilution): CG at 10^{-2} concentration; CG (1000 dilution): CG at 10^{-3} concentration; PBS: pulsed PBS sample.

According to **Figure 4.9**, and supporting aforementioned Petri results, non-supplemented Cow&Gate IMF is contaminated with an intrinsic bacterial strain that increases plate count and causes major turbidity, preventing PUV from being effective in decontaminating IMF.

4.2. E-beam

4.2.1. Background microbiological studies and the effect of time on reconstituted IMF

In order to assess the microbiological content of IMF without its supplementation, *Cow&Gate* and *SMA* powdered IMF was treated with e-beam at 2 and 10 kGy, still in its powder form.

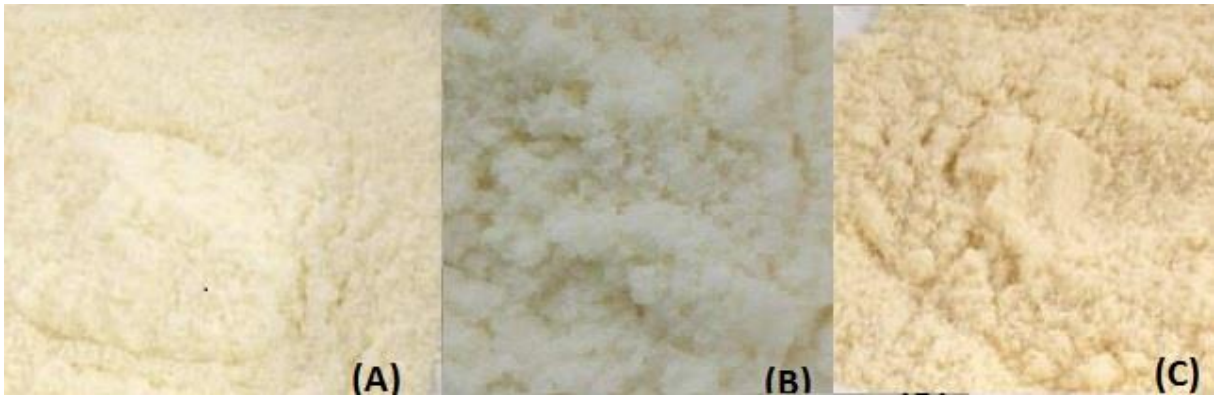


Figure 4.10. SMA IMF's powder (A) untreated sample; and after e-beam treatment at **(B)** 2 kGy and **(C)** 10 kGy. Powders were plated on Plate Count agar to assess their microbiological content, and results are shown in Figure 4.11.

As observed in **Figure 4.10**, there was a slight difference in the IMF colouring and texture between the untreated sample (A) and the sample which was subject to 10 kGy treatment (C); however, such a difference is not noticeable between samples (A) and (B) or (B) and (C). Figure 4.10A presented a finer texture and brighter colour comparing to Figure 4.10C.

10 g of these powders were reconstituted in 100 mL of sterile deionised water, plated and kept at 37°C overnight. The reconstituted IMF was left on the bench, and again plated after 24h and 48h.

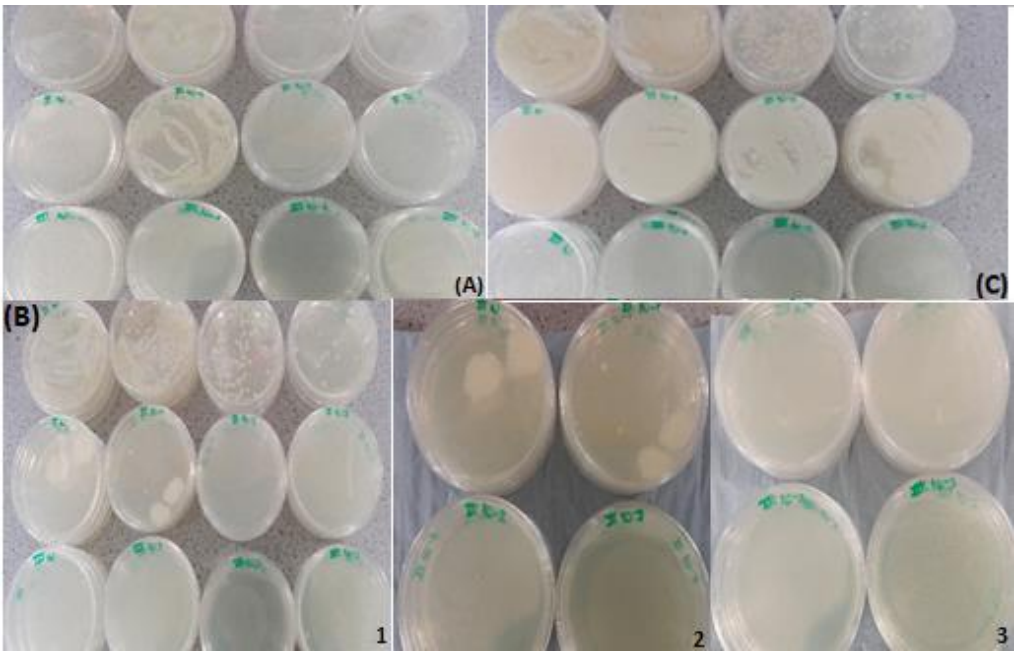


Figure 4.11. SMA's powder treated with e-beam at 0, 2 and 10 kGy. Samples were plated on Plate Count agar, after (A) 0h (B1) 24h and (C) 48h on benchtop. (B2) refers to samples treated at a 2 kGy intensity, while (B3) represents samples treated at 10 kGy, both after 48h on the benchtop at room temperature.

As seen in **Figure 4.11**, all samples plated immediately after reconstitution presented observable growth of mixed colonies, meaning possible contamination of media (Figure 4.11A). Samples plated after 24h on the benchtop showed individual colonies observable at 0 kGy and 2 kGy, and no observable growth at 10 kGy (Figure 4.11B). After 48h on the benchtop, plated samples present numerous colonies at 0 kGy and 2 kGy; however there is little growth at 10 kGy (Figure 4.11C).

4.2.2. Plate count results

E-beam results were expressed as $\log_{10}$ CFU/mL against e-beam intensity (in kGy) and the intensity taken to reduce the population of microorganisms by a single $\log_{10}$ was reported as the D-value for that particular microorganism and medium. D-values for microorganisms were read directly from the plots and are presented in **Table 4.2**.

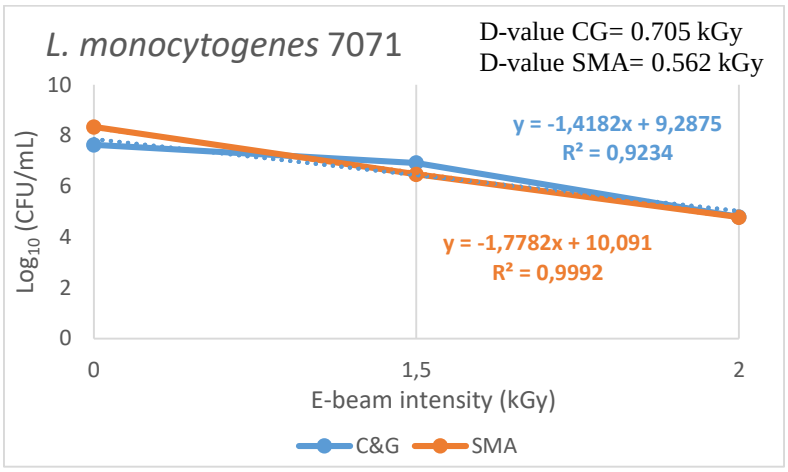


Figure 4.12. Log_{10} reduction of *L. monocytogenes* 7071 in Cow&Gate and SMA IMFs following treatment with e-beam intensities of 1.5 and 2 kGy. Following treatment, samples were diluted in 10% PBS buffer and plated on Plate Count Agar overnight, at 37°C. Results for PBS are not presented due to non-detectable levels in plate count.

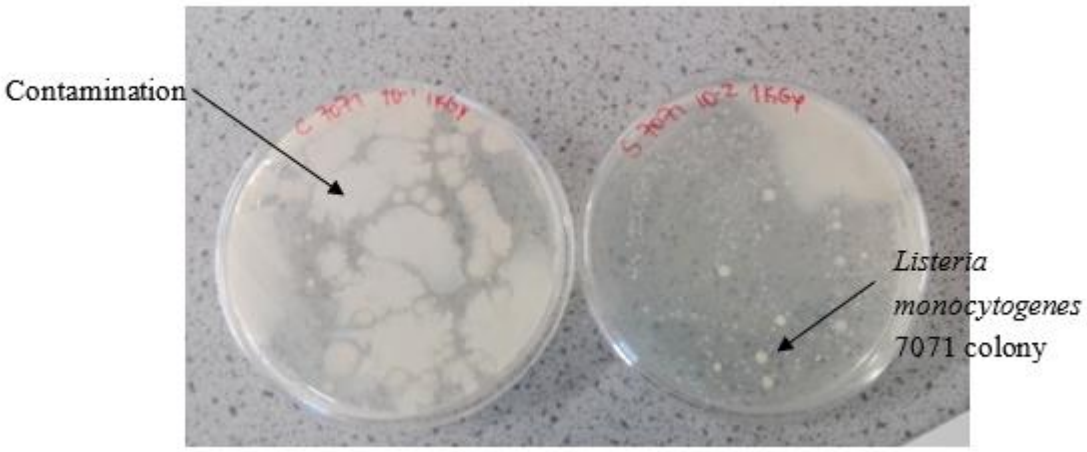


Figure 4.13. E-beam-processed *L. monocytogenes* 7071 plated on Plate Count agar showing contamination with intrinsic *Bacillus* strain (bigger colony), contrasting with *L. monocytogenes* colony (smaller in size). Since *L. monocytogenes*-enriched samples were only subjected to a maximum intensity of 2 kGy, *Bacillus* spores were not killed since this dose does not decontaminate samples.

Listeria monocytogenes 7071 was also diluted in PBS buffer, however, at 1.5 kGy there were no detectable levels of such colonies after 24h of incubation. Due to its limit of functionality, it is not possible to operate the e-beam device under 1.5 kGy.

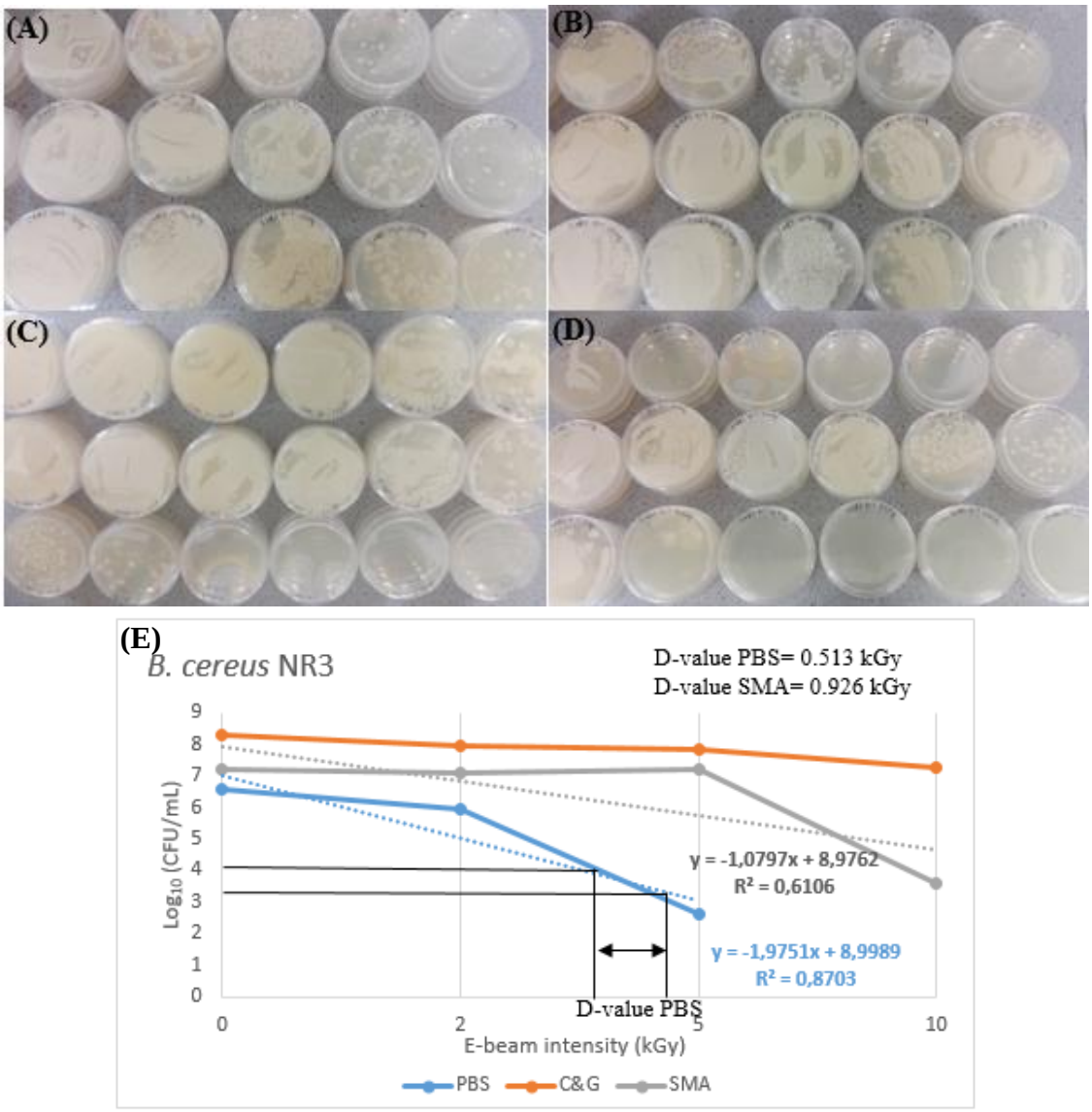


Figure 4.14. ***Bacillus cereus*** **NR3** control **(A)** and treated with e-beam irradiation at **(B)** 2 kGy, **(C)** 5 kGy and **(D)** 10 kGy, in PBS, Cow&Gate and SMA IMF, plated in 1 to 10^{-5} dilutions (triplicates). Petri dishes containing Plate Count agar were inoculated with a solution of either PBS, Cow&Gate, or SMA supplemented with *B. cereus* NR3 subjected to 0, 2, 5 or 10 kGy-e-beam irradiation. Each column represents a serial dilution (from left to right: 10^{0} to 10^{-5} concentrations). **(B)** $\mathbf{Log_{10}}$ **reduction of** ***Bacillus cereus*** **NR3 in PBS buffer, and Cow&Gate and SMA IMFs following treatment with e-beam intensities of 2, 5 and 10 kGy.** Irradiated samples were diluted in 10% PBS buffer according to the serial dilution method and plated on Plate Count agar, grown overnight at 37°C. Log_{10} (CFU/mL) data for samples diluted in PBS and irradiated with an intensity of 10 kGy not shown due to non-detectable levels in plate count.

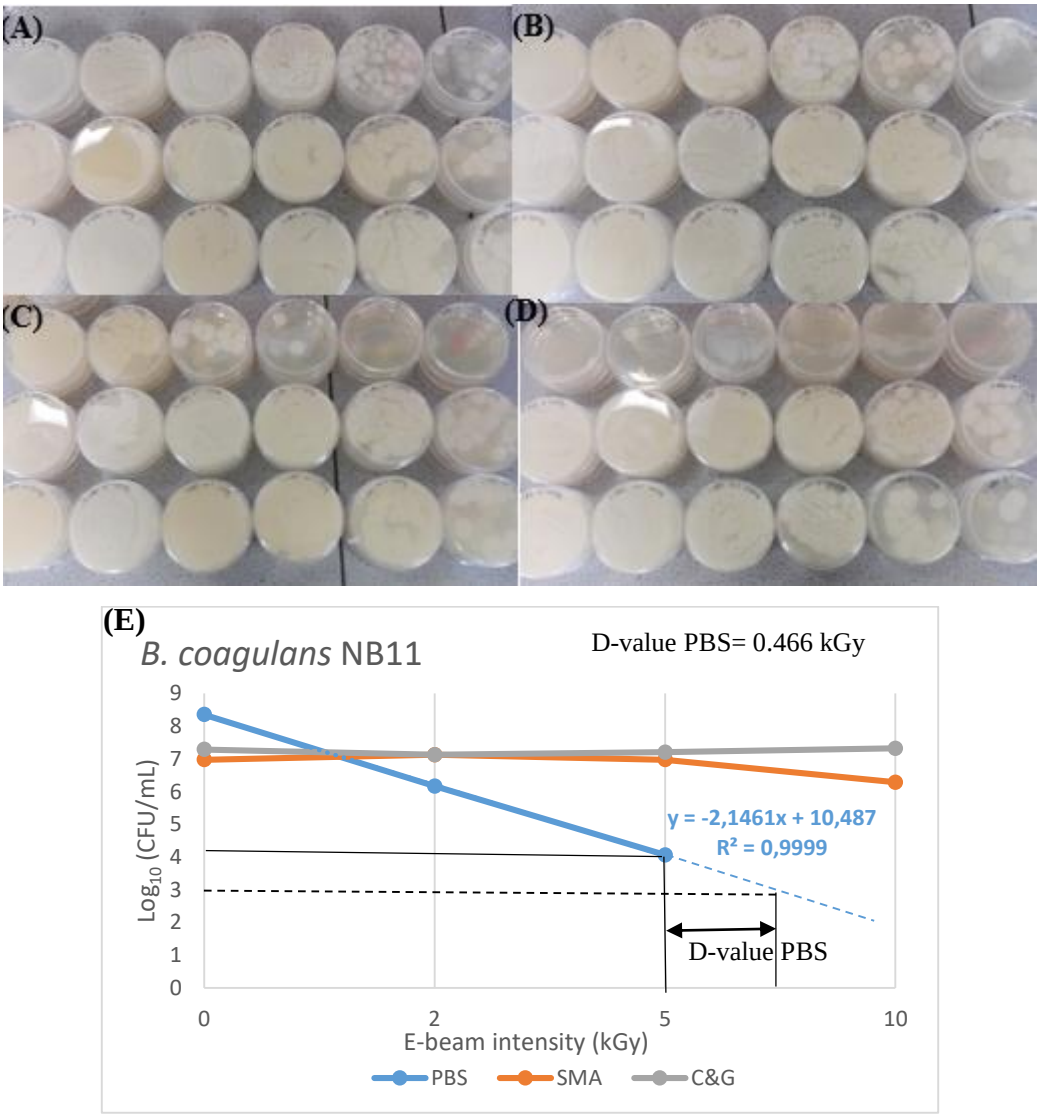


Figure 4.15. ***Bacillus coagulans*** **NB11** control **(A)** and treated with e-beam irradiation at **(B)** 2 kGy, **(C)** 5 kGy and **(D)** 10 kGy, in PBS, Cow&Gate and SMA IMF, plated in 1 to 10^{-5} dilutions (triplicates). Petri dishes containing Plate Count agar were inoculated with a solution of either PBS, Cow&Gate, or SMA supplemented with *B. coagulans* NB11 subjected to 0, 2, 5 or 10 kGy-e-beam irradiation (from top to bottom). Each column represents a serial dilution (from left to right: 10^{0} to 10^{-5} concentrations). **(E) Log_{10} reduction of** ***Bacillus coagulans*** **NB11 in PBS buffer, and Cow&Gate and SMA IMFs following treatment with e-beam intensities of 2, 5 and 10 kGy.** Irradiated samples were diluted in 10% PBS buffer according to the serial dilution method and plated on Plate Count agar, grown overnight at 37°C. Log_{10} (CFU/mL) data for samples diluted in PBS and irradiated with an intensity of 10 kGy not shown due to non-detectable levels in plate count.

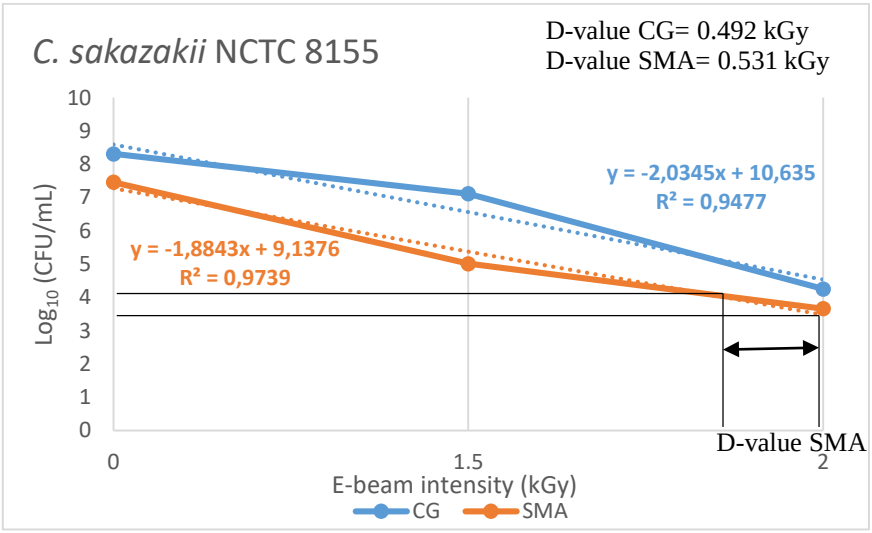


Figure 4.16. Log_{10} reduction of *Cronobacter sakazakii* NCTC 8155 in Cow&Gate and SMA IMFs following treatment with e-beam intensities of 1.5 and 2 kGy. Irradiated samples were diluted in 10% PBS buffer according to the serial dilution method and plated on VRBG agar, grown overnight at 37°C. Log_{10} (CFU/mL) data for samples diluted in PBS not shown due to non-detectable levels in plate count.

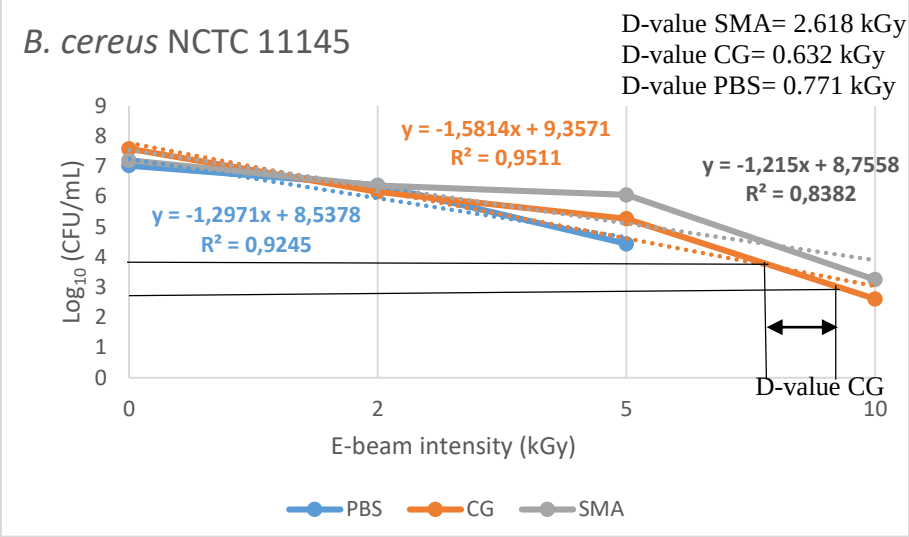


Figure 4.17. Log_{10} reduction of *Bacillus cereus* NCTC 11145 in PBS, Cow&Gate and SMA IMFs following treatment with e-beam intensities of 2, 5 and 10 kGy. Irradiated samples were diluted in 10% PBS buffer according to the serial dilution method and plated on Plate Count agar, grown overnight at 37°C. Log_{10} (CFU/mL) data for samples diluted in PBS and irradiated with 10 kGy not shown due to non-detectable levels in plate count.

The respective D-values are described in **Table 4.2**.

Table 4.2. D-values for four studied strains of bacteria, for e-beam irradiation.

Strain	D-value (kGy)					
	PBS	R^2	Cow&Gate	R^2	SMA	R^2
L. monocytogenes 7071	Dead at 1.5 kGy		0.705	0.9234	0.562	0.9992
C. sakazakii NCTC 8155	Dead at 1.5 kGy		0.492	0.9477	0.531	0.9739
B. cereus NCTC 11145	0.771	0.9245	0.632	0.9511	2.618	0.8382
B. cereus NR3	0.513	0.8703			0.926	0.6106
B. coagulans NB11	0.466	0.9999				

4.2.3. Nutritional Analysis

To determine if e-beam processing caused nutritional changes in Cow&Gate IMF, control and treated samples (10 kGy) were analysed for vitamin C, moisture, protein, total fat, ash, total carbohydrate and energy content by ALS Food & Pharmaceutical (Clonmel, Ireland). Results are depicted in **Table 4.3**.

Table 4.3. Nutritional values from untreated and treated powdered Cow&Gate IMF, attained from ALS Food&Pharmaceutical.

Parameter	Control (0 kGy)	Irradiated (10 kGy)
Moisture (Loss on Drying)	2.8 g/100g	2.8 g/100g
Protein (Nx6.25)	9.53 g/100g	9.58 g/100g
Total Fat (NMR)	23.4 g/100g	23.5 g/100g
Ash	2.2 g/100g	2.3 g/100g
Total Carbohydrate	62.1 g/100g	61.8 g/100g
Energy	497 kcal/100g 2083 kJ/100g	497 kcal/100g 2083 kJ/100g
Vitamin C	71.5 mg/100g	84.6 mg/100g

In **Table 4.4**, values presented in Table 4.3 are compared to standard guidelines for IMF in the European Union.

Table 4.4. Parameter range for IMF composition, according to the EU, and results attained from ALS's nutritional analysis.

Parameter	Minimum Value	Maximum Value	Irradiated IMF powder (10 kGy)
Energy	2500 kJ/100g	2950 kJ/100g	2083 kJ/100g
Protein	11.25 g/100g	17.5 g/100g	9.58 g/100g
Total Fat	26.25 g/100g	35 g/100g	23.5 g/100g
Total Carbohydrates	55 g/100g	85 g/100g	61.8 g/100g
Vitamin C	62.5 mg/100g	187.5 mg/100g	84.6 mg/100g

4.3.Gamma Irradiation

4.3.1. Background microbiological studies and the effect of time on reconstituted IMF

In order to assess the microbiological load of irradiated IMF powder (at 5 kGy) without its supplementation, 10 g of these powders were reconstituted in 100 mL of sterile deionised water, plated and kept at 37°C overnight. The reconstituted IMF was left on the bench, and again plated after 24h and 48h.

Samples plated immediately after reconstitution presented observable no noticeable growth, as well as samples plated after 24h on benchtop. After 48h on the benchtop, Petri dishes presented a countable number of colonies, corresponding to a 7 $\log_{10}$ CFU/mL.

4.3.2. Plate count results

Gamma irradiation results were expressed as $\log_{10}$ CFU/mL against gamma intensity (in kGy) and the intensity taken to reduce the population of microorganisms by a single $\log_{10}$ was reported as the D-value for that particular microorganism and medium. D-values for microorganisms was read directly from the plots and are presented in **Table 4.5**.

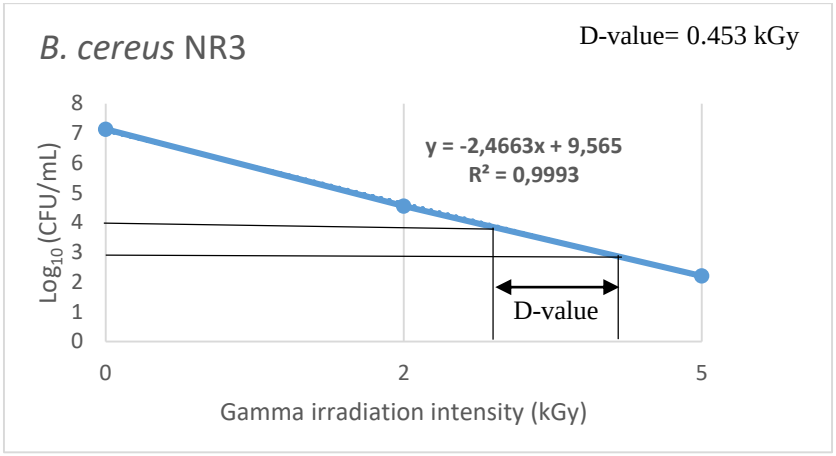


Figure 4.18. Log_{10} reduction of *Bacillus cereus* NR3 in PBS, following treatment with gamma irradiation intensities of 2, and 5 kGy. Irradiated samples were diluted in 10% PBS buffer according to the serial dilution method and plated on Plate Count agar, grown overnight at 37°C. Log_{10} (CFU/mL) data for with 10 kGy not shown due to non-detectable levels in plate count.

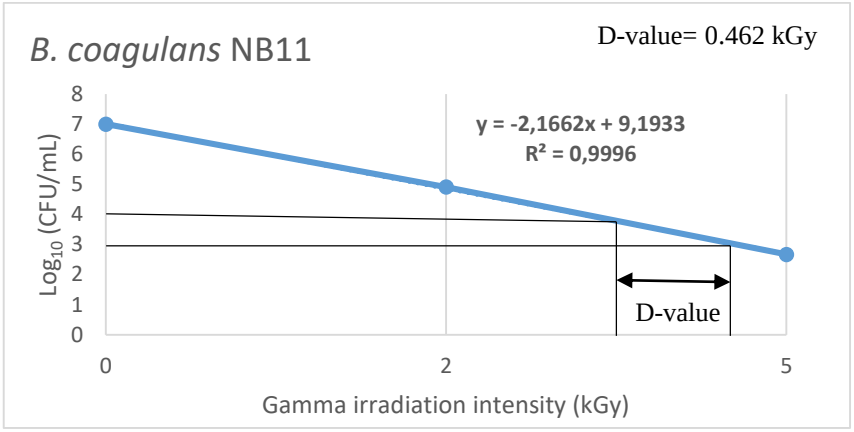


Figure 4.19. Log_{10} reduction of *Bacillus coagulans* NB11 in PBS, following treatment with gamma irradiation intensities of 2, and 5 kGy. Irradiated samples were diluted in 10% PBS buffer according to the serial dilution method and plated on Plate Count agar, grown overnight at 37°C. Log_{10} (CFU/mL) data for with 10 kGy not shown due to non-detectable levels in plate count.

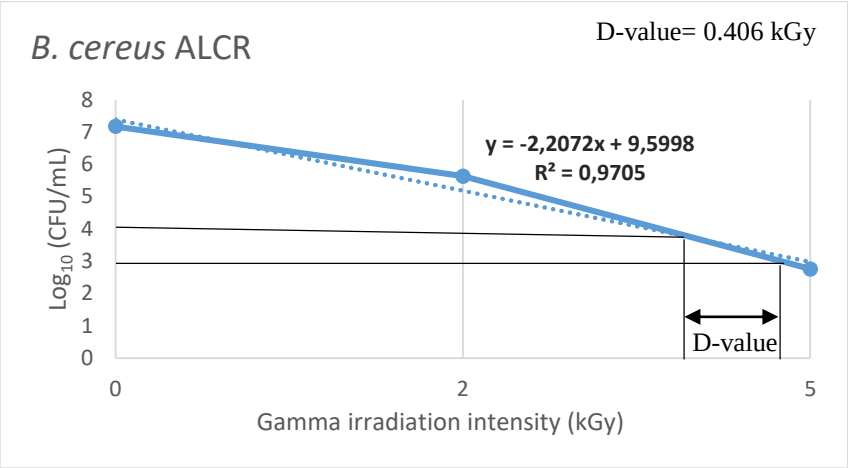


Figure 4.20. Log_{10} reduction of *Bacillus cereus* ALCR in PBS, following treatment with gamma irradiation intensities of 0, 2 and 5 kGy. Irradiated samples were diluted in 10% PBS buffer according to the serial dilution method and plated on Plate Count agar, grown overnight at 37°C. Log_{10} (CFU/mL) data for with 10 kGy not shown due to non-detectable levels in plate count.

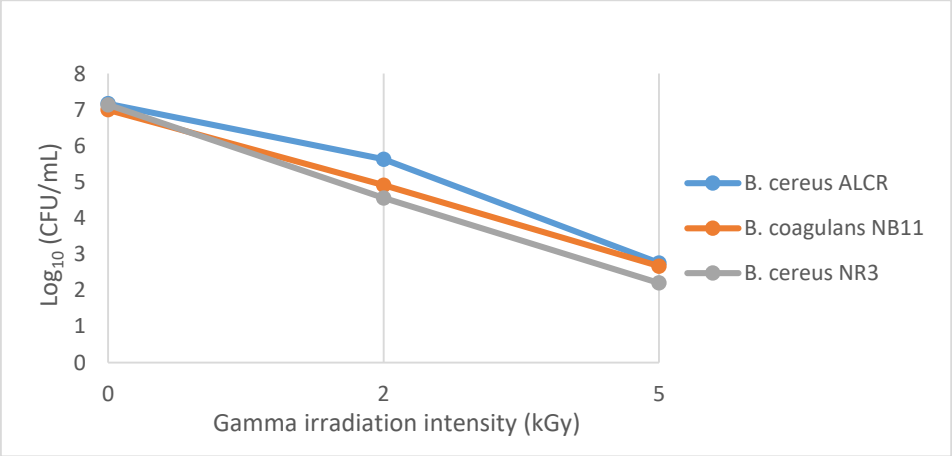


Figure 4.21. Comparison between log_{10} reduction of *B. cereus* ALCR, *B. coagulans* NB11 and *B. cereus* NR3 in PBS, following treatment with gamma irradiation intensities of 0, 2 and 5 kGy. Irradiated samples were diluted in 10% PBS buffer according to the serial dilution method and plated on Plate Count agar, grown overnight at 37ºC. Concentrations and D-values do not vary much among strains.

Corresponding D-values are described in **Table 4.5**.

Table 4.5. D-values for gamma-irradiated bacterial strains.

Strain	D-value (kGy)	R^2
B. coagulans NB11	0.462	0.9996
B. cereus ALCR	0.406	0.9705
B. cereus NR3	0.453	0.9993

4.3.3. Nutritional Analysis

As tested for e-beam-processed samples, gamma-irradiated samples at 10 kGy were also analysed to determine if processing caused nutritional changes in Cow&Gate IMF, by ALS Food & Pharmaceutical (Clonmel, Ireland). Results are depicted in **Table 4.6**.

Table 4.6. Nutritional values from untreated and treated (at 10 kGy) powdered Cow&Gate IMF, attained from ALS Food&Pharmaceutical, and comparison to European Commission's Guidelines for IMF composition.

Tested Parameter	Control (0 kGy)	Irradiated IMF (10 kGy)	European Commission's Guidelines	
			Minimum	Maximum
Moisture (Loss on Drying)	2.8 g/100g	3.8 g/100g	-	-
Protein (Nx6.25)	9.53 g/100g	9.27 g/100g	11.25 g/100g	17.5 g/100g
Total Fat (NMR)	23.4 g/100g	22.7 g/100g	26.25 g/100g	35 g/100g
Ash	2.2 g/100g	2.4 g/100g	-	-
Total Carbohydrate	62.1 g/100g	61.8 g/100g	55 g/100g	85 g/100g
Energy	2083 kJ/100g	2049 kJ/100g	2500 kJ/100g	2950 kJ/100g
Vitamin C	71.5 mg/100g	69.4 mg/100g	62.5 mg/100g	187.5 mg/100g

5. Discussion

Infant milk formula is the main source of nurturing for infants whose mothers cannot or do not wish to breastfeed. The powder is not sterile, becoming a source of foodborne disease and increasing the risk of premature death. Novel processing technologies, such as PUV, electron beam and gamma irradiation, are a viable solution to increase food safety, therefore their applicability to decontaminate food must be investigated, considering bacteria which cause foodborne disease in humans, without compromising nutritional value.

Table 5.1. All D-values for the six studied strains, in all three media and for each sterilisation technique.

Strain	PUV (pps)		E-beam (kGy)						Gamma Irradiation (kGy)	
	PBS	r^2	PBS	R^2	CG	R^2	SMA	R^2	PBS	R^2
Listeria monocytogenes 7071	0.516	0.9573	Dead at 1.5 kGy	-	0.705	0.9234	0.562	0.9992	-	-
C. sakazakii NCTC 8155	0.548	0.9234	Dead at 1.5 kGy	-	0.492	0.9477	0.531	0.9739	-	-
B. cereus NR3	0.783	0.8775	0.513	0.8703	-	-	0.926	0.6106	0.453	0.9993
B. coagulans NB11	0.66	0.993	0.466	0.9999	-	-	-	-	0.462	0.9996
B. cereus NCTC 11145	0.66	0.993	0.771	0.9245	0.632	0.9511	2.618	0.8382	-	-
Bacillus cereus ALCR	0.734	0.9955	-	-	-	-	-	-	0.406	0.9705

5.1. 2008 China's Milk Scandal and food irradiation acceptability

In 2013, China banned all IMF imports from New Zealand and Australia, due to Fonterra's produce contaminated with *Clostridium botulinum*. Fonterra announced the problem involved three batches of a whey protein concentrate produced. The company then conducted more intensive tests, and workers found signs of the presence of *Clostridium botulinum* in a sample. The problema lied in unsterilised pipes (Reuters 2013; Mullany 2013). However, this was not the first case of IMF contamination in China. In 2008, the Chinese dairy company *Sanlu*, held a large stake by Fonterra, was found to have added melamine. Nitrogen-rich melamine can be added to substandard or watered-down milk to increase protein content, since this is measured through the nitrogen content in a sample (Reuters 2008). Sixteen infants died from kidney stones and other kidney-related issues, but almost 300,000 children were affected (Mu Xuequan 2008; Braningan 2008).

As a consequence, it is now ilegal to transport more than 1.8 kg of IMF outside Hong Kong, and the person carrying said formula must be at least 16 years of age and must not have left the city in the last 24 hours. Offenders face up to two years in prison and a fine of up to HK$500,000 (Tsang & Nip 2013). Also, the EU announced a ban on imports of baby food containing Chinese milk, and the American FDA issued na alert against all finished food products from China (Jones 2008; Saputra et al. 2008).

Currently, it is very expensive to acquire IMF in China, since all these scandals have decreased the number of commercially available brands. Further studies on IMF irradiation ought to be conducted, in order to attain a potentially risk-free produce and to assure its safety, increasing its acceptability to consumers. (IAEA 2009). However, some nutritional losses are already known, even though these only result from high irradiation doses (>10 kGy). Of the water soluble vitamins, vitamins C and B_1 are least resistant to irradiation, while of the fat soluble vitamins, only vitamins A and E present some sort of radiation sensitivity. At low and medium doses, the effect of irradiation on the nutritional content of lipids is minimal, but the irradiation of lipids at high doses and in the presence of oxygen can lead to the formation of liquid hydroperoxides, substances with undesirable odours and flavours. Concerning proteins, while amino acids are relatively susceptible to free radical attack following irradiation, they are much less sensitive when in the rigid structure of a protein molecule (Mostafavi et al. 2010).

In 1993, a Resolution from IAEA selected four countries to introduce "commercial-scale food irradiation programmes": Chile, China, Morocco and Mexico. Food irradiation faces many acceptance challenges. Countries located in arid and semi-arid zones face more food losses due to the climate's ability to allow the growth of microorganisms during the processing chain. Irradiation is considered a safe way to reduce food losses and to increase the hygiene of exports. At the time, Canada, France, The Netherlands, Thailand, United Kingdom and The United States of America already irradiated food as an alternative to chemical fumigation of certain foods (IAEA 1993; Mostafavi et al. 2010). Currently, Mexico irradiates fruits; China irradiates spices, seafood and vegetables, while the United States of America feeds astronauts with irradiated food, and has approved the sale of meat and other produce. Worldwide, 55 countries approved food irradiation.

In spite of the fact that irradiated foods and the process of food irradiation have been carefully tested and the greater quality of treated products recognised, the quantity of irradiated foods in the global food trade is not significant. The lack of acceptance of food irradiation has been mostly because of misconceptions and irrational fear of nuclear connected technologies. Also, people are confused and fail to differentiate irradiated food from radioactive foods

(Johnson et al. 2004). There is growing public consciousness on food safety and quality combined with current incidences of foodborne pathogens (Patterson 2005). Attitude studies (mostly in the US) demonstrated most consumers expressed less concern about food irradiation than other food processing technologies. When given science-based information, from 60% to 90% of consumers prefer the advantages irradiation processing provides, and when information is accompanied by samples, acceptance may increase to 99% (Bruhn 1998; Johnson et al. 2004; Eustice & Bruhn 2012).

Far East markets, like China and the Republic of Korea, are critical IMF markets. A joint FAO/IAEA work showed that Asian consumers would accept irradiated foods and that trade benefits would ensue from the application of the technology. Also, commercial food irradiation is increasing significantly in Asia, but decreasing in EU (FAO & IAEA 2001; Kume et al. 2009). Particularly in China, the Ministry of Public Health approved irradiation of food in July 1998, establishing standards for six classes/groups of foods, namely vegetables and fruits, grains and beans, meats and poultry (fresh, chilled or frozen), cooked meat, spices and dehydrated vegetables and dried fruits and nuts. China established 50 large irradiators across the country, irradiating food and other products. In 1995–1998 more than 166 000 tons of different food products were irradiated and marketed through normal trading channels. The major products included rice, garlic, spices, dehydrated vegetables and health food. Between 1994 and 1996, more than 30 consumer acceptability tests covering 20 different products were diverse Chinese cities. More than 10 tons of irradiated pickled meat products with grain stillage were test marketed and evaluated for consumer acceptability. Consumer acceptance was favourable for the treated products marketed with the international logo for food irradiation. Market testing of irradiated rice, dehydrated vegetables and spices showed that 70–80% of consumers preferred the irradiated products based on quality considerations (FAO & IAEA 2001).

5.2. The influence of time on reconstituted, irradiated IMF

Studies conducted on IMF prove this infant feed is not sterile, housing an intrinsic *Bacillus* strain which was isolated and identified as *Bacillus cereus*. *Listeria monocytogenes* and *Cronobacter sakazakii* colonies were not found in the investigated IMF brands, as corroborated by previous studies (O'Brien et al. 2009; Haughton et al. 2010).

For e-beam-irradiated IMF, all samples plated immediately after reconstitution presented observable growth of mixed colonies, meaning possible contamination of media

(**Figure 4.11**). Samples plated after 24h on benchtop showed individual colonies observable at 0 kGy and 2 kGy, and no observable growth at 10 kGy. This possible means that non-spore forming bacteria are probably dead; colonies of spore-forming bacteria survive at 2 kGy because they probably had already pre-formed spores (Figure 4.11B). After 48h on benchtop, plated samples present numerous colonies at 0 kGy and 2 kGy; however, there is little growth at 10 kGy. It appears that spore-forming bacteria survive e-beam treatment at a 2 kGy intensity but not at 10 kGy (Figure 4.11C). Such results are corroborated by Rowan et al. (1997) and Haughton et al. (2010). When stored for 14h at 25°C, reconstituted PIF became unsafe for consumption because *Bacillus* spores increased from 10^2 spores/g to $1.3x10^3$ CFU/g. In addition, *B. cereus* inhibited the growth of other *Bacillus* species and other Gram-positive bacteria, such as *L. monocytogenes* (Rowan & Anderson 1997; Yilmaz et al. 2006; Haughton et al. 2010).

For gamma-irradiated IMF, samples plated immediately after reconstitution presented non-noticeable growth, as well as samples plated after 24h on benchtop. After 48h on the benchtop, Petri dishes presented a countable number of colonies, corresponding to a 7 $\log_{10}$ CFU/mL, meaning spores survived gamma irradiation and were capable of revert back to vegetative cells.

5.3. PUV inactivation

Results show PUV light does not decontaminate reconstituted IMF because of the media's turbidity. Such turbidity may be a result of formulae's components themselves (e.g. carbohydrates, proteins), but it may be also because of PUV induced-cell membrane damage, leading to leakage of cells' components. On the other hand, Farrell et al. (2009) and Rowan et al. (1999) have proved low UV light achieves only a 1 $\log_{10}$ reduction, contrasting to high UV light, reaching a 5-6 $\log_{10}$ reduction. Also, only UVC (low UV) light is capable of successfully damage cell walls (Gómez-López et al. 2007). This idea is not supported by evidence, since a 5-6 $\log_{10}$ reduction is accomplished when in PBS. Even though there is no efficient decontamination of IMF, PUV treatment affects the growth rate of *B. cereus* CECT 131/ATCC 10876 spores that survive irradiation (Aguirre et al. 2015).

In future research, it would be significant to do other assays, e.g. protein detection assays, metabolic activity assays (Kramer & Muranyi 2013), generation of reactive oxygen species (ROS) or comet assay to investigate nuclear damage (Farrell et al. 2011).

5.4. Novel processing technologies: e-beam and gamma radiation

The reference treatment intensities for ionizing radiation (**Table 1.6**), in order to delay spoilage and kill certain pathogenic bacteria e.g. *Salmonella*, are 1-7 kGy, the approximate range utilised in this work. Significant results were achieved at 5 and 10 kGy, so this seems to be the adequate intensity range for food decontamination.

Osaili et al. (2007) established the D-value for gamma irradiation of *C. sakazakii* in both dehydrated (DIMF) and rehydrated IMF (RIMF) using a maximum dose of 1 kGy. Two *C. sakazakii* strains isolated from IMF were used for media enrichment: IMF1 and IMF2. However, several authors mentioned *C. sakazakii* is not commonly found in IMF (Mullane et al. 2007; Mullane et al. 2008; Haughton et al. 2010) because it is not able to survive the pasteurisation step; it also was not identified in this study. Contamination with *Cronobacter sakazakii* can occur during the manufacturing process or during storage and reconstitution. If good hygiene practices are not being followed, it can lead to microorganism growth within the formula or introduction from the environment. For RIMF, the D-value for IMF1 and IMF2 are 0.37 kGy and 0.28 kGy, respectively; while for DIMF are 1.06 kGy and 1.15 kGy. It was concluded that ionizing gamma radiation is able to inactivate *C. sakazakii*. Lee et al. (2007) gamma-irradiated dried IMF, which has shown a significantly delay in the re-growth of *C. sakazakii* comparing to untreated samples. Lee et al. (2006) reported a D-value of 0.76 kGy for *C. sakazakii* after a 3 kGy-gamma irradiation followed by rehydration at 80°C. No recoverable bacteria were found in the PIF irradiated at 5 kGy, corroborating this study's results.

Tesfai et al. (2011) studied e-beam inactivation of *E. coli* DH5α after two cycles of low dose irradiation, followed by three cycles of increased but non-lethal irradiation. After the third cycle, D-values for non-selective and selective media were both 0.32 kGy; 0.39 and 0.40 kGy after the fourth cycle, respectively, and 0.46 kGy after the fifth cycle. Prakash et al. (2007) studied the effect of e-beam in *Salmonella spp.* in diced tomatoes dipped in calcium. D-values ranged from 0.26 to 0.39 kGy, indicating that a 5 $\log_{10}$ CFU/g reduction in *Salmonella* will require an irradiation dose of 1.3–1.95 kGy. Even though *E. coli* was not subjected to study, these D-values serve as a comparison term as *E. coli* belongs to the *Enterobacteriaceae* family, like *C. sakazakii*.

Velasco et al. (2015) e-beam-irradiated Camembert and Brie soft cheese varieties as a sanitation treatment. Food safety objective (FSO) according to EU and USDA criteria for *Listeria monocytogenes* were 1.27 and 2.59 kGy, respectively, and these doses allowed the control of *L. monocytogenes* growth. Calculated D-values for both Camembert and Brie were

0.34 kGy. These numbers are lower than the ones presented in Table 5.1, but one must keep in mind that IMF's turbidity might contribute to the need for a higher irradiation dose.

De Lara et al. (2002) obtained D-values in the 1.9-2.6 kGy for *Bacillus cereus*, Thayer & Boyd (1994) found D-values of approximately 2 kGy for spores of different strains of *B. cereus* gamma-irradiated in several food products. Monk et al. (1995) revised D-values of several foodborne microorganisms, including *B. cereus* spores and vegetative cells, and found D-values between 1.25 and 4.0 kGy when *B. cereus* spores were irradiated in different media. These numbers are considerably all higher than the ones attained from this study.

5.4.1. Nutritional analysis

In agreement with previous findings (Tesfai et al. 2014), gamma and e-beam irradiation do not alter IMF's quality even at doses higher than 10 kGy, from a nutritional point of view, since there are no significant changes in the tested parameters – protein content, total fat, ash, moisture, total carbohydrate, energy and vitamin C-, comparing to data from Yeung et al. (2006) related to heat sterilisation (autoclave). These numbers are also similar to E.C.'s guidelines, proving there is no significant difference when compared to untreated samples. Small differences may be due to losses during processing and/or due to equipment's operation limitations.

5.5. Final considerations

Bacillus cereus NCTC 11145, a HBL and BceT toxins-producer, seems to be more resistant to irradiation than other strains, considering higher calculated D-values. This suggests toxin-encoding gene clusters may be related to molecular damage repair mechanisms (Farrell et al. 2009). On the other hand, there is a possibility of a protective effect of IMF's carbohydrate/lipid layer on bacterial cells, preventing them from being damaged by radiation.

It is crucial to remember that this study was conducted on spores, and not vegetative cells. Spores' goal is to ensure bacterial survival in harsh environmental conditions. They are more UV-resistant than vegetative cells (Verhille et al. 2003), and it is known that they possess a spore-specific enzyme repair system (Setlow 2001) and specific proteins protecting spore DNA from UV damage. The next step is to work on gene silencing to infer the importance of said genes.

Another issue is the VBNC (viable but non culturable) state, in which a cell is metabolically active, but is being incapable of undergoing the cellular division required for

growth in or on a medium normally supporting grown of that cell (Olivier 1993). A great number of studies are conducted using a culture-based or kinetic methods. Therefore, these kinetic methods must be adapted to consider the VBNC state (Rowan et al. 2015). Live/dead cells ratio should be compared to plate count results, since significant discrepancies were found between conventional plate counts and viability staining parameters (Kramer & Muranyi 2013). Such state may represent a health risk for consumers, because bacteria retain their pathogenicity in the VBNC state (Rowan 2011).

This study's findings contribute to prove the importance of e-beam and gamma radiation not only for medical devices' sterilisation but also for food decontamination.

6. Conclusions

Studies conducted on IMF prove this infant feed is not sterile, housing an intrinsic *Bacillus* strain which was isolated and identified as *Bacillus cereus*. *Listeria monocytogenes* and *Cronobacter sakazakii* colonies were not found in the investigated IMF brands.

The influence of time on reconstituted, irradiated IMF was also investigated and pointed out to a survival of endospores 24 and 48 hours after reconstitution. This means reconstituted IMF should not be prepared earlier before consumption since it will cause disease in infants who have a weaker immune system.

Pulsed UV light was applied in treatment of supplemented PBS and IMF samples. Results show PUV does not reach a satisfactory decontamination level when in IMF due to high turbidity, therefore it cannot be applied to industrial decontamination of feeds.

E-beam and gamma radiation, both ionizing sterilisation techniques, were found to have potential to be applied to sterilisation at an industrial level. E-beam successfully decontaminated supplemented IMF samples. Gamma radiation seems more efficient because it achieves optimal decontamination at 5 kGy as opposed to e-beam's 10 kGy. On the nutritional point of view, there were no alterations in both IMF's composition comparing to control samples, even though a slight change in colouration when subjected to 10 kGy e-beam processing. All values were in accord with the European Commission's standard criteria for IMF composition. Further investigation to gamma radiation must be conducted on IMF, but so far results are convincing.

Bacillus cereus NCTC 11145, a toxin-producer bacterium that provokes vomiting in humans, seems to be more resistant to irradiation than other strains, considering higher calculated D-values.

7. Limitations to this study and future research

Goals set to this study were partially achieved, however some details might be investigated for further elucidation.

The irradiation equipment, both e-beam and gamma irradiator, were not located close to A.I.T., therefore there was a relatively long period of time between sample preparation, irradiation and culture. For the same reason, it was not possible to gamma-irradiate reconstituted IMF because of possible spoilage. Also, operation limitations of said equipment do not allow a more accurate calculation of D-values.

Considering all limitations and what has been done in IMF studies, the next steps in research are:

- Attempt to decrease e-beam/gamma processing intensities to non-toxigenic strains, in order to save energy, therefore saving money.
- Repeat treatment of toxigenic strains to assess the need for increased intensities.
- Apply vital stains and confocal microscopy to irradiated bacteria to assess damage to cell wall; compare live/dead cells ratio to plate count results, since significant discrepancies were found between conventional plate counts and viability staining parameters (Kramer & Muranyi 2013).
- Proceed to further analyse gamma and e-beam-irradiated samples to prove there are no nutritional alterations in IMF, concerning other nutritional aspects e.g. vitamins B and E.
- Useful bacteria, as lactic acid bacteria, are also affected by radiation so this problem must be solved.
- Continue studies on obligate anaerobic spore-forming bacteria, e.g. *Clostridium*.

8. References

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9. Annexes

Annex I

Essential composition of infant formulae when reconstituted as instructed by the manufacturer

1. Energy

Minimum	Maximum
250 kJ/100 mL (60 kcal/100 mL)	295 kJ/100 mL (70 kcal/100 mL)

2. Proteins

Protein content = nitrogen content x 6.25

2.1. Infant formulae manufactured from cows' milk proteins or goats' milk proteins

Minimum	Maximum
0.45 g/100 mL (1.8 g/100 kcal)	0.7 g/100 mL (3 g/100 kcal)

2.2. Infant formulae manufactured from protein hydrolysates

Minimum	Maximum
0.45 g/100 mL (1.8 g/100 kcal)	0.7 g/100 mL (3 g/100 kcal)

2.3. Infant formulae manufactured from soya protein isolates, alone or in a mixture with cows' milk or goats' milk proteins

Minimum	Maximum
0.56 g/100 mL (2.25 g/100 kcal)	0.7 g/100 mL (3 g/100 kcal)

3. Taurine

If added to infant formulae, the amount of taurine shall not be greater than 2,9 mg/100 kJ (12 mg/100 kcal).

4. Choline

Minimum	Maximum
1.7 mg/100 kJ (7 mg/100 kcal)	12 mg/100 kJ (50 mg/100 kcal)

5. Lipids

Minimum	Maximum
1.05 g/100 kJ (4.4 g/100 kcal)	1.4 g/100 kJ (6.0 g/100 kcal)

5.1. The use of the following substances shall be prohibited: sesame seed oil; cotton seed oil.

5.2. Lauric acid and myristic acid

Minimum	Maximum
-	Separately or as a whole: 20 % of the total fat content

5.3. Trans fatty acid: shall not exceed 3 % of the total fat content.

5.4. Erucic acid: shall not exceed 1 % of the total fat content.

5.5. Linoleic acid (in the form of linoleates)

Minimum	Maximum
70 mg/100 kJ (300 mg/100 kcal)	285 mg/100 kJ (1200 mg/100 kcal)

α-linolenic acid: shall not be less than 12 mg/100 kJ (50 mg/100 kcal).

Linoleic:α-linolenic acid ratio: shall not be less than 5 nor greater than 15.

Long-chain (20 and 22 carbon atoms) polyunsaturated fatty acids (LCP) may be added. In that case their content shall not exceed:

- 1 % of the total fat content for n-3 LCP, and
- 2 % of the total fat content for n-6 LCP (1 % of the total fat content for arachidonic acid (20:4 n-6))

The eicosapentaenoic acid (20:5 n-3) content shall not exceed that of docosahexaenoic (22:6 n-3) acid content. The docosahexaenoic acid (22:6 n-3) content shall not exceed that of n-6 LCP.

6. Phospholipids

Shall not be greater than 2 g/l.

7. Inositol

Minimum	Maximum
1 mg/100 kJ (4 mg/100 kcal)	10 mg/100 kJ (40 mg/100 kcal)

8. Carbohydrates

Minimum	Maximum
2.2 g/100 kJ (9 g/100 kcal)	3.4 g/100 kJ (14 g/100 kcal)

Only the following carbohydrates may be used: lactose, maltose, sucrose, glucose, maltodextrins, glucose syrup or dried glucose syrup, pre-cooked starch (naturally gluten-free), and gelatinised starch (naturally gluten-free).

8.1. Lactose

Minimum	Maximum
1.1 g/100 kJ (4.5 g/100 kcal)	-

8.2. Sucrose

Sucrose may only be added to infant formulae manufactured from protein hydrolysates. If added, the sucrose content shall not exceed 20% of the total carbohydrate content.

8.3. Glucose

Glucose may only be added to infant formulae manufactured from protein hydrolysates. If added, the glucose content shall not exceed 0.5 g/100 kJ (2 g/100 kcal).

8.4. Pre-cooked starch and/or gelatinised starch

Minimum	Maximum
-	2 g/100 mL, and 30 % of the total carbohydrate content

9. Fructo-oligosaccharides and galacto-oligosaccharides

Fructo-oligosaccharides and galacto-oligosaccharides may be added to infant formulae. In that case their content shall not exceed: 0.8 g/100 mL in a combination of 90% oligogalactosyl-lactose and 10 % high molecular weight oligofructosyl-saccharose.

10. Mineral substances

10.1. Infant formulae manufactured from cows' milk or goats' milk proteins or protein hydrolysates

Table annex I 1. Minimum and maximum allowed values for mineral substances in infant formulae manufactured from cows' milk or goats' milk proteins or protein hydrolysates.

	Per 100 kJ		Per 100 kcal	
	Minimum	Maximum	Minimum	Maximum
Sodium (mg)	5	14	20	60
Potassium (mg)	15	38	60	160
Chlorine (mg)	12	38	50	160
Calcium (mg)	12	33	50	140
Phosphorus (mg)	6	22	25	90
Magnesium (mg)	1.2	3.6	5	15
Iron (mg)	0.07	0.3	0.3	1.3
Zinc (mg)	0.12	0.36	0.5	1.5
Copper (µg)	8.4	25	35	100
Iodine (µg)	2.5	12	10	50
Selenium (µg)	0.25	2.2	1	9
Manganese (µg)	0.25	25	1	100
Fluorine (µg)	-	25	-	100
The calcium:phosphorus ratio shall not be less than 1 nor greater than 2.				

10.2. Infant formulae manufactured from soya protein isolates, alone or in a mixture with cows' milk or goats' milk proteins

Table annex I 2. Minimum and maximum permitted values for mineral substances for infant formulae manufactured from soya protein isolates, alone or in mixture with cows' milk or goats' milk proteins.

	Per 100 kJ		Per 100 kcal	
	Minimum	Maximum	Minimum	Maximum
Sodium (mg)	5	14	20	60
Potassium (mg)	15	38	60	160
Chlorine (mg)	12	38	50	160
Calcium (mg)	12	33	50	140
Phosphorus (mg)	7.5	25	30	100
Magnesium (mg)	1.2	3.6	5	15
Iron (mg)	0.12	0.5	0.45	2
Zinc (mg)	0.12	0.36	0.5	1.5
Copper (µg)	8.4	25	35	100
Iodine (µg)	2.5	12	10	50
Selenium (µg)	0.25	2.2	1	9
Manganese (µg)	0.25	25	1	100
Fluorine (µg)	-	25	-	100

11. Vitamins

Table annex I 3. Minimum and maximum permitted values for vitamins in infant milk formulae.

	Per 100 kJ		Per 100 kcal	
	Minimum	Maximum	Minimum	Maximum
Vitamin A (µg all trans retinol equivalent)	14	43	60	180
Vitamin D (µg)	0.25	0.65	1	2.5
Thiamine (µg)	14	72	60	300
Riboflavin (µg)	19	95	80	400
Niacin (µg)	72	375	300	1500
Pantothenic acid (µg)	95	475	400	2000
Vitamin B6 (µg)	9	42	35	175
Biotin (µg)	0.4	1.8	1.5	7.5
Folic acid (µg)	2.5	12	10	50
Vitamin B12 (µg)	0.025	0.12	0.1	0.5
Vitamin C (mg)	2.5	7.5	10	30
Vitamin K (µg)	1	6	4	25
Vitamin E (mg α-tocopherol equivalent)	0.5/g of polyunsaturated fatty acids expressed as linoleic acid but in no case less than 0.1 mg/100 kJ	1.2	0.5/g of polyunsaturated fatty acids expressed as linoleic acid but in no case less than 0.5 mg/100 kcal	5

12. Nucleotides

Table annex I 4.Maximum permitted values for added nucleotide in infant milk formula.

	Maximum[1]	
	(mg/100 kJ)	(mg/100 kcal)
cytidine 5′-monophosphate	0.6	2.5
uridine 5′-monophosphate	0.42	1.75
adenosine 5′-monophosphate	0.36	1.5
guanosine 5′-monophosphate	0.12	0.5
inosine 5′-monophosphate	0.24	1
[1] The total concentration of nucleotides shall not exceed 1.2 mg/100 kJ (5 mg/100 kcal).		

Annex II

Table annex II 1. Indispensable and Conditionally Indispensable Amino Acids in Breast Milk.

	Per 100 kJ[1]	Per 100 kcal
Cystine	9	38
Histidine	10	40
Isoleucine	22	90
Leucine	40	166
Lysine	27	113
Methionine	5	23
Phenylalanine	20	83
Threonine	18	77
Tryptophan	8	32
Tyrosine	18	76
Valine	21	88
[1] 1 kJ = 0.239 kcal		

Annex III

Specification for the protein content and source and the processing of protein used in the manufacture of infant formulae and follow-on formulae with a protein content less than 0.56 g/100 kJ (2.25 g/100 kcal) manufactured from hydrolysates of whey proteins derived from cows' milk protein

1. Protein content

   Protein content = nitrogen content × 6.25

Minimum	Maximum
0.44 g/100 kJ (1.86 g/ 100 kcal)	0.7 g/100 kJ (3 g/100 kcal)

2. Protein source

   Demineralised sweet whey protein derived from cows' milk after enzymatic precipitation of caseins using chymosin, consisting of:

   (a) 63% caseino-glycomacropeptide free whey protein isolate with a minimum protein content of 95 % of dry matter and protein denaturation of less than 70% and a maximum ash content of 3%; and

   (b) 37% sweet whey protein concentrate with a minimum protein content of 87% of dry matter and protein denaturation of less than 70% and a maximum ash content of 3.5%.

3. Protein processing

   Two-stage hydrolysis process using a trypsin preparation with a heat-treatment step (from 3 to 10 minutes at 80 to 100 °C) between the two hydrolysis steps.

4. Protein quality

Table annex III 1. Specifications for amino acids' content in infant milk formula and follow-on formula manufactured from hydrolysates of whey proteins derived from cows' milk protein.

	Per 100 kJ[1]	Per 100 kcal
Arginine	16	69
Cystine	6	24
Histidine	11	45
Isoleucine	17	72
Leucine	37	156
Lysine	29	122
Methionine	7	29
Phenylalanine	15	62
Threonine	19	80
Tryptophan	7	30
Tyrosine	14	59
Valine	19	80
[1] 1 kJ = 0.239 kcal		

Annex IV

Table annex IV 1. Microbiological criteria established for three types of infant and follow-on formulae.

Product	**Microorganism**	**Limits**		**Reference Analytical Method**	**Legislation**
		Minimum	Maximum		
Dehydrated infant formulae and dietary foods for special medical purposes intended for infants younger than 6 months-old	*Salmonella*	Absence in 25 g		EN/ISO 6579	E.C. Regulation 1441/2007
	Cronobacter spp. (Enterobacter sakazakii)	Absence in 10 g		TS/ISO 22964	E.C. Regulation 365/2010
	Enterobacteriaceae	Absence in 10 g		ISO 21528-1	E.C. Regulation 1441/2007
	Bacillus cereus	50 CFU/g	500 CFU/g	EN/ISO 7932	E.C. Regulation 1441/2007
Dehydrated transition formulae	*Salmonella*	Absence in 25 g		EN/ISO 6579	E.C. Regulation 1441/2007
	Enterobacteriaceae	Absence in 10 g		EN/ISO 21528-1	E.C. Regulation 1441/2007
Ready-to-eat foods intended for infants and ready-to-eat foods for special medical purposes	*Lysteria monocytogenes*	Absence in 25 g		EN/ISO 11290-1	E.C. Regulation 1441/2007

Annex V

Table annex V 1. API®20E tests, reactions and positive/negative result identification of *Enterobacteria*.

Tests	Substrates	Reaction tested	Negative results	Positive results
ONPG	ONPG	Beta-galactosidase	Colourless	Light yellow
ADH	Arginine	Arginine dihydrolase	Yellow	Bright pink
LDC	Lysine	Lysine decarboxylase		Orange
ODC	Ornithine	Ornithine decarboxylase		Red-pink
CIT	Citrate	Citrate utilisation		Blue
H2S	Na thiosulfate	H_2S production	Colourless	Black
URE	Urea	Urea hydrolysis	Yellow	Bright pink
TDA	Tryptophan	Deaminase		Dark red
IND	Tryptophan	Indole production	Colourless	Red-pink
VP	Na pyruvate	Acetoin production		Light pink
GEL	Charcoal gelatin	Gelatinase	Black deposit	Black
GLU	Glucose	Fermentation/oxidation	Blue	Yellow
MAN	Mannitol	Fermentation/oxidation		
INO	Inositol	Fermentation/oxidation		
SOR	Sorbitol	Fermentation/oxidation		
RHA	Rhamnose	Fermentation/oxidation		
SAC	Sucrose	Fermentation/oxidation		
MEL	Melibiose	Fermentation/oxidation		
ARA	Arabinose	Fermentation/oxidation		

Annex VI

Table annex VI 1. API® 50CHB tests, reactions tested and positive/negative identification of *Bacillus*.

Tests	Substrates	Negative result	Positive result
O	Control	Red	Orange
GLY	Glycerol		Yellow
ERY	Erythytrol		
DARA	D-arabinose		
LARA	L-arabinose		
RIB	Ribose		
DXYL	D- xylose		
LXYL	L-xylose		
ADO	Adonitol		
MDX	B-Methyl-D-Xyloside		
GAL	Galactose		
GLU	Glucose		
FRU	Fructose		
MNE	Mannose		
SBE	Sorbose		
RHA	Rhamnose		
DUL	Dulcitol		
INO	Inositol		
MAN	Mannitol		
SOR	Sorbitol		
MDM	α-Methyl-D-Mannoside		
MDG	α-Methyl-D-Glucoside		
NAG	N-Acetyl-Glucosamine		
AMY	Amygdalin		
ARB	Arbutin		
ESC	Esculin		Black
SAL	Salicin		Yellow
CEL	Cellobiose		
MAL	Maltose		
LAC	Lactose		
MEL	Melibiose		
SAC	Sucrose		
TRE	Trehalose		
INU	Inulin		
MLZ	Melezitose		
RAF	Raffinose		
AMD	Starch		
GLYG	Glycogen		
XLT	Xylitol		
GEN	Gentobiose		
TUR	D-Turanose		
LYX	D-Lyxose		

TAG	D-Tagatose		
DFUC	D-Fucose		
LFUC	L-Fucose		
DARL	D-Arabitol		
LARL	L-Arabitol		
GNT	Gluconate		
2KG	2-Keto-Gluconate		
5KG	5-Keto-Gluconate		