Characteristics, behaviour and toxigenic potential of bacterial dairy contaminants

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by

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Abstract

The impacts of foodborne bacterial pathogens on human health and the economy are a great concern for public health authorities and food manufacturers worldwide. To ensure consumer protection, knowing the characteristics, behaviour and diversity of foodborne pathogenic bacteria is an essential task for food industries. The dairy industry is an important contributor to the Australian economy, thus, product quality is critical for consumer confidence and safety. Several studies have revealed the characteristics and behaviour of some important pathogenic bacterial in milk and dairy products, but information about other important bacteria such as *Escherichia coli* and *Bacillus cereus* group is lacking. Therefore, the current study aimed to evaluate the prevalence, characteristics, and diversity of *E. coli* as a representative of gram negative, non spore forming bacteria in raw milk and *B. cereus group* as a representative of gram positive, spore-forming bacteria in raw and pasteurised milk samples in Victoria, Australia.

According to the the results obtained, *E. coli* was recovered from 90% of raw milk samples, but only 4.2% of isolates carried a Shiga toxin (*stx*) gene and can be considered pathogenic. Antibiotic susceptibility testing showed that all raw milk *E. coli* isolates were resistant to at least one antibiotic, and 16.7 % of isolates were multi-drug resistant. Most of the isolates also could form a biofilm, although most had weak biofilm-forming capacity. *B. cereus* group was also recovered from 42.3% of raw and pasteurised milk samples, with a greater level of recovery from pasteurised milk. Virulence studies identified genes *nheA*, *nheB*, *hblA* and *nheC* in most isolates and *cyk* gene in 46% of all isolates, indicating that many potentially diarrhoeal strains exist in the collected samples. Antimicrobial resistance of *B. cereus* group was considerably higher in pasteurised milk samples than raw milk samples. The biofilm forming capacity within *B. cereus* group showed that 53.7% of the isolates had the ability to form a biofilm, with a higher prevalence of biofilm producers observed in pasteurised milk isolates. However, the current study showed that peracetic acid (PAA) could be considered as an effective sanitiser against both bacteria. However, greater concentrations of PAA were required to be effective against *B. cereus* group isolates. It was observed that resistance to PAA increased with greater biofilm formation ability which may be a challenge for maintaining hygienic conditions during dairy production. Overall, the virulence and antimicrobial resistance characteristics, together with the ability of isolates to produce biofilm, clearly indicate the importance of *B. cereus* group in the Australian dairy industry.

In the current study, the capability of two commercially available MALDI-TOF MS systems as rapid detection techniques was evaluated. Results indicated that MALDI Biotyper® displayed greater accuracy

than Vitek® MS for both *E. coli* and *B. cereus* group isolates. The difference between the identification level of these instruments could be due to different reference spectral profiles of food bacteria in their databases.

The diversity of isolates was evaluated using genetic (ERIC-PCR), proteomic (MALDI-TOF MS) and chemical (FT-IR) methods. Analysis of genetic profiles generated by ERIC-PCR showed that *E. coli* isolates could be divided into five major clusters. All three STEC isolates belong to the same ERIC-PCR cluster. The clusters showed a strong correlation with antimicrobial resistance phenotypes and biofilm formation ability of *E. coli* isolates. ERIC-PCR also showed a correlation between clusters and source of *E. coli* Isolates. ERIC-PCR placed most *B. cereus* group isolates from pasteurised milk in the same cluster, indicating that they probably originated from a similar source. In contrast, the raw milk isolates showed greater diversity indicating various sources. Overall results showed that ERIC-PCR could discriminate *E. coli* and *B. cereus* group isolates based on their characteristics and origin with acceptable discriminatory power.

In the current study, a low agreement was observed between MALDI-TOF MS clusters and ERIC-PCR clusters for *E. coli* and *B. cereus* group isolates. In contrast, chemical profiling with the new IR Biotyper®, which uses FT-IR spectroscopy technique to classify the isolates, showed high agreement with ERIC-PCR profiling. Moreover, results did not show a strong correlation between FT-IR and MALDI-TOF clusters. The present study showed that FT-IR typing has greater capability than MALDI-TOF MS for typing and source tracking, and FT-IR could be adopted as a rapid and low-cost tool for the typing of these bacteria. However, genomics methods like ERIC-PCR provide greater discrimination and are the preferred typing method.

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Declaration

I hereby declare, that to the best of my knowledge, this thesis contains neither material which has been accepted for the award of any other degree or diploma, or any material previously published or written by another person, except where due reference is made in the text of the thesis.

Behrad Radmehr

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List of Abbreviations:

AFLP	Amplified fragment length polymorphism
AIEC	Adherent Invasive *E. coli*
ANOVA	Analysis of variance
ARE	Antibiotic resistant bacteria
ATCC	American Type Culture collection
ATP	Adenosin three phosphate
BHI	Brain heart infusion
BPW	Buffered peptone water
CFU	Colony forming units
CHCA	α-cyano-4-hydroxy cinnamic acid
HCCA	α-cyano-4-hydroxy cinnamic acid
dH2O	Distilled water
DAEC	Diffusely Adherent *E. coli*
Da	Dalton
DMSO	Dimethyl sulphoxide
DNA	Deoxy ribosenucleic acid
EAEC	Enteroaggregative *E. coli*
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ERIC	Enterobacterial repetitive intergenic consensus sequence
ETEC	Entrotoxigenic *E. coli*
ExPEC	Extraintestinal Pathogenic *E. coli*
FSANZ	Food Standards Australia and New Zealand
FTIR	Fourier transform infrared
GMPs	Good manufacturing practices

HACCP	Hazard analysis and critical control points
Kbp	Kilo base pairs
LAMP	Loop-mediated isothermal amplification
MALDI	Matrix assisted laser desorption/ionisation
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
MLVA	Multilocus variable number of tandem repeat analysis
MS	Mass Spectrometry
NGS	Next Generation Sequencing
OD	Optical density
PBS	Phosphate buffered saline
PAA	Peracetic acid
PCA	Principal component analysis
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
rpm	Revolutions per minute
RAPD	Random amplified polymorphic DNA
REP	Repetitive extragenic palindromic
RTE	Ready-to-eat
SARAMIS	Spectral ARchiving and Microbial Identification System
SD	Standard Deviation
SPC	Standard plate count
STEC	Shiga Toxin–producing *E. coli*
TLC	Thin layer chromathography
TOF	Time of flight
TSB	Tryptone soya broth
UHT	Ultra high temperature
WGS	Whole genome sequencing

Chapter 1

Literature Review

1. Literature review

1.1 Importance of milk and dairy products

The connection between food, nutrition, and health is well proven. Recent studies have shown that nutritional factors seem to have a more significant impact on human health than previously anticipated. In current medicine, prevention is as important as the treatment of diseases. Nowadays, consumers are highly aware of the health properties of food, and the market for nutritious food and food with extra health benefits is growing (Haug, Hostmark & Harstad, 2007; Rozenberg et al., 2016).

Milk and dairy products have a long history in human nutrition. They have been an important part of the human diet for over 8000 years and are part of the formal nutritional recommendations in many countries globally. These products are beneficial foods that deliver many nutritional advantages (Jenness, 1988; Rozenberg et al., 2016).

Milk is a very complex food containing essential nutritional components, which may positively affect health. Milk contains the nutrients required for the growth and development of the newborn animal and is a source of proteins, amino acids, lipids, vitamins and minerals. It contains essential growth factors, immunoglobulins, hormones, peptides, polyamines, enzymes and many trace nutrients. The proteins are in colloidal dispersals as micelles. The casein micelles are present as colloidal complexes of salts and protein, mainly calcium. The milk lipids are emulsified in globules covered with membranes. Lactose and most minerals are in soluble form (Haug et al., 2007; Jenness, 1988; Nickerson, 1995).

The quantities of the main milk ingredients vary depending on the specific animal, animal breed, age, stage of lactation and health status. Herd management practices and environmental conditions similarly influence milk composition. The average composition of cow's milk is shown in Table 1.1. Milk structure has a dynamic nature, and the composition varies with the period of lactation, nutrition, energy balance, age, breed, and animal health condition. The variation in milk ingredients during lactation seems to match the changing requirement of the growing newborn, providing different amounts of components essential for infant health, growth and development. Particular milk proteins are involved in the initial development of immune response, while others like lactoferrin take part in the non-immunological defence. Milk contains many diverse types of fatty acids. These components make milk and dairy products a rich nutrient food item (Haug et al., 2007; Nickerson, 1995; Rozenberg et al., 2016).

Principally, milk products are rich in calcium, protein, potassium and phosphorus. They provide around 52–65 % of the recommended calcium intake and 20–28 % of the protein requirement, subject to the consumer's age. The contribution of dairy products to the recommended calcium intake has driven the dietary recommendations for dairy consumption in most nutritional guidelines. More than 60% of the population's calcium intake in developed countries is supplied by dairy products (Jenness, 1988; Nickerson, 1995; Rozenberg et al., 2016).

Table 1.1 Composition of cow's milk main ingredients (Jenness et al., 1988)

Main ingredient	**Range (%)**	**Mean (%)**
Water	85.5 – 89.5	87.0
Total solids	10.5 – 14.5	13.0
Fat	2.5 – 6.0	4.0
Proteins	2.9 – 5.0	3.4
Lactose	3.6 – 5.5	4.8
Minerals	0.6 – 0.9	0.8

In addition to the health benefits, the food and dairy industries are important contributors to the economies of many countries. World dairy production has passed 800 million tonnes of milk annually and there is an increasing worldwide demand for milk and other dairy products. Global competitiveness has led to improvement in the quality of dairy products and increased product shelf life. A very sharp rise in world dairy prices was driven, primarily, by the huge global demand for dairy products. In recent years, trade has also been facilitated by refrigeration and transportation technologies. The international dairy trade has been controlled for many years by the European Union, New Zealand and Australia. In particular, Australia and New Zealand, with their low-cost dairy production and dynamic international marketing, are prominent producers of cheese and milk powder to Asia-Pacific markets (More, 2009; Sheng, Chancellor & Jackson, 2020).

In Australia, the food industry is one of the main sectors in terms of economic revenue. The dairy industry is a significant sector of the Australian food industry, producing more than 9 billion litres of milk annually, half of which is exported to the international market. The

Australian dairy industry consists of a large number of diverse farming systems with farmers striving to produce the best quality milk products. An understanding of international competitors, and of global developments in milk quality, will play an important role in the efforts of the dairy industry to strategically plan for future market. Therefore, the Australian dairy industry continues to invest in technology, quality programs and resources to maintain and improve milk quality (Bethune & Armstrong, 2004; Pagotto & Halog, 2016; Sheng et al., 2020).

1.2 Milk microbiology

Besides being a nutritious food for humans, milk provides a favourable environment for the growth of ranges of microorganisms. A broad spectrum of bacteria, yeasts and moulds can transfer to and grow in milk and dairy products. Microbes can enter milk through the udder, feedstuffs, milk handling equipment and processing facilities. Microorganisms that get into the milk can increase in number quickly. Thus, it is more effective to minimise contamination from microorganisms than to control microbial growth once they have entered the milk. Bacteria that are usually associated with milk and milk products are given in Table 1.2.

Table 1.2. Bacterial types are commonly associated with milk and milk products (Lorenzo et al., 2018)

Microorganism	**Role**
Bacillus cereus group.	Pathogenic and spoilage
Brucella	Pathogenic
Carnobacterium spp.	Pathogenic and spoilage
Clostridia	Pathogenic and spoilage
Enterobacteriaceae	Pathogenic and spoilage
Lactobacillus spp.	Spoilage
Lactococcus spp.	Spoilage
Listeria spp.	Pathogenic
Mycobacterium	Pathogenic
Pseudomonas	Spoilage
Staphylococcus	Pathogenic
Streptococcus	Pathogenic and spoilage

The initial microbiota of raw milk shows microbial contamination during production in the farm environment. When milk leaves the farm, the microbiota is affected by the temperature at which the milk has been chilled and stored. The initial bacterial count of raw milk may range from less than 1000 cfu/ml to 10^6 cfu/ml. High microbial counts are evidence of inadequate production hygiene. Milk microbial growth can be controlled by cooling the milk. Most microorganisms grow slower in colder environments. Raw milk leaves the cow's udder at around 38°C. Bacteria multiply rapidly in warm milk, and milk quickly becomes sour if it is held at these temperatures. If the milk is not cooled, an average air temperature of 16°C means that the milk temperature will have only decreased to 28°C after three hours. Cooling the milk container with running water can help to reduce the temperature to 16°C after one hour. At lower temperatures, microbial growth will be reduced and enzyme activity delayed. Therefore, milk will keep longer if cooled (Marth & Steele, 2001; Porcellato et al., 2018; Robinson, 2005). Microbial contamination in the dairy production chain can also occur during transportation and in the processing facilities. Thus, the composition of the raw and treated milk microbiota is significantly affected by several factors, such as farm management practices, hygienic practices and storage conditions throughout the production chain. However, naturally soured milk is used to produce several dairy products like sour cream, yoghurt, buttermilk and cheese. These products historically provide methods of milk preservation and provide pleasant foods to consume produced through the process of fermentation (Porcellato et al., 2018; Robinson, 2005).

Knowing the actual bacterial community in milk and milk products throughout the production chain will ensure the production of high quality and safe products. To avoid milk spoilage and ensure safe production, the dairy industry uses thermal treatment to remove pathogenic bacteria and reduce the milk microbial load. The most used thermal treatments in dairy production are pasteurisation and sterilisation. Pasteurisation is a treatment used to destroy and decrease the microbial count in food products. In milk pasteurisation, milk is heated to a temperature necessary to kill all pathogenic and some non-pathogenic bacteria, below the milk's boiling point. Pasteurisation, by killing many non-pathogenic microorganisms, extends the storage stability and shelf life of milk. However, some spore-formers and heat-resistant bacteria can endure this treatment. Storage of processed milk and dairy products at low temperatures is also essential to minimize microbial growth and to decrease enzymatic activities, thus maintaining product shelf life (Nickerson, 1995; Sukumar, 1980).

The most common temperature for milk pasteurisation is 72°C for 15 seconds followed by prompt cooling stage to below 4°C. This is generally named as High Temperature Short Time

(HTST) treatment. Milk is usually pasteurised prior to dispatch for sale as liquid milk. Pasteurisation may has a minor effect on the nutritional value of milk as the main nutrients are not changed. There is some loss of vitamin, but this is not significant. This process kills many fermentative organisms as well as pathogens (Chandan, Kilara & Shah, 2009; Robinson, 2005). Another method of milk heat treatment is sterilisation. In this method, milk is subjected to high heat treatment to ensure almost the whole destruction of the milk microbial population. This product is then considered to be commercially sterile. In conventional treatments, a temperature above 100°C is used for 15 to 40 minutes. Another method of sterilisation is called ultra-heat treatment, or UHT. In this method, milk is heated under high pressure to about 140°C for 4 seconds. The product is effectively sterile. UHT preserves more of the properties of fresh milk than conventionally sterilised milk. Sterile products have a longer shelf life than pasteurised milk (Robinson, 2005; Sukumar, 1980).

1.3 Foodborne pathogens

1.3.1 Foodborne pathogens and public health implications

As living standards develop, concerns about food safety and probable contamination of foods will continue to be an important issue. Foodborne diseases have been recognized for over 1000 years and more than 200 different food-borne diseases have been identified (Bintsis, 2017; Fung, Wang & Menon, 2018). Foodborne pathogens continue to have a high prevalence with significant impacts on health and the economy. Foodborne disease occurs when a pathogen is consumed with food and established in the human body or when a toxigenic pathogen establishes in food and produces toxins. Consequently, foodborne illness is generally classified into foodborne infection and foodborne intoxication. (Bintsis, 2017; Martinovic, Andjelkovic, Gajdosik, Resetar & Josic, 2016; Morris, 2013). The most common food pathogenic bacteria are *Bacillus cereus, Campylobacter jejuni, Clostridium botulinum, Clostridium perfringens, Cronobacter sakazakii, Esherichia coli, Listeria monocytogenes, Salmonella* spp*., Shigella* spp*., Staphylococccus aureus, Vibrio* spp. and *Yersinia* spp. (Artursson et al., 2018; Bintsis, 2017; Christner et al., 2017; Marrollo, 2016; Morris, 2013). The implicated food vehicles were generally of animal origin, in particular eggs, meat, milk and dairy products and crustaceans (Morris, 2013; Sumner et al., 2003).

1.3.2 Foodborne pathogens in milk and the dairy industry

The dairy industry is an important part of the global food business, which plays a significant role in the sustainability of the economy of many countries. Besides dairy production, dairy producers must ensure that processing meets the expected quality and customer expectations. Thus, quality issues affecting products will need to be assessed to maintain product quality and safety (Gopal et al., 2015; Oliver, Jayarao & Almeida, 2005; Porcellato et al., 2018).

Milk and dairy products can transfer a range of microorganisms and can be significant sources of foodborne pathogens. The presence of foodborne pathogens in raw milk can be due to direct contact with contaminated sources in the dairy farm environment (Gopal et al., 2015; Oliver et al., 2005). Pasteurisation reduces the number of bacteria in milk but a number of factors, like the consumption of raw milk or consumption of several types of cheeses manufactured from raw milk, and the presence of foodborne pathogens through contaminated raw milk into the dairy food processing chain, can lead to the persistence of these pathogens in biofilms, and following contamination of processed milk products, pasteurisation may not be effective in destroying all foodborne pathogens (Artursson et al., 2018; Ombarak et al., 2016). Moreover, pathogens such as *Listeria monocytogenes* can survive cleaning procedures in the processing environment, leading to recontamination of dairy products. These ways can increase the risk of the presence of foodborne pathogens in dairy products (Baylis, 2009; Mugadza & Buys, 2017; Oliver et al., 2005).

The Australian dairy industry has developed a reputation for the production of safe, high quality products that are favourably preferred around the world. These industries have introduced high standard safety programs that ensure the products meet all requirements of food safety. Of the ten most important pathogenic bacteria relevant to food safety according to the Department of Health of the Australian government, many, including *E. coli, L. monocytogenes, S. aureus* and *B. cereus*, are very important to the dairy industry. There is some information available about *L. monocytogenes* and *S. aureus* in the dairy sector, but information about *E.coli* (representing Gram negative bacteria) and *B. cereus group* (representing Gram positive and spore-forming bacteria) is lacking in the Australian dairy industry (McAuley et al., 2014). Because of significant impact of dairy pathogens on human health and the economy, the ability to implement epidemiological investigations to define the primary sources of bacterial contamination is essential to improve food safety and public health in dairy industry.

1.4 *Escherichia coli*

1.4.1 Bacterial characterization

Escherichia coli belongs to the family *Enterobacteriaceae,* which consists of rod-shaped, gram negative, facultative anaerobic, non-sporeforming bacteria. This group includes the foodborne pathogens, *Cronobacter* spp., *Salmonella enterica*, *Shigella* spp., *Yersinia* spp. and *Escherichia coli* pathotypes (Keane, 2016; Smith & Fratamico, 2016).

Escherichia is a type genus of the *Enterobacteriaceae* family, and *E. coli* is the type species of the genus. *E. coli* are catalase-positive, oxidase-negative, fermentative, short rod, gram-negative, non-sporeforming bacteria. *E. coli* is closely related to the genus *Shigella*, although the former can ferment lactose and is more active biochemically (Adams & Moss, 2008; Baylis, 2009). The gastrointestinal tract of humans and animals is a common source of *E. coli*. It can also be isolated from water, soil, food and animal feed as a result of faecal contamination (Smith & Fratamico, 2016). Its frequent presence in faeces; simple isolation methods, the mostly non-pathogenic character and survival features in water led to the establishment of *E. coli* as an indicator of faecal contamination and the possible incidence of enteric pathogens in food and water. Thus, *E.coli* is a typical indicator of potential faecal contamination of food and water (Adams & Moss, 2008; Baylis, 2009).

E. coli are divided into four groups regarding phylogenetic typing: A (A0 and A1), B1, B2 (B21 and B22), and D (D1 and D2). It is well accepted that extraintestinal pathogenic strains mainly belong to groups B2 and, to a minor extent, D and have numerous virulence genes that are lacking in commensal strains (Escobar-Páramo et al., 2006). *E. coli* strains are also classified based on the O antigen in the lipopolysaccharide on the outer envelope of the cell, the flagellar H-antigen, and the capsular K-antigen. Most microbiology laboratories do not have the capability to type K-antigens, thus serotyping using O- and H-antigen specific antibodies has been used as the gold standard for typing *E. coli.* There are more than 185 identified O-groups and 53 different H-groups. The combination of O- and H-antigen describes the *E. coli* serotype (Nandanwar et al., 2014; Smith & Fratamico, 2017). Most *E. coli* serotypes are harmless, but some serotypes have different virulence genes, and thus they cause various types of disease (Smith & Fratamico, 2017).

1.4.2 *E. coli* pathogenesis and foodborne disease

E. coli are universal inhabitants of the intestinal tract of humans and other warm-blooded animals. Many *E. coli* strains are harmless, but they can be opportunistic pathogens causing a variety of infections (Awadallah et al., 2016; Smith & Fratamico, 2016). The pathogenic strains can be divided into diarrhoea-inducing strains and strains that reside in the intestines but only cause disease in biological sites outside of the gastrointestinal tract, the so-called extraintestinal pathogenic *E. coli* (Adams & Moss, 2007; Smith & Fratamico, 2017).

Based on pathogenic phenotypes and the diseases that *E. coli* cause, they have been classified into eight groups: Enteropathogenic (EPEC), Enterotoxigenic (ETEC), Enteroinvasive (EIEC), Enteroaggregative (EAEC), Diffusely adherent (DAEC), and Shiga toxin producing (STEC) and enterohemorrhagic (EHEC) (Morris, 2013; Smith & Fratamico, 2017).

Various virulence factors are known in *E. coli*, including toxins, invasins, adhesions, capsule production, iron scavenging and resistance to serum complement. *E. coli* strains with a combination of some of these virulence factors may cause disease. All these factors help bacteria to fight against the host's immune system. Hence, the treatment might be problematic due to the pathogen's virulence, especially if they are resistant to routine antibiotics (Fernandes et al., 2011; Kaipainen et al., 2002).

1.4.2.1 *E. coli* pathotypes and disease

1.4.2.1.1 Entropathogenic *E. coli* (EPEC)

EPEC infections are a leading cause of diarrhoea, vomiting, and fever in infants and children in developing countries. Diarrhoea may be persistent and potentially fatal. Both EPEC and Shiga toxin producing *E. coli* (STEC) have the Locus of Enterocyte Effacement (LEE) pathogenicity island, but EPEC does not produce Shiga toxin (Stx) (Huasai et al., 2012; Rey et al., 2006). Attaching and Effacing (A/E) lesions in the gut mucosa associated with microvilli and intimate attachment of host cells and bacteria are characteristic of EPEC like STEC. A/E lesion formation in EPEC depends on LEE island genes encoding the type III secretory system, T3SS, which allows close attachment between the bacteria and the gut cells. However, it is not completely clear how EPEC produces a diarrheic response (Bintsis, 2017; Smith & Fratamico, 2017).

EPEC infections occur via the fecal-oral route, mostly in children less than two years old. EPEC strains are a major cause of infantile diarrhoea in developing countries, but they are rare in developed countries. The disease causes severe watery stool with a large amount of mucus but

is infrequently bloody. Disease treatment involves rehydration therapy. Humans are the reservoir for typical EPEC, whereas humans and animals are the reservoirs for atypical EPEC (Bintsis, 2017; Smith & Fratamico, 2017). However, the serotypes of animal origin are not usually those that cause human illness. Foods like raw meats or vegetables contaminated by food handlers are associated with EPEC diarrhoea. The infections are usually sporadic and the specific food vehicles are often unidentified. However, consumption of any food or water exposed to infected human faeces may cause diarrhoea (Escobar-Páramo et al., 2006; Smith & Fratamico, 2017).

1.4.2.1.2 Entrotoxigenic *E. coli* (ETEC)

ETEC induce a mild self-limiting or sometimes severe cholera-like watery diarrhoea. Children in undeveloped countries or regions with poor hygiene and unsuitable water are vulnerable to ETEC infections, which cause remarkable morbidity and mortality (Smith & Fratamico, 2017). Moreover, this organism is a leading cause of traveller's diarrhoea for visitors to developing countries. Toxins produced by ETEC induce non-inflammatory watery diarrhoea without blood, mucus and leukocytes. If the diarrhoea continues, rehydration therapy is necessary (Bintsis, 2017; Sabaté et al., 2008).

ETEC colonise the epithelial surface of the small intestine by using adhesins and colonisation factors, and diarrhoea is through the action of plasmid-borne enterotoxins. Colonisation factors can be fimbrial, nonfimbrial, helical or fibrillar. Both colonisation factors and flagella help the initial attachment of ETEC to the cells, but the intimate attachment is caused by the Tia and TibA outer membrane proteins (Smith & Fratamico, 2017).

ETEC has two types of plasmid-encoded diarrhoea inducing enterotoxins: heat-labile enterotoxins (LT-1, LT-2) and heat-stable enterotoxins (STa, STb). LT-1 (designated as LT) and STa (designated as ST) are associated with human disease. ETEC strains may produce LT, ST or both (Donnenberg, 2014; Smith & Fratamico, 2017).

Humans are the reservoir of ETEC, and consumption of contaminated food or water is often the probable cause of illness. Foods associated with infections include cheese, turkey, crab, deli foods, mayonnaise, salads and fresh vegetables. The infective dose is very high (10^6 to 10^8 organisms). Remarkably, newborns and young animals, including calves, lambs, and piglets, are also susceptible to ETEC diarrhoea, resulting in economic problems to the livestock industry (Smith & Fratamico, 2017; Suojala et al., 2011).

1.4.2.1.3 Shiga Toxin–producing *E. coli* (STEC)

STEC is also known as enterohemorrhagic *E. coli* (EHEC) when associated with severe illness and hemolytic-uremic syndrome (HUS). STEC serotype O157:H7 and certain non-O157 STEC serogroups are major foodborne pathogens that cause diarrhoea, haemorrhagic colitis (HC), HUS, and death after severe disease (Awadallah et al., 2016; Rey et al., 2006; Zhang et al., 2017). Watery diarrhoea and abdominal cramps are initial symptoms of STEC infection. Almost 90% of STEC infections result in HC, 5–15% of the HC cases develop to HUS, and 5–15% of the HUS cases result in death. Patients with HUS may suffer long-term consequences affecting the renal system, gastrointestinal tract, cardiovascular system, or central nervous system. Most HUS patients recover without major consequences; however, renal failure can occur in a small number of patients who will require long-term dialysis or a kidney transplant (Donnenberg, 2014; Smith & Fratamico, 2017).

The infective dose for STEC O157:H7 is in the range of 10–100 cells; however, the dose for some non-O157 STEC may be higher and may be related to their level of acid sensitivity. The most susceptible people to the complications due to HUS are the very young and the elderly. The most crucial virulence factor in O157 and non-O157 STEC is a bacteriophage-encoded cytotoxin which is similar to the Shiga toxin produced by *Shigella dysenteriae*. STEC produce two closely related Shiga toxins, Stx1 and Stx2; Stx2 has approximately 60% sequence homology to Stx1. There is only a single amino acid difference between the Shiga toxin of *S. dysenteriae* and Stx1 of *E. coli* (Li et al., 2016; Smith & Fratamico, 2017).

After ingestion, STEC adhere to the intestinal mucosa and secrete Stx, which enters the bloodstream. Toxin injury to the endothelial cells results in the activation of platelets and leukocytes, leading to the formation of fibrin thrombi in the kidney capillaries. Narrowing of the capillary lumina leads to a reduced blood supply to glomeruli with loss of kidney function resulting in HUS. HUS is characterised by haemolytic anemia that is caused by the destruction of red blood cells, acute renal failure (uremia), and low platelet number in the blood (thrombocytopenia). Moreover, STEC has mechanisms to resist the extremely acidic conditions encountered during bacterial transit through the acid stomach and the mild acid conditions of the gut. The acid resistance systems give STEC the ability to survive and grow in acidic environments such as acidified or fermented foods and survive in the host during passage through the acidic gastrointestinal tract (Smith & Fratamico, 2016; Smith & Fratamico, 2017).

STEC are spread via the fecal-oral route. The most important reservoir for STEC is the intestinal tract of cattle. Contact with cattle and food products originating from cattle deliver major risk factors for STEC infection to humans. It is probable that all ruminants can act as reservoirs for STEC. Nonruminants, including cats, dogs, pigs, horses, rabbits, and chickens, also can carry STEC. Infected persons can transmit STEC, and STEC-containing food may result from cross-contamination or by contact from an infected food handler (Christner et al., 2017; Ombarak et al., 2016; Smith & Fratamico, 2017).

A wide range of foods inlcuding meats, dairy products, fruit, vegetables, and nuts have been associated with STEC infections. Interestingly, foodborne outbreaks are more commonly associated with STEC O157:H7, whereas non-O157 STEC is more often associated with sporadic cases (Bintsis, 2017; Hosseini et al., 2013; Rey et al., 2006; Sumner et al., 2005). However, foodborne outbreaks caused by various serogroups or serotypes of non-O157 STEC have occurred in the United States, Europe, Japan, and Australia. Information on STEC isolates from cases in the United States, the European Union, and Australia indicates that STEC O157 was the most common cause of illness. Strict hygiene during animal slaughtering, vegetable and fruit growth and harvesting, product handling, shipping, and food preparation are the most important factors to consider in the control and prevention of STEC disease (Rugeles et al., 2010; Smith & Fratamico, 2016).

Shiga Toxin–producing Entroaggregative *E. coli* (STEAEC) is a very similar pathotype to STEC. STEAEC are not pathogenic *E. coli* with different virulence factors, rather they have combined the virulence properties of the EAEC pathotype with the Shiga toxin-producing ability of the STEC pathotype. STEAEC outbreaks are associated with a higher severity of illness. Increased virulence of STEAEC is not related to increased Stx production but rather to more efficient colonisation by aggregative adherence fimbriae, thus allowing sustained delivery of toxin into the host intestinal cells. Most cases involved middle-aged adults, and there was a predominance of illness in females (Li et al., 2016; Smith & Fratamico, 2017).

1.4.2.1.4 Enteroinvasive *E. coli* (EIEC)

EIEC cause invasive inflammatory colitis with profuse watery diarrhoea. EIEC are intracellular pathogens that are less similar to other types of *E. coli* and physiologically and taxonomically more related to *Shigella* spp. EIEC do not produce Stx (Michelacci et al., 2016; Parsot, 2005). The O-antigen of many EIEC serogroups is antigenically similar to *Shigella* serovars. The site of infection of both EIEC and *Shigella* is the colonic mucosa. EIEC strains may carry the *sen*

gene, which encodes *Shigella* enterotoxin-2 (ShET- 2) and may cause inflammation of intestinal epithelial cells (Michelacci et al., 2016; Smith & Fratamico, 2017).

When EIEC are ingested in contaminated food or water and spread to the colon, the pathogen accesses the colonic submucosa through M cells and is taken up by macrophages. The bacterial cells escape from the macrophages and enter and replicate in colonocytes. While most individuals infected by EIEC develop watery diarrhoea, a few people show symptoms similar to *Shigella dysenteriae*: abdominal cramps with blood, fever, mucus and white blood cells in the stool, and tenesmus. EIEC infections are most probable with foods contaminated by infected food handlers (Michelacci et al., 2016; Ombarak et al., 2016). Outbreaks associated with EIEC in several countries have been associated with water, cheese, vegetables, and potatoes. Treatments recommended for EIEC infection are rehydration and antibiotic therapy. Humans are the only identified reservoir of EIEC; therefore, most foodborne infections are due to foods prepared by individuals infected with the pathogen (Michelacci et al., 2016; Ombarak et al., 2016; Smith & Fratamico, 2017).

1.4.2.1.5 Enteroaggregative *E. coli* (EAEC)

EAEC strains cause watery diarrhoea that can be associated with mucus or blood. Diarrhoea may be of long duration. There are three stages in EAEC pathogenesis: initial adherence of bacteria to the intestinal mucosa, increased mucus production causing a thick bacterial biofilm on the mucosal surface, and production of toxins that damage the mucosa and induce intestinal secretion (Choi et al., 2001; Nataro & Steiner, 2002; Seo et al., 2017).

The main feature of EAEC virulence is colonisation of the intestinal mucosa by mucoid biofilm formation, amplification of enterotoxins, and mucosal inflammation. The virulence plasmid pAA encodes the AAF fimbriae essential for adhesion. Adhesion is followed by the secretion of PET and EAST1 toxins. PET upsets the actin cytoskeleton and induces epithelial cell rounding. The EAST1 enterotoxin is a plasmid-mediated toxin that shows *in vitro* properties like the heat-stable toxin of enterotoxigenic *E. coli* (ETEC). Some EAEC strains produce the *Shigella* enterotoxin ShET1, but it is not clear that the enterotoxins found in EAEC strains have a role in inducing diarrhoea (Nataro, Steiner, & Guerrant, 1998; Seo et al., 2017).

Children and immunocompromised people living in both developed and undeveloped countries are susceptible to EAEC diarrhoea. EAEC strains are a significant cause of travellers' diarrhoea. Infected food handlers are the most likely source of the pathogen, but the reservoir

of EAEC is still unknown (Choi et al., 2001; Nataro & Steiner, 2002; Smith & Fratamico, 2017).

1.4.2.1.6 Diffusely Adherent *E. coli* (DAEC)

The term DAEC comes from the bacteria's ability to diffusely attach to cell surfaces; "diffuse adherence" describes the pattern of cell attachment in which bacteria cover the entire surface of cells, whereas "localised adherence" is bacterial attachment as microcolonies to only a few sites on the cell surface (Nataro & Steiner, 2002a; Ombarak et al., 2016).
DAEC is a poorly characterised and heterogeneous population of *E. coli* that colonise the small intestine and may be responsible for watery diarrhoea in children under five years. A secreted autotransporter toxin (SAT) has been verified in DAEC strains associated with diarrhoea. SAT induces lesions on tight junctions of epithelial cells, causing increased cellular permeability and may be a virulence mechanism involved in the induction of watery diarrhoea (Nataro & Steiner, 2002a; Smith & Fratamico, 2017).

1.4.2.1.7 Adherent Invasive *E. coli* (AIEC)

AIEC strains recovered from Crohn's disease (CD) lesions and able to adhere to and invade cultured intestinal epithelial cells. AIEC has been involved in inflammatory bowel diseases (IBD) like CD and ulcerative colitis (UC). However, a single causative agent has not been positively identified for IBD, and the disease may be caused by a combination of human genetics, environmental factors, intestinal microbiota, and enteric pathogens, including *Mycobacterium paratuberculosis*, *Campylobacter* spp., and AIEC. At present, AIEC is poorly defined as disease agents and may only be found in the intestinal tracts of patients with IBD (Michelacci et al., 2016; Parsot, 2005; Smith & Fratamico, 2016).

1.4.2.1.8 Extraintestinal Pathogenic *E. coli* (ExPEC)

ExPEC are found in the intestine, but they do not induce diarrhoea and cause illness outside of the gut. ExPEC are facultative pathogens that reside in the gut and behave as harmless commensal organisms until they escape the intestinal tract and invade other areas of the body (Martinovic et al., 2016; Smith & Fratamico, 2017). The fatality rates for ExPEC infections are increasing globally due to increased resistance to antibiotics in ExPEC, resulting in an enormous human public health problem. It is estimated that ExPEC causes more than 40,000 deaths yearly in the United States linked to sepsis related to urinary tract infections (UTIs),

which is more than the total deaths associated with *Salmonella*, *Campylobacter*, and *E. coli* O157:H7 combined. In general, the intestinal pathogenic *E. coli* have well-defined virulence traits, whereas ExPEC strains are very diverse and have few common virulence factors among them. The definition of ExPEC by virulence properties is complicated. Molecular epidemiological techniques cannot differentiate ExPEC and commensal nonpathogenic *E. coli*, and, therefore, there is a limited understanding of the biology of ExPEC (Nandanwar et al., 2014; Smith, Fratamico, & Gunther, 2007).

Diseases caused by ExPEC include UTIs, neonatal meningitis, necrotising fasciitis, pneumonia, surgical site infections, sepsis and infections in other extraintestinal locations. Additionally, ExPEC strains cause extraintestinal infections in domestic animals and pets. ExPEC that cause disease in poultry are known as Avian Pathogenic *E. coli* (APEC). Many virulence factors are common in human and animal ExPEC, indicating that the organisms are potential zoonotic pathogens. It is probable that animals, especially chickens, act as reservoirs for human ExPEC strains. ExPEC infection is expensive regarding medical costs and productivity losses for humans, as well as for farm animals. ExPEC strains have been isolated from food products, in particular from retail raw meats and poultry, indicating that these *E. coli* strains may represent a new class of foodborne pathogens (Nandanwar et al., 2014; Smith & Fratamico, 2017; Smith et al., 2007).

1.4.3 Detection of pathogenic *E. coli*

Conventional methods for *E. coli* detection involve enrichment of the sample to allow the target *E. coli* to grow to necessary levels for detection, and this is continued by isolation on selective and differential agars, followed by confirmation of the isolates using confirmation methods, such as biochemical tests and serotyping, are necessary to support in the identification (Ntuli, Njage, & Buys, 2016; Samet-Bali et al., 2013; Vivek Prabhu, 2010). The sensitivity of methods is insufficient to detect pathogens in foods and other types of samples without an enrichment step in a selective or non-selective growth medium. The medium used for enrichment will vary depending on the type of sample being analysed (Smith & Fratamico, 2017; Zhang et al., 2017a).

E. coli isolates are generally identified biochemically; in addition, methods such as PCR are used to test for virulence factors associated with the respective pathogenic group. Unlike conventional culture-based methods, rapid methods use molecular techniques or immunoassays to detect genes or antigens that are specific for the *E. coli* pathogroup of interest.

The use of rapid methods often requires sample preparation to remove sample components that may inhibit assay performance (Clermont, Bonacorsi, & Bingen, 2000; Rugeles et al., 2010; Santos et al., 2015). For example, prior to PCR, the use of a kit or reagent to extract the DNA from the sample is required. Other than kits for detection of STEC and detection of ETEC toxins, there are very few commercially available methods that have been developed for detecting pathogenic *E. coli* groups in foods. However, PCR-based assays targeting genes linked to different pathogroups have been described and can be used to detect specific *E. coli* groups (Awadallah et al., 2016; Rey et al., 2006; Wang et al., 2017).

There are a number of commercial kits available for use by the food industry for testing *E. coli* O157 and other non-O157 STEC. Non-O157 STEC strains are a heterogeneous group that do not share biochemical phenotypes, except for the production of Shiga toxin, which can be used for detection of specific serogroups, and it is difficult to differentiate them from nonpathogenic *E. coli* on available culture media (Enriquez-Gomez et al., 2017; Gundogan & Avci, 2014; Zhang et al., 2017). Thus, a combination of assays must be used for their detection and identification. The method described for testing for these pathogens involves enrichment of the food sample, followed by extraction of genomic DNA that is used in PCR-based screening assays. More research is needed in the development of rapid methods that can differentiate between the pathogenic and other types of *E. coli* (Smith & Fratamico, 2017; Zhang et al., 2016).

The development of methods for the detection and identification of pathogenic *E. coli* requires knowledge of their phenotypic and genotypic characteristics. There are phenotypic and genetic differences among pathogenic *E. coli*, specifically, the virulence genes they possess. These variations can be the subject of detection and identification of specific pathogroups (Paauw et al., 2015; Zhang et al., 2017). Conventional methods for *E. coli* detection involve enrichment of the sample to allow the target *E. coli* to grow to necessary levels for detection, and this is followed by isolation on selective and differential agars, followed by confirmation of the isolates (Smith & Fratamico, 2017; Zhang et al., 2017). *E. coli* isolates are generally identified biochemically; in addition, methods such as PCR are used to test for virulence factors associated with the respective pathogenic group. PCR-based assays targeting genes linked to different pathogroups have been described and can be used to detect specific *E. coli* groups (Kaipainen et al., 2002; Suojala et al., 2011).

E. coli detection and identification using mass spectrometry techniques are relatively new, but Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) is already accepted as a reliable diagnostic method that is increasingly used, particularly

in clinical microbiology (Santos et al., 2015; Weiss et al., 2019). Some research confirmed that the MALDI-TOF MS is capable of identifying *E. coli* isolates from pathotypes to the species level and, additionally, MALDI-TOF MS with developed evaluation criteria is effective for differentiating most pathotypes. Conversely, many studies have stated that the accuracy of MALDI-TOF MS is limited in *E. coli* and *Shigella* species. Therefore, traditional methods, such as biochemical tests and serotyping, are still necessary to support the identification (Clark et al., 2013; Feng et al., 2020; Ojima-Kato et al., 2014). Other newly developed methods, such as Fourier transform infrared (FT-IR) spectroscopy, are also used for *E. coli* typing. Some studies indicate that FT-IR is a better technique for typing *E. coli* than MALDI-TOF MS, but the bacterial typing accuracy was significantly improved by combining the data from MALDI-TOF MS and FT-IR spectroscopy. These studeis suggest FT-IR could be used to complement MALDI-TOF MS for the identification and typing of microorganisms (Feng et al., 2020; Martak et al., 2019; Wenning et al., 2014).

1.5 *Bacillus cereus* group

1.5.1 Bacterial characterization

The *Bacillus* genus is a part of the *Bacillaceae* family, and is probably the oldest and the most diverse genus of bacteria. *Bacillus cereus* sensu lato, commonly known *Bacillus* cereus group, contains at least eight species: *Bacillus cereus* (*sensu strico*), *Bacillus anthracis*, *Bacillus cytotoxicus, Bacillus mycoides, Bacillus pseudomycoides, Bacillus thuringiensis, Bacillus toyonensis, and Bacillus weihenstephanensis* (Gdoura-Ben Amor et al., 2018; Gopal et al., 2015; Porcellato et al., 2019) *Bacillus cereus* group members are Gram positive, aerobic and spore-forming bacteria. *Bacillus* is a highly heterogeneous group of bacteria that includes species with wide variations in phenotypes, physiological and metabolic characteristics (Adams & Moss, 2008; Granum, Arnesen, & From, 2014). Their taxonomy is very complex and has been revised in recent years. *B. cereus* group is a highly homogenous subdivision. This group includes the species *B. cereus*, *Bacillus weihenstephanensis, Bacillus anthracis, Bacillus pseudomycoides, Bacillus mycoides* and *Bacillus thuringiensis* (Abee et al., 2011; Adams & Moss, 2007). *B. weihenstephanensis* was suggested to comprise the psychrotrophic *B.cereus* group strains. Growth ability under 7°C, but not above 42°C, enables discrimination of *B. weihenstephanensis* from the other species of the *B. cereus* group (Stenfors Arnesen,, O'Sullivan, & Granum, 2007).

B. cereus is facultatively anaerobic with large vegetative cells. It grows in a wide range of temperatures from 8 to 55ºC, optimally around 28–35ºC. It produces ellipsoidal, central to subterminal spores and, as a spore-former, can be widely distributed in the environment. The spores show variable heat resistance (Adams & Moss, 2007; Minnaard et al., 2007). *B. cereus* group can be distinguished from the other *Bacillus* by positive egg-yolk lecithinase activity, ability to grow anaerobically, inability to produce acid from mannitol, positive Voges–Proskauer test and resistance to 0.001% lysozyme. The cells of *B. cereus* are typically large and reach 3–5 μm in length and 1.0–1.2 μm in width. They often make chains. While most B. cereus group are motile, non-motility is a distinguishing feature of *B. anthracis* (Griffiths & Schraft, 2017).

B.cereus group can contaminate food from the environment. The spores are present in soil and can easily contaminate raw milk by from the cow's udder. *B. cereus* attaches to solid surfaces like pipelines in the dairy processing environment. The heat-resistant spores survive pasteurisation and can cause problems in a wide range of dairy products (Li et al., 2016; Montville, 2012). No simple and reliable methods are available to discriminate pathogenic *B.cereus* group strains from non-pathogenic ones. The toxin production of *B. cereus* strains can be assessed by cell culture assays (Stenfors Arnesen,, O'Sullivan, & Granum, 2007).

B. cereus spores associated with food poisoning typically have a D95°C of approximately 24 minutes (D-value or decimal reduction time is the time required at a given condition (e.g. temperature) to kill 90% (or 1 log) of the exposed microorganisms), although a wide range of heat resistance (D95°C of 1.5–36 min) is observed. Strains involved in food poisoning may have greater heat resistance and be expected to survive cooking temperatures. Spores are also resistant to irradiation. The dose for a 90% reduction is between 1.25 and 4 kGy (Griffiths & Schraft, 2017). Spore germination occurs in the temperature range between −1°C and 59°C in laboratory media. The hydrophobic nature of spores enables them to adhere to several types of surfaces. This adhesiveness ability makes them difficult to remove during the cleaning and sanitation of food processing surfaces (Griffiths & Schraft, 2017; Marrollo, 2016).

1.5.2 Pathogenesis and foodborne disease

B. cereus group is recognised as a foodborne pathogen. The importance of *Bacillus cereus* group in food illness has been recognised since the beginning of the 20th century. In Europe, *Bacillus cereus* group is the second most common cause of food poisoning outbreaks. However, the impact of *B. cereus* group in food poisoning is underestimated as a result of the

lack of systematic surveillance (Radmehr et al., 2020; Ramarao et al., 2020; Stenfors Arnesen, O'Sullivan, & Granum, 2007).

B. cereus causes two types of disease according to their symptoms, an emetic and a diarrhoeal syndrome, which are usually mild and self-limiting problems. However, severe systemic infections have also been linked with this foodborne pathogen (Organji et al., 2015). The diverse distribution between countries may be observed for the diarrhoeal and the emetic diseases caused by *B. cereus*, which possibly reflects the different food vehicles (Griffiths & Schraft, 2017; Marrollo, 2016). In the United Kingdom and Japan, the emetic syndrome is more prevalent, whereas in Europe and North America, the diarrhoeal form seems to dominate (Marrollo, 2016). Dairy products, meat and desserts are common vehicles for the transmission of the diarrhoeal form of the illness, while rice is the main source of the emetic illness. *B. cereus* emetic toxin causes vomiting, while diarrhoea caused by enterotoxins is the main symptom of the second type of disease. In a limited number of cases, both symptoms have been seen (Granum et al., 2014; Griffiths & Schraft, 2017).

The diarrhoeal disease is supposed to represent a toxic infection caused by *B. cereus* vegetative cells that, after ingestion of spores or viable cells, produce enterotoxins in the small intestine. The disease typically presents with watery diarrhoea, abdominal pain, and sometimes nausea and vomiting (Bintsis, 2017; Griffiths & Schraft, 2017). The incubation time is 8–16 hours, although in some cases, a longer incubation period has been seen. The disease duration is usually 12–24 hours, but sometimes lasts several days. *B. cereus* group food infection symptoms are generally mild and self-limiting and would not usually require treatment (Marrollo, 2016; Zhou et al., 2008).

B. cereus group can be isolated from different sources of food and can survive many kinds of food preservation methods as there is no way to ensure the removal of all of the spores from foods. Moreover, many kinds of food may be the vehicle for *B. cereus*, such as rice, pasta, meats, spices, and sprouts (Fogele et al., 2017; Granum et al., 2014). Also, different foods are more frequently associated with either of the two types of the syndrome: the emetic type has frequently been related to fried and cooked rice, as well as pastry, pasta and noodles. In contrast, the diarrheal type is usually linked with proteinaceous foods like meat and milk products, along with sauces and vegetables, puddings and soups (Griffiths & Schraft, 2017). Control against food poisoning is only possible at preventing germination of spores in food and decreasing the probability of vegetative bacterial cell growth. Therefore, foods should be kept above 60°C or quickly chilled to less than 7°C, and be carefully reheated before use (Griffiths & Schraft, 2017; Marrollo, 2016).

Based on reports, *Bacillus cereus* group accounts for 1.4%–12% of foodborne illness outbreaks globaly, but this is likely to be an underestimate. The bacteria are capable of contaminating a wide range of food products, including rice, chicken, vegetables, spices, and dairy products. Australia's network for foodborne disease surveillance reported that 18 of the 741 outbreaks of foodborne foodborne disease that happened in Australia between 2001 and 2007 were associated with *B. cereus*. The most commonly associated food vehicles in Australian outbreaks of *B. cereus* were cooked foods with a chicken or rice component (Eglezos et al., 2010). However, foodborne *B. cereus* illness occurs and continues to increase in Australia. (Berger, 2014). *B. cereus* group were the most prevalent bacteria isolated in the dairy farm environment, although *B. cereus* did not widely contaminate raw milk. Moreover, no food outbreaks in Australia have been attributed to dairy products since 1995 (Grutsch et al., 2018; McAuley et al., 2014).

1.5.3 Detection of pathogenic *B. cereus* group

The conventional method for detection of *B. cereus* group is culture-based, for which the ISO standards provide the guidelines. For this method, several steps are needed, including sample preparation, serial dilution preparations, then plating steps using specific media. Plates needed to be incubated at 30 °C for 24 h to 48 h. After incubation, colonies have to be streaked onto the non-selective agar medium (Bintsis, 2017; Li et al., 2016; Ramarao et al., 2020). Furthermore, an additional confirmation assay is required to discriminate bacteria of the *B. cereus* group. This last step takes an extra 24 hours. So, traditional methods are time-consuming, laborious, expensive and require skilled staff to ensure the correct procedure (Eglezos et al., 2010; Gdoura-Ben Amor et al., 2018; Marrollo, 2016; Te Giffel et al., 1997).

The Australian standard method specifies a horizontal test for the detection and enumeration of presumptive *B. cereus* cells. This method involves a serial dilution of the samples and subsequent plating on *B. cereus* selective mannitol egg yolk medium agar (MYP). Incubation at 30 °C for 48 hours is required. On MYP plates, colonies of *B. cereus* group have a pink colour, surrounded by a characteristic halo shaped of pink precipitation. Testing for haemolysis activity is performed on presumptive bacterial colonies to confirm *B. cereus* group isolates (The Australian Institute of Food Safety, 2016; Berger, 2014; Eglezos et al., 2010; Fricker, Reissbrodt, & Ehling-Schulz, 2008; Vithanage et al., 2014).

Recently, alternative methods for the detection and enumeration of *B. cereus* group in food have been validated. The AFNOR methods use selective chromogenic medium, COMPASS

and BACARA, separately. *B. cereus* colonies appear green on COMPASS and orange surrounded by an opaque halo on BACARA. These selective agars stop the majority of the background microbiota and allow easy identification. Their specificity and selectivity are similar to the reference method. Moreover, they are more rapid and do not involve further confirmation (Fricker et al., 2008; Ramarao et al., 2020; Silva et al., 2018).

Molecular methods used for *B. cereus* group identification have many advantages compared to the traditional methods, such as flexibility, lower turnaround time (TAT) and cost, and higher specificity. In the two past decades, different PCR techniques have been developed for *B. cereus* group detection and typing (Chaves, Pires, & Vivoni, 2011; Merzougui et al., 2013; Porcellato et al., 2019). Some procedures, such as Pulse Field Gel Electrophoresis (PFGE) and DNA amplification fingerprinting methods like rep-PCR methods such as ERIC-PCR, have been applied to allow profiling of *B. cereus* isolates. The discriminative profiles enable sourcing and tracing of *B. cereus* contamination in food. Despite the benefits of the molecular techniques, they have some limitations: trained analysts are required to perform the assays, amplification errors can occur, or false-negative results may be acquired when using complex mediums like food samples (Gao et al., 2018; Merzougui et al., 2013; Mugadza & Buys, 2017; Porcellato et al., 2019).

In recent years, biosensors have been developed as ideal methods for the detection of foodborne pathogens. The success of these methods is generally due to their rapidity to provide results, simplicity, and the low cost and high consistency of the analysis. So far, several types of biosensors have been used for the detection of *B. cereus* group. DNA-based biosensors have had great success, as they give the possibility for selective identification of different *B. cereus* group strains (Bintsis, 2017; Ramarao et al., 2020).

MALDI-TOF MS was also shown promise as a straightforward and sensitive approach to differentiate and typing of *B. cereus* group as it does not involve complicated sample preparation. Recently, it has been proposed that MALDI-TOF MS represents an applicable method for identifying *B. cereus* group. Some recently published studies showed MALDI-TOF MS, as a proteomics-based technology, can be more informative in differentiating between species, particularly when the genetic analysis is inadequate, such is the differentiation of *B. cereus* group (Fernandez et al., 2013; Jadhav et al., 2015; Lasch et al., 2016; Welker & Moore, 2011).

Fourier-Transform Infrared (FT-IR) spectroscopy has also emerged as a potential rapid, cost-effective and straightforward method for discrimination of *B. cereus* group members. In this

method, the entire chemical and biochemical composition of bacterial cells can be obtained by the interaction of infrared light and the molecules existing in the cells, which provides species-specific features. These spectral fingerprints are exclusive for each microorganism and allow the identification and differentiation at different taxonomic levels. It was shown that assisted FT-IR spectroscopy is a suitable method for the identification of *B. cereus* group members in routine diagnostics laboratories and outbreak investigations. Moreover, FT-IR is a promising tool for investigation of *B. cereus* group in different kinds of samples requiring high throughput capabilities, such as water, soil and food environments (Bagcıoglu et al., 2019; Feng et al., 2020; Martak et al., 2019).

1.6 Rapid microbial identification and typing methods

Phenotypic characterization of bacteria is based on colony morphology, staining, microscopic evaluation and ability of growth on selective culture media. In general, these phenotypic methods involve elaborate and time-consuming culturing procedures (Forghani et al., 2015; Jadhav et al., 2015; Montville, 2012; Rohde et al., 2017). Therefore, rapid microbiological identification methods have been developed to decrease the time required to detect and identify microbes in food. Various phenotypic and molecular methods have been established for the identification of bacteria. In the last 25 years, significant progress has been made in the development of rapid bacterial detection methods, including immunological methods and molecular methods (de Koster & Brul, 2016; Fung et al., 2018; Rohde et al., 2017; Zhang et al., 2017). Although the method selected may be rapid and able to be completed within a reasonable time frame, other factors, like sample preparation, accuracy, and cost, must be considered. Many factors can affect the cost of the method, including the training of personnel, the purchase of equipment to conduct the assay, service and maintenance, disposable supplies, and reagents (Clermont et al., 2000; Montville, 2012; Ramarao et al., 2020; Rohde et al., 2017). Beyond genus and species level identification of bacteria, the diversity of isolated organisms may require them to be classified into subtypes or strains. The process of subtyping is very important for identifying virulent strains of organisms (Li, Raoult, & Fournier, 2009; Scott et al., 2002; Uyttendaele et al., 2014). Subtyping has been performed by a number of rapid methods. All of these methods must meet several criteria in order to be useful, like high differentiation power, and reproducibility, and all organisms within a species must be typeable by the method (Dinkelacker et al., 2018; Leung, Rob Mackereth, Tien, & Topp, 2004; Olive &

Bean, 1999; Vivek Prabhu, 2010). Nowadays, multiple methods are available for bacterial sub typing and source tracking and determining the distribution of pathogens isolated from food. The molecular-based typing methods available can be divided into three general categories: methods based on restriction analysis of the bacterial DNA, methods based on polymerase chain reaction (PCR) amplification of particular genetic targets, and methods based on identifying DNA sequence polymorphisms (Foleya, Lynne & Nayak, 2009; Uyttendaele et al., 2014)

1.6.1 DNA-based methods

There has been an explosion in the past two decades in the introduction of nucleic acid-based assays for the identification and differentiation of foodborne pathogens. DNA-based detection methods are well known to most scientists dealing with food microbiology (Elmerdahl Olsen, 2000; Uyttendaele et al., 2014). DNA methods such as Polymerase Chain Reaction (PCR), pulsed-field gel electrophoresis, ribotyping, plasmid typing, amplified polymorphic DNA, and restriction fragment length polymorphism are applied in the detection of various foodborne pathogens. Some of these techniques have been automated, and kits are available to facilitate the recovery of pure DNA (Figure 1.1). Nowadays, the most widely used method is PCR (Montville, 2012; Postollec et al., 2011).

1.6.1.1 Polymerase Chain Reaction (PCR)

PCR-based techniques are used increasingly in food microbiology research since they offer sensitive and specific detection of pathogens. They are the most widely used of all molecular genetic methods for detecting and identifying bacteria in foods (Elmerdahl Olsen, 2000b; Fung et al., 2018; Uyttendaele et al., 2014). The increasing use of PCR is due to its high sensitivity, specificity and the commercial availability of PCR-based methods in kit formats. It is more applicable to the identification of food organisms than to their enumeration. PCR can be used in microbiology not only as a diagnostic method but also for the identification and typing of isolates. Typical PCR assays used in bacterial detection involve several critical steps, such as DNA extraction from the sample, DNA amplification, and detection of amplicons. In particular, when dealing with samples , in which the target bacteria are present in low abundance, such as a food sample, all steps must be cautiously designed and performed (Bagheripoor-Fallah et al., 2015; Elmerdahl Olsen, 2000a; Innis, 1999; Uyttendaele et al., 2014).

Nowadays, multiplex PCR is also used for the detection of different foodborne pathogens. Multiplex PCR has great potential, as it is more cost-effective and time saving than conventional PCR. One problem with multiplex PCR is that it cannot be used when the target amplicons are the same in size as the results must be checked by agarose gel electrophoresis. This method also requires a significant amount of optimisation of the primer pairs ,Mg^{2+}, dNTPs and DNA polymerase. In particular, the annealing temperatures of each primer needed to obtain good amplification of each target is critical; however, when optimised, multiplex PCR is a very sensitive and selective detection technique (Elmerdahl Olsen, 2000; Uyttendaele et al., 2014).

In the last decade, real-time PCR has also been developed to enhance the advantages of PCR. In real-time PCR, the PCR amplicons are monitored as they accumulate. In contrast, in conventional PCR, amplicons are checked at the end of the reaction. There are different types of real-time PCR systems available, but the TaqMan real-time PCR and SYBR Green methods are the most commonly used. In real-time PCR, the progress of the PCR is measured by observing the change in fluorescence levels, which is relative to the amount of PCR product. The fluorescent molecule is also a target-specific probe labelled with a fluorescent dye and a quencher or a non- specific DNA binding dye such as SYBR Green (Postollec et al., 2011; Uyttendaele et al., 2014).

1.6.1.2 Loop-mediated isothermal amplification (LAMP)

LAMP is a relatively new molecular technique of nucleic acid amplification. Unlike PCR, LAMP is a DNA amplification method that includes an auto-cyclic isothermal amplification of target DNA by Bst DNA polymerase, which is a DNA polymerase extracted from *Bacillus stearothermophilus*. The Bst polymerase does not have the exonuclease activity of Taq polymerase but instead has a strand displacement activity. The metods uses a set of four or six diverse primers, each of which binds to six or eight different regions on the target gene, making it extremely specific. The LAMP reaction can be completed using an isothermal condition by using Bst DNA polymerase which has high displacement activity. In a LAMP assay, the reaction occurs in a single tube having buffer, DNA polymerase, target DNA, and primers. The tube is incubated at a constant temperature. Amplified products can be detected by he naked eye as a white precipitate or a yellow to green colour solution with the addition of SYBR green to the reaction tube (Li et al., 2009; Wong et al., 2018).

1.6.1.3 DNA based fingerprinting and subtyping methods

Several microbial genomic fingerprinting and source tracking methods have shown some success in differentiating and predicting the sources of different bacteria (Figure 1.1). The most widely used methods include Pulsed-Field Gel Electrophoresis (PFGE), Amplified Fragment Length Polymorphism (AFLP) and Repetitive sequencing-based PCR (REP-PCR). The AFLP and REP-PCR genomic fingerprinting methods have been used comprehensively to classify several bacterial species at the subspecies and strain levels (Li, Raoult, & Fournier, 2009; Wang et al., 2017).

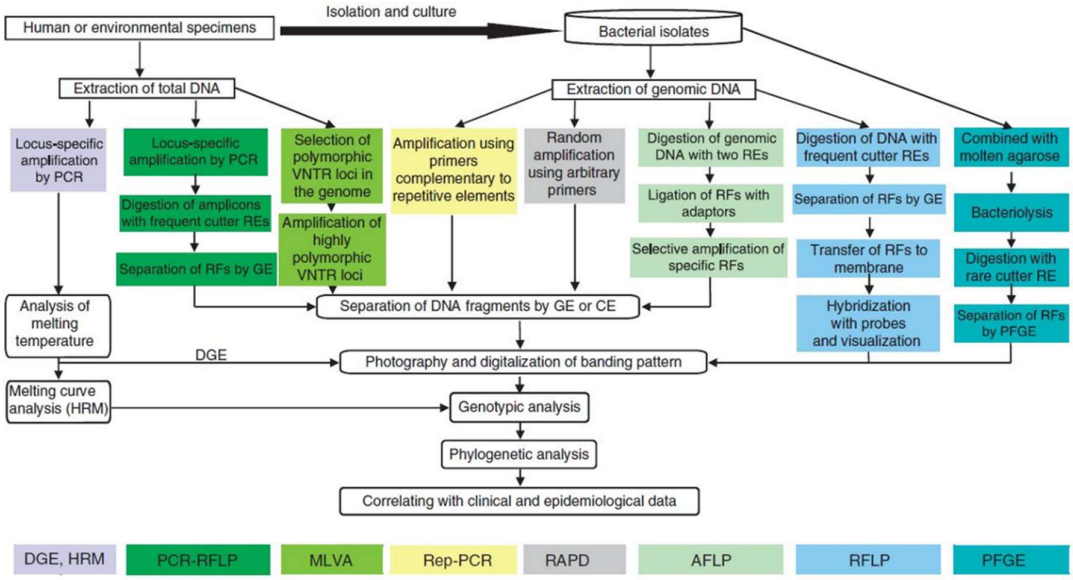

Figure 1.1 Flow chart of DNA genotyping methods for bacterial strains (Li et al., 2009).

1.6.1.3.1 Pulsed-Field Gel Electrophoresis (PFGE)

PFGE is recognised as a gold standard for bacterial typing. This method includes enzyme restriction of bacteria DNA, separation of the restricted DNA bands in a pulsed-field electrophoresis chamber and clonal assignment of bacteria based on PFGE banding patterns. Several PFGE procedures have been developed for typing of bacteria. This method is one of the most commonly used methods for phylogenetic studies, food safety surveillance, infection control and outbreak research. PFGE uses restriction enzymes to cut the genomic DNA into 8 to 25 fragments ranging between 40 to 600 kbp. In this technique, the bacteria are lysed in the first step, and the genomic DNA is digested using enzyme in agarose plugs. The DNA banding

patterns obtained after restriction digestion are compared to discriminate the isolates into different groups. PFGE can be readily standardised and is a very sensitive method that can detect genetic changes like insertions, mutations, deletions and transpositions (Jadhav et al., 2015; Neoh et al., 2019).

1.6.1.3.2 Amplified Fragment Length Polymorphism (AFLP)

AFLP offers an effective method of bacterial genotyping, mainly when not enough information is available about an organism's genome. In this method, restriction enzymes cut the bacterial DNA and adaptors are attached to the ends of the DNA fragments. Then DNA fragments are amplified, and their variable lengths can be visualized on capillary electrophoresis-based platforms or conventional gel electrophoresis. AFLP is highly sensitive for identifying genetic polymorphisms but needs relatively large quantities of high quality DNA and has challenging mixture analysis. In AFLP, the genomic DNA is digested with two restriction enzymes, and double stranded adaptors are ligated to the fragments produced on denaturing polyacrylamide gels. With an automatic sequencer, these fragments are amplified using PCR and subjected to capillary gel electrophoresis. AFLP has high specificity, but as a result of the added step of adding adaptors to the digested DNA, this method is expensive and time-consuming. AFLP has been used in source-tracking of food processing environment contamination along with other subtyping methods (Leung et al., 2004; Li et al., 2009).

1.6.1.3.3 Restriction Fragment Length Polymorphism (RFLP)

In this method, bacterial DNA can be compared by digesting their chromosomal DNA with a restriction endonuclease and separating their DNA fragments by gel electrophoresis. The frequent cutting restriction enzyme is used for the analysis in this method. The DNA fingerprints are typically challenging to interpret because there are often more than 100 fragments that can be used in the comparison between the bacterial isolates (Li et al., 2009; Foleya, Lynne & Nayak, 2009).

There are two conventional approaches to solving this difficulty in RFLP analysis. The first uses a rare cutting restriction enzyme and specialized electrophoresis methods to separate the large DNA fragments. This methodology is described in the section on pulsed-field gel electrophoresis (PFGE). The second approach is to transfer a large number of DNA fragments to membranes and hybridize the DNA fragments with a labelled probe for specific repetitive DNA fragments present in the bacterial genome. The size and number of restriction fragments

homologous to the probe are used to compare bacterial strains. Several studies have found RFLP appropriate tool for typing foodborne pathogens (Foleya, Lynne& Nayak, 2009; Uddin et al., 2021).

1.6.1.3.4 Repetitive sequencing-based PCR (REP-PCR)

Repetitive DNA sequences are found in multiple copies of bacterial genomes, and their presence is very beneficial for DNA bacterial fingerprinting using different kinds of REP-PCR methods. In REP-PCR, primers complementary to repetitive consensus sequences (found dispersed through the genome) are used to amplify DNA fragments between the repetitive elements (Banyko & Vyletelova, 2009; Leung, Mackereth & Tien, et al., 2004).

Different types of repetitive sequences, including the 35–40-bp repetitive extragenic palindromic (REP) sequence, the 124–127-bp enterobacterial repetitive intergenic consensus (ERIC) sequence and the 154-bp BOX element sequences, have commonly been used in REP-PCR assays. The procedures are referred to as REP-PCR, ERIC-PCR, and BOX-PCR genomic fingerprinting, respectively. Detection of DNA fragments is achieved by agarose gel electrophoresis or capillary electrophoresis. The optimisation of electrophoresis conditions can increase the resolution of amplified DNA fragments (Gillings & Holley, 1997; Li et al., 2009; Wilson & Sharp, 2006).

1.6.1.3.4.1 Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR)

Enterobacterial repetitive intergenic consensus (ERIC) sequences, described as intergenic repetitive units, vary from most other bacterial repeats in being distributed across a broader range of species. ERIC sequences were first described in *Escherichia coli*, *Salmonella* Typhimurium and other members of the *Enterobacteriaceae* (Vivek Prabhu, 2010; Wilson & Sharp, 2006). Nowadays, the ERIC sequences are known in a large number of bacterial genomes. The incomplete palindrome sequences are commonly detected within transcribed areas associated with intergenic consensus. Additionally, there are different numbers of ERIC sequence copies among bacterial species (Ranjbar et al., 2017).

ERIC sequences have been commonly used for DNA typing. ERIC sequences are 124–127-bp elements that contain a highly conserved central inverted repeat and are placed in extragenic regions of the genome. They have been defined principally based on sequence data acquired from *Salmonella* Typhimurium and *E. coli* (Chen et al., 2010; Olive & Bean, 1999; Wilson & Sharp, 2006).

Many studies have proved that REP-PCR is a very sensitive methods for characterising a broad range of bacterial species from diverse bacterial genera (Banyko & Vyletelova, 2009; Leung et al., 2004). Because of the rapidity, ease of use, low cost of materials, and sensitivity, ERIC-PCR is a suitable method for bacterial strain typing. ERIC-PCR is very reproducible because specific primers are used for amplification (Elmerdahl Olsen, 2000; Li et al., 2009; Olive & Bean, 1999).

The amplification-based and non-amplification-based methods described here are used to explore small fragments of the bacterial genome, which limits these approaches to species-dependent protocols. Whole Genome Sequencing (WGS) based typing of bacterial pathogens contains mobile genetic elements and could provide unique resolution in discriminating even highly related species, thereby preventing the use of species-dependent protocols. By sequencing the entire genome, WGS immediately provides information on pathogen detection and identification and epidemiological typing, which is crucially important information that in conventional outbreak management is possible only through multiple methods. However, concerns about high operational costs associated with WGS limit this method's use in routine laboratory and epidemiological practice in the food industry (Quainoo et al., 2017; Peng, Ed-Dra & Yue, 2022).

Nowadays, advancements in some methods, like Next Generation Sequencing (NGS) technology, have significantly reduced the cost and improved the ease of microbial whole genome sequencing. However, NGS still needs the traditional laboratory practice of selecting and culturing bacterial isolates (Billington, Kingsbury & Rivas, 2022).

1.6.2 Mass spectrometry based methods

Mass spectrometry (MS) is a fast growing technique in molecular biology. The mass spectrometric analysis of proteins and metabolites has revolutionised medical and biological research (Böhme, Barros-Velazquez, & Calo-Mata, 2017; Clark et al., 2013; Vertes, Telekes, & Vekey, 2008). The development of MS as a bacterial identification tool has become very powerful and practical during the last two decades. These methods offer fast analysis of the sample, providing reliable information on bacteria characterisation even at the subspecies level. Consequently, these methods have been recognised as routine techniques, alongside conventional tests and genome sequencing methods (Clark et al., 2013; Rohde et al., 2017; Vertes et al., 2008). MS can detect a broad range of biomarkers with a variety of ionisation techniques. Nowadays, proteomic profiling is the most successful method such that MS has the

potential to displace classic microbiological diagnostic biochemical methods in the future (Granlund et al., 2011; Krasny, Hynek, & Hochel, 2013; Sloan, Wang, & Cheng, 2017).

1.6.2.1 Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF-MS)

MALDI-TOF MS was developed in the 1980s and has since been applied to several areas like protein and proteome analysis, polymer analysis, recombinant protein studies, DNA sequencing, and analysis of body fluid and tissues. However, in recent decades, this technology has been developed as a diagnostic tool for microorganisms and is a well-documented tool for the identification of clinical pathogens (Carbonnelle et al., 2011; Clark et al., 2013; Lasch et al., 2016).

A mass spectrometer is made of several functional units, an ion source to ionize and transfer sample molecules ions into a gas phase, a mass analyser that seperates ions based on their mass-to-charge ratio (*m*/*z*), and a detection tool to monitor separated ions. Different ionization methods have been established, including chemical, atmospheric pressure, electrospray, and MALDI. The method of ionization is defined according to the nature of the sample and the purpose of the MS analysis. Electrospray ionisation (ESI) and MALDI are soft ionization techniques that allow ionization and vaporization of large non-volatile biomolecules like proteins. Unlike ESI, MALDI generates mainly singly charged ions, and therefore MALDI-derived spectra include larger numbers of proteins. Time of flight (TOF) mass analysers have also been used for detection of microorganisms for many years since they are suitable for interfacing with pulse laser ionization and offer the possibility of rapid analysis. (Seng et al., 2009; Wieser & Schubert, 2016).

In MALDI-TOF MS analysis, bacterial samples are prepared by mixing the samples with a matrix that results in the crystallization of the sample. The matrix is made of small acid molecules that have a great optical absorption in the range of the used laser wavelength. The matrix composition differs according to the biomolecule to be analysed and the type of laser used. The most frequently used matrices are α-cyano-4-hydroxycinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (DHB), ferulic acid (FA), sinapinic acid (SA), and 2,4-hydroxy-phenyl benzoic acid. CHCA, FA, and SA have been shown to be effective for the detection of protein biomarkers (Lasch et al., 2016; Lay Jr, 2001; Seng et al., 2009).

MALDI-TOF MS is progressively recognised as a molecular characterization method for the identification of genera, species and isolates of microbial food spoilers and pathogens. Nowadays, MALDI-TOF MS is a rapid, cost-effective and straightforward method to detect

important pathogenic bacteria in food and clinical laboratories (Clark et al., 2013; de Koster & Brul, 2016; Sandrin, Goldstein, & Schumaker, 2013; Veloo et al., 2011).

MALDI-TOF MS bacterial identification is based on the acquisition of a protein fingerprint of the microorganism. This fingerprint is searched against a library of reference spectra (Paauw et al., 2015; Wieser & Schubert, 2016). This method usually involves simple sample preparation involving deposition of the sample with matrix molecules on a target plate, placing the plate in a MALDI-TOF mass spectrometer, desorption and ionisation of proteins assisted by a laser, analysis of mass polypeptide ions and database searching of the mass spectra (Figure 1.2). Among the compounds identified in the spectrum, some peaks (molecular masses) are specific to genus, species, and sometimes to subspecies (Carbonnelle et al., 2011; de Koster & Brul, 2016; Ghyselinck et al., 2011; Lasch et al., 2016).

Microorganisms can be directly applied to MALDI-TOF without pre-treatment because most bacteria are lysed after contact with water and organic solvent or strong acid in the matrix. However, for resistant microorganisms like fungi and yeast cells, the use of potent organic acids or alcohols are recommended. In practice, a single microbial isolate is deposited and dried out on a conductive plate before the addition of the matrix. The microbial sample is mixed with a matrix on the plate. After matrix and sample crystallisation, the target plate is loaded into the mass spectrometer, where it is bombarded with laser pulses from, generally, a nitrogen laser. The matrix absorbs energy from the laser, resulting in the desorption of the vaporised and ionised analytes to the gas phase. This matrix-assisted desorption and ionization of the analytes leads to the formation of ions. The desorbed and ionized molecules are accelerated through an electrostatic field and ejected through a flight tube exposed to a vacuum until they reach a detector. Smaller ions travel faster than larger ions. The time of flight (TOF) is determined by the mass (m) and charge (z) of the analytes and is correlated to the square root of m/z. Therefore, analytes with different m/z that make a complex sample are separated according to their TOF and create a mass spectrum that is characterised by the m/z and the amount of the ions (Carbonnelle et al., 2011; Croxatto, Prodhom, & Greub, 2012; Seng et al., 2009).

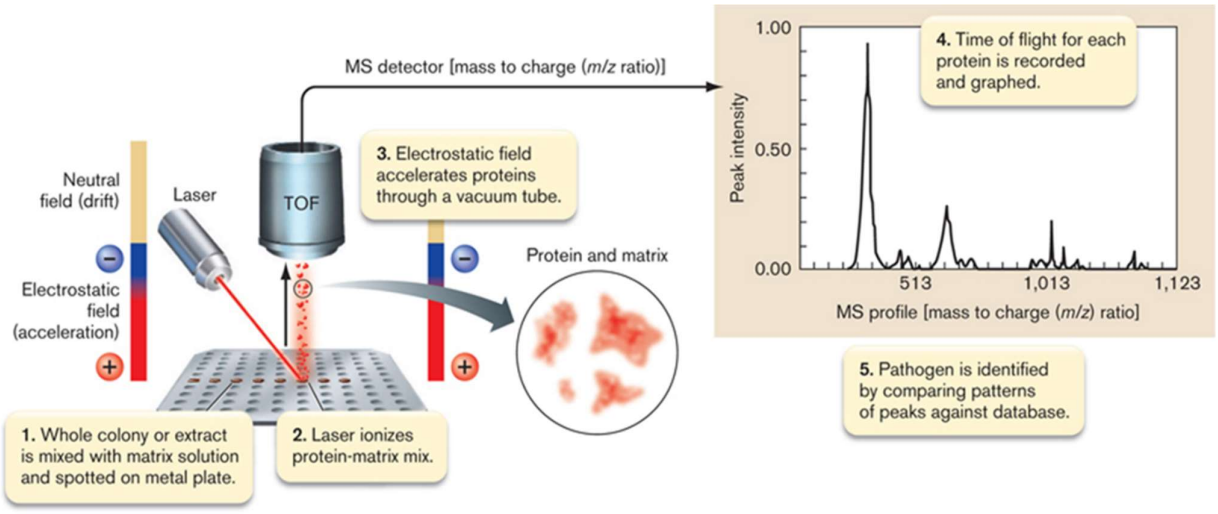

Figure 1.2 Matrix-Assisted Laser Desorption Ionization – Time of Flight process (Carbonnelle et al., 2011)

Several studies compared the efficacy of available commercial MALDI-TOF MS systems with conventional methods for microbial identification. Veen et al (2010) used the Bruker Biotyper system and tested 327 clinical samples previously identified by biochemical techniques. They observed a 95.1% correct identification at the genus level and an 85.6% at the species level. Veen et al (2010) also performed a prospective study on 980 clinical isolates of bacteria and yeasts that showed a 92.2% accurate identification by MALDI-TOF MS, a performance significantly better than the 83.1% identification obtained with conventional biochemical systems. Accurate species identification by MALDI-TOF MS was observed in 97.7% of *Enterobacteriaceae*. In that study, misidentification was associated with insufficient spectra from suitable reference strains in the reference spectra database (Croxatto et al., 2012; Veen, Claas, & Kuijper, 2010).

Seng et al. (2009) have conducted a prospective MALDI-TOF MS identification analysis with the Bruker system on 1660 bacterial isolates in parallel with conventional phenotypic bacterial identification. They showed that 95.4% of the isolates were correctly identified using MALDI-TOF MS, among which 84.1% were at the species level, and 11.3% were at the genus level. The absence of identification (2.8% of isolates) and incorrect identification (1.7% of isolates) were mainly due to improper database entries. The authors estimated that MALDI-TOF MS

identification required an average time of 6 min for an estimated 70–80% reduced cost compared with conventional identification methods (Croxatto et al., 2012; Seng et al., 2009).
Since, accuracy, costs and speed are critical aspects of microbial diagnostics, many studies have focused on these parameters when comparing the differences between MALDI-TOF MS and conventional identification methods. In a study performed by Seng *et al* (2009), the cost of bacterial identification by MALDI-TOF MS was estimated only 17–32% of the costs of conventional identification methods. The same study showed the turnaround time for bacterial identification with MALDI-TOF MS was 6–8.5 minutes compared to 5–48 hours for conventional identification. Thus, MALDI-TOF MS is considered as a highly accurate method suitable for high-throughput microbial identification from culture plates and is a powerful detection technique that succeeds the prior conventional biochemical or molecular identification methods (Sandrin et al., 2013; Seng et al., 2009).

However, MALDI-TOF results may be compromised by technical problems and poor maintenance of the MALDI-TOF MS instrument. To avoid any possible technical problems, many laboratorties regularly use the calibration control proposed by some suppliers. For example, the Bruker control, Bacterial Test Standard (BTS), contains lyophilized *E. coli* extracts and two additional proteins. Proper maintenance is also important to assure accurate bacterial identification. Vacuum failure might be observed due to the presence of dust on joints or to the aging of these plastic joints. Dust exposure might be reduced by placing the mass spectrometer in a dust-free area (Bruker, 2020; Seng et al., 2009; Welker & Moore, 2011). In spite of sufficient maintenance and correct procedures, some microbial groups will frequently be misidentified due to the content of some spectral databases. Therefore, it is important to implement a quality control program targeting routine procedures and improve the quality of the database (Ghyselinck et al., 2011; Guembe et al., 2016; Seng et al., 2009).
In summary, MALDI-TOF MS represents a novel technique that has the potential to substitute conventional identification techniques for most routine isolates in microbiology laboratories, such as food microbiology laboratories. MALDI-TOF-MS is an effective method in bacterial identification, and several studies have shown that its application is well-suited for identification in the food microbiology laboratory. This method is currently the fastest method to accurately identify microorganisms (Lasch et al., 2016; Vithanage et al., 2014).
Despite numerous studies on the use of MALDI-TOF-MS in bacterial confirmation at genus and species level, few studies have focussed on its ability to perform strain level discrimination.

Unlike microbial identification to the species level using MALDI-TOF MS, strain level subtyping involves more detailed investigations as changes in culture conditions, sample preparation methods, quality of the spectra and the type of data analysis tools used can impact the outcomes. However, independent methods can identify close bacterial subgroups by unrelated peaks (Clark et al., 2013; Jadhav, Bhave, & Palombo, 2012; Lay Jr, 2001; Ojima-Kato et al., 2014; Sauget et al., 2016).

1.6.3 Fourier-transform infrared spectroscopy (FT-IR)

Infrared (IR) spectroscopy evaluates the effect of interactions between material and emitted energy in the IR range. This effect is calculated by the measurement of the absorption of various IR frequencies by a sample placed in the IR beam path. While IR radiation is passing through a sample, depending on its frequency and the structure of the molecule, the radiation can be absorbed or transmitted. Therefore, IR radiation can stimulate certain molecular groups. The absorption of energy happens at different frequencies matching the molecular mode of vibration of the corresponding molecules. As diverse molecules absorb particular frequencies of IR radiation, the technique can be used in sample identification (Alvarez-Ordonez & Prieto, 2012; Berthomieu & Hienerwadel, 2009; Cordovana et al., 2021b; Mariey et al., 2001).

Different spectroscopic techniques have been used to acquire IR spectra for the characterization of the molecular composition of foodborne bacteria. Two types of IR spectrometers are utilized in analytical practices for the characterization of organic compounds: IR-dispersive spectrometers and Fourier transform IR (FT-IR) spectrometers. IR radiation in both techniques is acquired from the thermal emission of a source (Alvarez-Ordonez & Prieto, 2012; Berthomieu & Hienerwadel, 2009). There are different sources available: rods of silicon carbide heated electrically, the construction of rare-earth oxides in the form of a hollow cylinder, carbon dioxide lasers, high-pressure mercury arcs, and Nichrome coils. They all make continuous radiation but have diverse radiation energy profiles and, accordingly, different applications. Nowadays, there are several spectroscopic devices that are able to analyse several preparations as samples in the form of liquid, solid or gas (Alvarez-Ordonez & Prieto, 2012; Berthomieu & Hienerwadel, 2009; Martak et al., 2019).

FT-IR spectroscopy is used to analyse the molecular vibrations caused by the absorption of IR light. Different chemical structures vibrate at different frequencies; the carboxyl unit in fatty acids and lipids vibrate at 2800–3000 cm^{-1}, proteins vibrate at 1500–1800 cm^{-1}, and polysaccharides vibrate at 900–1200 cm^{-1} (Figure 1.3).

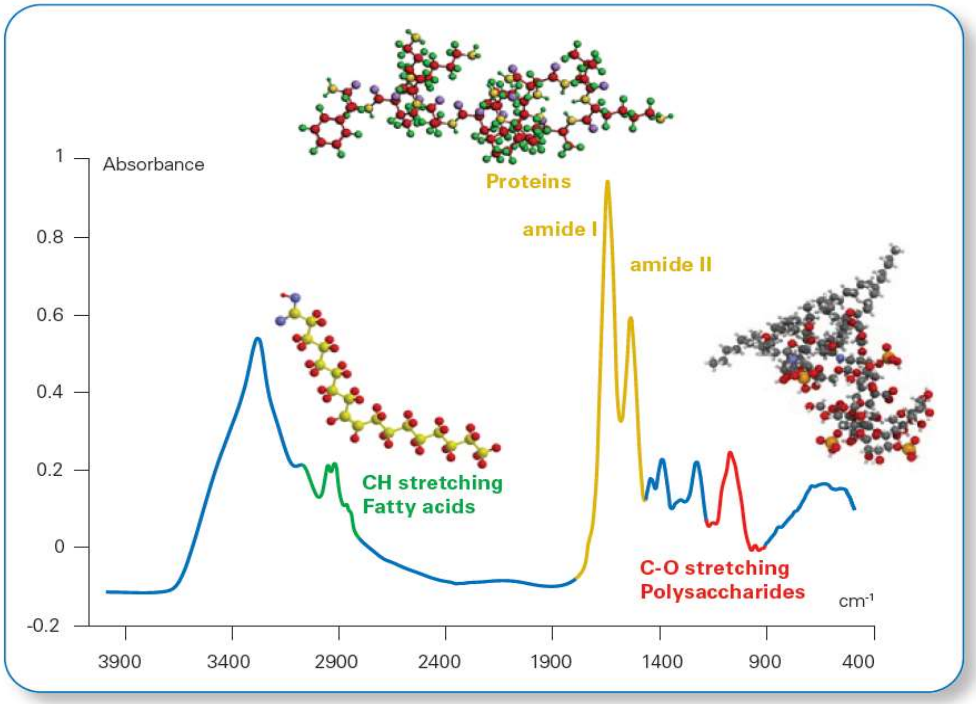

Figure 1.3 FT-IR spectra in the wavenumber range typical for carbohydrates (red), fatty acids (green) and proteins (yellow)

FT-IR spectroscopy offers the option to use data from all these ranges to provide information about the full range of diagnostic molecules present in the sample. The polysaccharide region is the most useful in routine use since this part of the spectrum is like a 'fingerprint', providing information about the carbohydrates present in many molecules and allowing microorganisms to be classified. This method is based on the differential vibrational modes of distinct chemical bonds when exposed to an infrared beam. FT-IR is a rapid, simple, low-cost, and high-throughput analytical method. FT-IR spectroscopy is a phenotypic method usually used in chemistry to determine the molecular composition (Cordovana et al., 2021; Feng et al., 2020; Novais et al., 2019; Wenning et al., 2014). The principle of the technique in microbiology is the absorption of the IR radiation by bacterial cells that causes excitation and vibration of the different chemical compounds of the cell. Different functional groups absorb radiation at different wavenumber ranges and thus originate characteristic spectral peaks. Thus, an IR spectrum of a bacterial cell provides a specific fingerprint that reflects its composition of nucleic acids, proteins, lipids, and carbohydrates, so each microorganism has a highly specific

IR absorption signature which correlates with its genetic characteristics (Feng et al., 2020; Martak et al., 2019; Novais et al., 2019).

The advantages of FT-IR spectroscopy and the specific applications in food microbiology are now well recognized, and many research groups are working on this technique. This technique has many different applications in food microbiology, such as identification and discrimination of bacteria, detection of spores, identification of bacterial capsules, and properties such as drug resistance. Bacteria in several physiological forms, like biofilms or planktonic cells, can also be analysed. IR spectroscopy has also shown reasonable agreement with other taxonomic tools, such as several types of genotyping methods established for microbial groups. Classification can be done at very diverse taxonomic levels like genus, species, and even strain levels. However, classifications achieved at the genus level are not taxonomically relevant in all experiments, showing disagreements with other standard procedures based on genotypic data. Therefore, up till now, the most general applications of FT-IR spectroscopy in food microbiology have been the identification and characterization of bacteria (Alvarez-Ordonez & Prieto, 2012; Dinkelacker et al., 2018; Feng et al., 2020; Mietke et al., 2010; Radmehr et al., 2020). Therefore, this method can revolutionise the bacterial typing and analysis of foodborne microbial pathogens, helping expedite the detection and evaluation of foodborne outbreaks, minimising public health concerns, and limiting costly food recalls.

Table 1.3 Advantages and disadvantages of some typing techniques used for bacterial characterisation

Method	Advantage	Disadvantage
Pulse Field Gel Electrophoresis (PFGE)	• High resolution • Established DNA-based method • Relatively simple • Established criteria for interpretation • Moderate price	• Time-consuming (up to a week) • Issues with standardisation • No automated analysis • Inter-laboratory variations • Skilled labour required
Multi Locus Sequence Typing-PCR (MLST)	• High resolution • Sequence-based with a central database • Good reproducibility • Inter-laboratory comparisons feasible • Highly discriminative	• Time-consuming • High technical and instrumental needs • Not established for all species • Needs specific MLST genes for each species

Whole Genome Sequencing (WGS)	• Highest resolution • Ssuitable for different types of analysis of, e.g. antimicrobial resistance • Accurate detection of specific strains diversity	• Extreme technical and instrumental needs • Highest computing power and bioinformatics knowledge needed • Time-consuming • Expensive • Cut-offs not established
Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR)	• Rapid, inexpensive and simple technique • Minimal labour • No need to design species-specific primers	• Amplification between two ERIC elements may not be genuine with use of low annealing temperatures • Inter-laboratory variation
Fourier Transform Infrared (FT-IR)	• High resolution • Easy to handle • Real-time results (less than three hours to result) • Automation • Low consumable costs	• Cultivation needs to be standardised • High cost of equipment • Cut-offs not established

1.7 Antimicrobial resistance of pathogens; a global public health concern

From their discovery until the present day, antibiotics have saved millions of lives, and their use has contributed to improving human health. Antibiotics support natural defences, like the immune system, to eliminate harmful bacteria. They usually act by preventing the synthesis of a bacterial cell, proteins, DNA, RNA or other specific bacterial cell actions (Aminov, 2010; Oliver, Murinda, & Jayarao, 2011; Ventola, 2015). Despite the benefits, there is concern from the public health and food safety perspectives about the potential development and transmission of antimicrobial resistance that could impact the treatment of diseases affecting humans and animals. Management of bacterial infections is becoming more difficult every year. Infections persist as antibiotics gain resistance, and treatment failure is now more common due to single antibiotic and multi-drug resistance. Treatment costs as a result of multidrug resistant bacterial infection result in extra healthcare costs (Chaves et al., 2011; Fernandes & Ackerman, 1989; Oliver et al., 2011).

The extensive use of antibiotics has given rise to antibiotic resistance in different bacteria via the distribution of the antibiotic resistance genes, in particular among bacterial strains causing

infectious diseases. Antibiotic resistance occurs when an antibiotic fails in its capability to efficiently inhibit bacterial growth. As a result, resistant bacteria are able to continue to multiply in the presence of therapeutic levels of the antibiotics. The distribution of the antibiotic resistance genes is usually due to mobile genetic elements such as transposons, plasmids and integrons (Aminov, 2010; Dolejska et al., 2008; Ventola, 2015; Zaman et al., 2017).

Nowadays, antibiotic resistance is at all time high in most parts of the world. In Australia, the prevalence of antibiotic-resistant bacteria is on the rise. Where prevalance has been studied, resistance varied from state to state; it was highest in the Eastern states of New South Wales, Victoria, and Queensland, and lowest in Tasmania and Western Australia (Fernandes & Ackerman, 1989; Hardy-Holbrook et al., 2013; Zaman et al., 2017). No comprehensive data are available on the antimicrobial properties of dairy isolates of *E.coli* and *B.cereus* in Australia. Therefore, this study aimed to determine the prevalence and antimicrobial resistance patterns of the *E.coli* and *B.cereus* group isolated from various dairy products.

1.7.1 Methods for *in vitro* evaluating antimicrobial activity

1.7.1.1 Diffusion methods

1.7.1.1.1 Agar disk diffusion method

Agar disk diffusion is the approved method used in many microbiology laboratories for standard antimicrobial susceptibility testing. Nowadays, many approved standards are issued by the Clinical and Laboratory Standards Institute (CLSI) for bacteria antimicrobial (CLSI, 2017; Iniguez-Moreno et al., 2017).

In this method, agar plates are inoculated with a consistent inoculum of the test microorganism. Then, paper discs containing the test compound at a preferred concentration are positioned on the agar surface. The plates are incubated under suitable time and temperature conditions. Antimicrobial agents diffuse into the agar and inhibit the growth of the test microorganism, and then the diameters of inhibition growth zones are evaluated. This method provides qualitative results by classifying bacteria as susceptible, intermediate or resistant. Accordingly, it is a typing method based on the resistance phenotype of the tested microorganism. Results also help clinicians in the appropriate selection of appropriate treatments and antibiotics used for individual patients. This method offers many advantages: simplicity and low cost, the ability to test vast numbers of microorganisms and antimicrobial agents, and easy results interperation. However, since the bacterial growth inhibition does not imply bacterial death,

this method cannot differentiate bactericidal and bacteriostatic effects of antimicrobial. Moreover, this method is not applicable to defining the minimum inhibitory concentration (MIC), as it is impossible to measure the amount of the antimicrobial agent diffusing into the agar medium (Balouiri, Sadiki, & Ibnsouda, 2016; CLSI, 2017).

1.7.1.1.2 Agar well diffusion method

Agar well diffusion method is generally used to assess the antimicrobial activity of plants or microbial extracts. Like the disk diffusion method, the agar plate surface is inoculated by spreading an microbial inoculum. A hole with a 6 to 8 millimetre diameter is punched aseptically into the agar and 20–100 µL of the antimicrobial agent or extract solution at preferred concentration is transferred into the well. Plates are incubated under appropriate conditions of the test microorganism. The antimicrobial agent diffuses into the agar medium and prevents the growth of the microbial strain measured (Balouiri et al., 2016).

1.7.1.1.3 Antimicrobial gradient method

The antimicrobial gradient method merges the principle of diffusion method and dilution methods to define the MIC value. This method is based on the probability of producing a concentration gradient of the antimicrobial agent tested in the agar medium. In this method, a strip saturated with an increasing concentration gradient of the antimicrobial agent from one end to the other end is placed on the agar surface that is inoculated with the test microorganism. This method is utilized for the MIC determination of antibiotics. MIC is determined at the junction of the strip and the growth inhibition. The method is simple to use, and it is usually used to meet the needs of clinicians. This technique can also be used to study the antimicrobial interaction between two agents (Balouiri et al., 2016; Othman et al., 2011).

1.7.1.2 Thin-layer chromatography (TLC)

Direct bioautography is the most used method among TLC methods. The prepared TLC plate is dipped to or sprayed with a microbial suspension. Then, bioautogram is incubated at 25 °C for 48 hours. Tetrazolium salts, such as p-Iodonitrotetrazolium violet, are converted to corresponding intensely coloured formazan by the dehydrogenases of live cells are frequently used for the visualization of microbial growth.

Another frequently used TLC method is the agar overlay bioassay, also known as immersion bioautography. In this method, the TLC plate is covered with a molten seeded agar medium.

To allow a good diffusion of the tested mixes into the agar medium, the plates can stay at a low temperature for a few hours before incubation. After incubation and under suitable conditions for testing microorganisms, staining is made with tetrazolium dye. Similar to direct bioautography, this method can be applied to all microorganisms, including yeast and moulds. This method gives good growth inhibition zones and is not sensitive to contamination. Overall, TLC methods are simple, effective and inexpensive techniques for separating a complex mixture and localising the active constituents on the TLC plate (Balouiri et al., 2016; Horvath et al., 2010).

1.7.1.3 Dilution methods

Dilution methods are the most suitable methods for the determination of MIC values because they provide the possibility to estimate the concentration of the tested antimicrobial agent in the agar or broth medium. Broth or agar dilution methods can be used to measure the antimicrobial activity against bacteria. MIC is recorded as the lowest concentration of the assayed antimicrobial agent that inhibits the visual growth of the tested microorganism. There are many approved guidelines for dilution antimicrobial susceptibility testing. The most accepted standards are provided by the CLSI and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). These guidelines establish a consistent procedure for testing that is suitable for standard microbiology laboratories (Balouiri et al., 2016; CLSI, 2017).

1.7.1.3.1 Broth dilution method

Broth dilution is one of the fundamental antimicrobial susceptibility testing methods. The procedure includes preparing two-fold serial dilutions of the antimicrobial agent in a liquid medium dispensed in tubes or wells. Then, each tube or well is inoculated with a microbial inoculum after dilution of standardized microbial suspension. After mixing, the inoculated tubes are incubated under appropriate conditions depending on the test microorganism. The MIC is the lowest concentration of antimicrobial agent that completely inhibits the growth of the organism in tubes or wells as detected visually (Baker, Thornsberry, & Hawkinson, 1983; Balouiri et al., 2016).

The EUCAST broth dilution method is similar to CLSI with modifications generally concerning some of the test parameters like inoculum size, inoculum preparation and the MIC reading method, which is spectrophotometric in EUCAST procedures and visual in the CLSI assay. The determination of minimum bactericidal concentration (MBC) is also the most

common estimation of bactericidal activity. The MBC is defined as the lowest concentration of antimicrobial agent required to kill 99.9% of the final inoculum after incubation for 24 h under a standardized set of conditions (CLSI, 2017; Jonasson, Matuschek, & Kahlmeter, 2020).

1.7.1.3.2 Agar dilution method

The agar dilution method includes the incorporation of variable concentrations of the antimicrobial agent into an agar medium, usually using serial two-fold dilutions, followed by the inoculation of microbial inoculum on the agar plate surface. The MIC endpoint is recorded as the lowest concentration of antimicrobial agent that totally inhibits growth in suitable incubation conditions (Dolejska et al., 2008; Othman et al., 2011).

1.7.1.4 ATP bioluminescence assay

ATP bioluminescence assay is established on the ability to measure adenosine triphosphate (ATP) produced by bacteria. ATP is the chemical form of energy of all living cells, and it is present in a constant amount in a cell. Therefore, its quantification is used to estimate the microbial population in a sample. D-luciferin in the presence of ATP undergoes transformation by luciferase to oxyluciferin that produces light. The amount of the emitted light is measured by a luminometer and expressed as relative light. Consequently, there is a linear relationship between cell viability and luminescence measured. Bioluminescence assays have a large range of applications. Moreover, they have been used for antibacterial testing. The rapidity is the main benefit of this technique that provides quantitative results (Balouiri et al., 2016; Othman et al., 2011).

17.1.5 Flow cytofluorometric method

Several studies were reported on the effectiveness of the flow cytometer as a tool for antibacterial testing. This method based on the rapid detection of damaged cells depends on the use of appropriate dyes staining. Moreover, in this method, dead, viable and injured cells can be clearly discriminated. The injured cells are described as stressed cells displaying damage to cellular components and subsequent damage of reproductive growth. Quantification of injured cells is an imprtant outcome, especially in food microbiology, because these bacteria can be problematic if cell recovery is possible during, for example, inappropriate temperature conditions during food storage. Therefore, the flow cytofluorometric method allow the detection of antimicrobial resistance and approximations of the impact of the molecule tested

on the viability and microorganism. However, the use of this methodology for antimicrobial susceptibility testing currently is not common due to the inaccessibility of the required flow cytometry equipment in many laboratories (Balouiri et al., 2016; Reller et al., 2009).

1.8 Biofilm formation in food industries: A food safety concern

Biofilms are an aggregation of microorganisms attached to and growing on a surface. Biofilm formation is a dynamic process, and several mechanisms are involved in the attachment, development and colonisation of microorganisms on the contact surfaces. The development of biofilms is affected by various factors, like the particular bacteria strain, surface properties, environmental parameters, nutrient levels and temperature (Speranza & Corbo, 2017; Srey, Jahid, & Ha, 2013). Biofilm cells are very resistant to antimicrobial agents, as they have a barrier that prevents or lessens contact with antimicrobial agents. Biofilm is problematic in a wide range of food industries, including brewing, seafood processing, meat processing, and dairy processing (Speranza & Corbo, 2017).

Biofilm formation in dairy manufacturing is a well-known threat that can affect product safety. Consequently, it is considered a developing public health concern all over the world. Dairy products are very susceptible to contamination by biofilm, and it is challenging to eliminate those microorganisms. Biofilms can form on milk processing equipment and other contact surfaces and act as a persistent source of bacterial contamination and effect the microbial quality and safety of milk products, and sometimes may result in food-borne disease. Biofilms can also create several complications for manufacturing operations like mechanical blockages, the impedance of heat transfer, and bio-corrosion of metallic and polymeric systems components (Marchand et al., 2012; Mogha et al., 2014).

1.8.1 Mechanism of biofilm formation

Biofilm formation depends on many factors in the dairy industry. It is speculated that the type of bacteria in the milk samples may cause biofilm formation. (Srey et al., 2013). Microorganism can naturally attach to solid surface containing nutrients that are sufficient for their viability and growth. Primarily, these microorganisms are placed on the surface and then attach, grow, and multiply to form a colony of cells. Biofilm formation is a dynamic process, and several steps are involved in their attachment and growth:

Step 1: Conditioning of surface

In the dairy industry scenario, organic molecules, like proteins, are adsorbed to the surface, forming a conditioning film. These molecules, alongside the microorganisms, are moved to the surface by diffusion or by a turbulent flow of the liquid and are accumulated at the solid-liquid interface of the milk contact surface that has a greater concentration of nutrients compared to the fluid phase. The microtopography of the milk contact surface is essential, mainly if the surface consists of deep channels to trap microorganisms (Beloin, Roux, & Ghigo, 2008; Mogha et al., 2014).

Step 2: Adhesion of cells

In the second phase of biofilm formation, bacterial cells are transported to surfaces by diffusion or fluid dynamic forces from the surrounding fluid. The bacterial adhesion is affected by the nutrient accessibility in the surrounding medium and the growth phase of the bacterial cells. The initial weak interaction developed between the cell wall and the substratum is referred to as reversible adhesion. During this stage, fluid forces still can easily remove bacteria. In the irreversible attachment phase, the repulsive forces inhibit the bacterial cells in the production of direct contact with the surface; still, the bacterial attachment is aided by flagella, fimbriae, pili, and bacterial extracellular polymeric substances (EPSs) that can form a connection between bacteria and the film (Mogha et al., 2014; Speranza & Corbo, 2017; Tang et al., 2010). Spores show a greater rate of adhesion than vegetative bacterial cells. Upon adhesion to a surface, spores germinate and the vegetative cells grow to produce EPS. At this stage, removal of cells involves much stronger forces such as scrubbing. The chemical structure of the EPS is different among different types of bacteria and is also dependent on environmental conditions (Hussain & Oh, 2017; Momba et al., 2000).

Step 3: Formation of microcolony

These irreversibly attached bacterial cells can grow by using the nutrients in the conditioning film and the surrounding environment. This forms microcolonies, which increase and merge to form a layer of cells covering the surface. During this time, the attached cells also produce polymer (EPS), which helps stabilize the colony against the environment's fluctuations (Marchand et al., 2012; Mogha et al., 2014).

Step 4: Biofilm formation

This continuous attachment of the bacterial cells to the surface and following growth along with EPS production forms a biofilm. Multilayers of bacterial cells are entrapped within the EPS. Additional growth of biofilms happens by the deposition or attachment of other organic

and inorganic material to the biofilm from the surrounding liquid phase (Kumar & Anand, 1998; Mogha et al., 2014).

Interest in the study of biofilms in the dairy industry has grown as a result of consumer demand for quality products with longer shelf life and higher safety level. For example, high levels of thermoduric streptococci and *Bacillus* species in milk could be caused by contamination via the distribution of biofilm. Many studies have focused on the role of specific bacteria in the formation of biofilm in the dairy industry. Some research has been performed to increase understanding of biofilms and to identify solutions in order to avoid contamination of dairy products (Oosthuizen, Steyn, Lindsay, Brözel, & von Holy, 2001; Tang et al., 2010; Teh et al., 2014b). The ability of biofilm formation varies not just between species; biofilm formation of different strains and serovars of the same species can vary significantly (Oosthuizen et al., 2001; Srey et al., 2013). So, investigation of the biofilm formation ability of dairy bacteria is an important part of their characterisation and behaviour.

1.9 Efficacy of commercial sanitisers in the dairy industry

The dairy processing environment can be a significant source of bacterial contamination, and it is recommended that appropriate actions be taken to identify and remove the sources of contamination. The sanitation of the food processing environment is essential for the control of cross-contamination during production. However, cleansing and disinfection can only eliminate some foodborne microorganisms (Rossoni & Gaylarde, 2000; Tang et al., 2010). All food processing surfaces are possible sites for attachment of bacteria and biofilm formation that may happen even when hygiene and sanitation programmes are correctly applied. Therefore, the selection of a sanitiser must be carefully made with regard to potential microbial contaminants (Marchand et al., 2012; Rossoni & Gaylarde, 2000).

On the other hand, some pathogenic bacteria, especially spore-forming bacteria like *B. cereus* group, are a challenge for cleaning and disinfection. Physical or chemical treatments are insufficient in inactivating spores. In addition, spore germination can be increased by exposure to heat. So, adequate disinfection against bacteria as a part of Good Hygienic Practices includes adequate disinfection against bacterial spores, and it is essential to develop the quality and shelf life of food. Only a few commonly applied chemical disinfectants that are appropriate for use in food areas are sporicidal. Among spore formers, *B. cereus* group are relatively highly resistant against chemical disinfectants (Sudhaus et al., 2014).

Nowadays, the “clean-in-place” (CIP) system is generally used by industries to control biofilms in dairy processing. It is a sanitization procedure that invovles regular cleaning of equipment by using acid and alkaline solutions applied at high temperatures. Some of the approaches that can be applied to prevent and control biofilm are alteration in the existing CIP procedure, using improved detergents and a sanitisers, and attention to biofilm prone areas in the processing line (Mogha et al. To choose a good sanitiser, properties like low corrosiveness, greater microbial lethality and biodegradability need to be considered (Gawande et al., 2013; Marchand et al., 2012; Mogha et al., 2014).

1.9.1 Peracetic acid in the dairy industry

Peracetic acid ($C_2H_4O_3$) (PAA) is known by many names, inlcluding Peroxyacetic acid, Ethaneperoxic acid, Acetyl Hydroperoxide, Acetic peroxide, Desoxon, Peroxyethanoic acid and Persan but, basically, it is an aqueous mixture of hydrogen peroxide and acetic acid (Bessalah et al., 2016; Cheng et al., 2020). PAA is a strong oxidizing agent with stronger oxidation potential than chlorine and chlorine dioxide. Liquid PAA is colourless without foaming capability, and it has a strong acetic acid odour. PAA is effective and stable between pH 1 to 9. The degradation products of PAA are acetate and water and finally biodegraded to carbon dioxide, oxygen and water. It has several advantages over chlorination, which can harm aquatic life by developing carcinogenic chlorinated hydrocarbons. It is also more persistent than chlorine-based disinfectants but less than quaternary ammonium compounds (Cheng et al., 2020; Micciche et al., 2019). PAA does not contribute to the odour and taste of the food product due to the very low concentrations at which it is used. The efficacy of PAA is unaffected at temperatures as low as 2.2 ºC for most bacterial species (Cheng et al., 2020; Gawande et al., 2013).

PAA was patented in 1950 to sanitize fruits and vegetables and reduce bacteria spoilage. In recent years, it has been known to be bacteriocidal and applicable in food areas due to its chemical composition. Nowadays, PAA is a stabilized equilibrium solution that is approved for several uses, including cleaning and industrial sanitization of equipment such as tanks, pipelines, evaporators, pasteurizers, fillers, aseptic machines and, most importantly, all types of process equipment in dairy processing, including farm equipment. PAA has gained dairy industry acceptance in recent years and is being promoted as a potent chlorine replacement (Cheng et al., 2020; Silva et al., 2018; Sudhaus et al., 2014).

As PAA is known as a highly active sanitiser against both gram-positive and gram-negative microorganisms and regarding its usage in the dairy industry, the current study aimed to

investigate its antibacterial effects against *E. coli* and *B. cereus* group isolated from raw and pasteurised milks. In this study, the emphasis is on the biofilm formation ability of isolates as a contamination source, and to comprehend the efficacy of the most frequently used sanitiser to eradicate two important pathogenic bacteria of concern in the dairy industry.

1.10 Research Aims and Objectives

The main aims and objectives of this project were:

I. To investigate the prevalence of *E. coli* contamination in raw milk and *B. cereus* group contamination in raw and pasteurised milks obtained from Victorian dairy producers
II. To compare the ability of of two available commercial MALDI-TOF MS (VITEK® MS and MALDI Biotyper®) systems to identify and characteris *E. coli* isolated from raw milk and *B. cereus* group isolated from raw and pasteurized milks
III. To determine antimicrobial resistance patterns of *E. coli* and *B. cereus* group isolates
IV. To assess the biofilm formation ability of *E. coli* and *B. cereus* group isolates
V. To investigate the pathogenic potential of *E. coli* and *B. cereus* group isolates
VI. To investigate the diversity of *E. coli* and *B. cereus* group isolates using genomic (ERIC-PCR), proteomics (MALDI-TOF MS) and stereoscopic (FT-IR) fingerprinting methods
VII. To compare the capability of ERIC-PCR, MALDI-TOF MS and FT-IR fingerprinting methods to determine the diversity of *E. coli* and *B. cereus* group isolates
VIII. To assess the effectiveness of Preacetic Acid (PAA) disinfectant against *E. coli* and *Bacillus cereus* group isolates

1.11 Thesis outline

The thesis is comprised of six chapters:

Chapter 1 provides a comprehensive review of the important characteristics of the *E. coli* and *Bacillus cereus* group in the food and dairy industry. It also provides an insight into the importance of antimicrobial resistance and the biofilm formation ability of these bacteria. In this chapter, a comprehensive review of available rapid bacterial detection and identification and methods for bacterial fingerprinting and source tracking is provided.

Chapter 2 describes materials and methods adopted in this research.

Chapter 3 investigates the prevalence, characterisation and diversity of *E. coli* isolated from raw milk and also provides a comparison of the capability of ERIC-PCR, MALDI-TOF MS and FT-IR fingerprinting methods to determine the diversity of *E. coli* isolates based on the place of sampling.

Chapter 4 investigates the prevalence, characterisation and diversity of *B. cereus* group isolated from raw and pasteurised milk, and also provides a comparison of the capability of ERIC-PCR, MALDI-TOF MS and FT-IR fingerprinting methods to determine the diversity of *B. cereus* group isolates based on their sources.

Chapter 5 studies antibacterial properties of Preacetic Acid (PAA) disinfectant against *E. coli* and *Bacillus cereus* group with regards to the isolates biofilm formation ability.

Chapter 6 summarises the main findings of this research and suggests certain future directions.

Chapter 2

Experimental Design and Methodology

2. Experimental design and methodology

2.1 Materials and equipment

2.1.1 Microbiological media

Several microbiological media were used in this study (Table 2.1). Dehydrated and pre-prepared microbiological media were purchased from different suppliers. Dehydrated media were prepared using deionised water (dH_2O) and sterilised by autoclaving at 121 °C for 20 minutes.

Table 2.1 List of microbiological media used

Medium	Supplier	Selectivity
Brain Heart Infusion Broth (BHIB)	Oxoid®	Non-selective
Brain Heart Infusion Agar (BHIA)	Oxoid®	Non-selective
Tryptone Soy Broth (TSB)	Oxoid®	Non-selective
Tryptone Soy Broth (TSA)	Oxoid®	Non-selective
Chromocult® Agar	Merk®	*E. coli*
Mannitol Egg Yolk Polymicyn Agar (MEYP)	Edwards®	*B. cereus* group

2.1.2 Equipment

The equipment used in this study is listed in Table 2.2.

Table 2.2 List of equipment used in this study

Equipment	Manufacturer	Application
Nanodrop®	ThermoScientific	DNA quantification
Bioanalyzer®	Agilent Technology	DNA sizing
VITEK® MS	bioMérieux	Mass spectrometric sample analysis

MALDI Biotyper®	Bruker Daltonics GmbH	Mass spectrometric sample analysis
IR Biotyper®	Bruker Daltonics GmbH	FT-IR sample analysis
FastPrep® bead beater	MP Biomedicals	Physical lysis of bacterial cells for DNA extraction
Finnpipette	ThermoScientific	Dispensing liquids
Gel DOC® XR system	Bio-Rad	Imaging of agarose gels
GELAIRE® laminar flow cabinet	Latch	Aseptic work
Inverted microscope	Olympus Imaging corporation	Observation of cell lines
Microtiter plate reader	Omega	Measuring absorbance of 96-well plates
Mini Spin® centrifuge	Eppendorf	Centrifugation of solutions
MyCycler®	Bio-Rad	PCR
Orbital shaker	Ratek	Incubation of cultures
PowerPac power supply	Bio-Rad	Agarose electrophoresis
Water bath	Labec	Incubation of samples
Thermo Spectronic® Spectrophotometer	ThermoScientific	Measuring absorbance
Orbital Shaker	Ratek	Incubation of cultures
Vortex	Ratek	Mixing solutions
SimpliAmp	Applied Biosystems	PCR reaction

2.1.3 Chemicals, enzymes and kits

The chemicals, enzymes, and kits used in this study are listed in Table 2.3.

Table 2.3 Enzymes, commercial kits and chemicals used in this study

Enzyme/Kit/Material	Manufacturer	Purpose
Antibiotic susceptibility discs	Oxoid	Antibiotic susceptibility testing
CHCA	bioMérieux	Mass spectrometric analysis of samples
HCCA	Bruker Daltonics GmbH	Mass spectrometric analysis of samples
BTS	Bruker Daltonics GmbH	Mass spectrometric analysis of samples
Bruker standard solvent	Sigma Aldrich, Australia	HCCA and BTS preparation
IR Biotyper extraction kit	Bruker Daltonics GmbH	FT-IR analysis of samples
Mangomix	Bioline, Australia	PCR reactions
1000 and 100 bp DNA ladders	Promega, Australia	Molecular weight standards
Lysozyme	Sigma Aldrich, Australia	Bacterial cell wall lysis
Oligomix	Bioline, Australia	PCR
Proteinase K	Sigma Aldrich, Australia	Protein hydrolysis in bacterial cells
Bioanalyser DNA 12000 kit	Integrated Sciences	DNA sizing

2.1.4 Buffers and reagents

The buffers and reagents used in this study are listed in Table 2.4. All chemicals used for the preparation of buffers and reagents were of analytical grade and supplied by Sigma Aldrich. All buffers were prepared using Milli-Q water and were sterilised by autoclaving or filtering through a 0.22 µm filter.

Table 2.4 Buffers used in this study

Buffers and solutions	**Composition**
Phosphate buffered saline (PBS) 10x	80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4 and 2.4 g KH_2PO_4 in 1000 mL Milli-Q water, pH 7.4
0.5 M EDTA buffer	18.61 g EDTA disodium salt, dehydrate in 100 mL Milli-Q water, pH 8.0
TAE (tris-acetate EDTA buffer) 50x*	242 g Tris base, 100 mL of 0.5 M EDTA and 57.1 mL glacial acetic acid in 1000 mL Milli-Q water, pH 8.2–8.4
Saline solution	0.85 g NaCl in 100 mL Milli-Q water
TE buffer	1 mL of 1 M Tris-HCl and 0.2 mL of 0.5 M EDTA in 1000 mL Milli-Q water, pH 7.9–8.0

*To make 1x TAE from 50x TAE stock, 20 mL of stock was diluted with 980 mL of dH_2O water

2.2 Sample collection

Between August 2016 and June 2018, 177 samples—including 140 raw and 37 pasteurised milk samples—were collected. Raw milk samples were collected from three Victorian milk producers and pasteurised samples were collected from four different commercial brands. All samples were transported in a cool box at below 4 ºC to the Department of Chemistry and Biotechnology laboratories at the Swinburne University of Technology and analysed immediately upon arrival. Eighty raw milk samples were used to isolate *E. coli* and 60 raw milk and 37 pasteurised milk samples were used for isolation of the *B. cereus* group. As per Australian standards, pasteurised milk must be *E. coli* free, and all producers must check their products to certify that they are not contaminated with *E. coli*. Therefore, pasteurised milk samples were not used for the isolation of *E. coli*.

2.3 Isolation and identification of *E. coli* from raw milk

The isolation of *E. coli* from 80 raw milk samples was carried out according to Rugeleset al. (2010) and Awadallah et al. (2016). Briefly, 1 mL of the raw milk sample was spread over three Eosin Methylene Blue (EMB) agar plates (Oxoid, Australia) and incubated at 37 °C for 18–24 hours. Then, the presumptive *E. coli* colonies were subjected to Gram staining and biochemical tests using the IMViC method (Awadallah, Ahmed, Merwad, & Selim, 2016a; Laura Cristina Rúgeles et al., 2010). To increase the likelihood of isolating diverse strains of bacteria from each milk sample, only one colony was picked from those growing on the agar plates. This reduced the possibility of selecting clones of individual isolates. The confirmed *E. coli* isolates were stored in cryovials containing Brain Heart Infusion (BHI) broth (Oxoid, Australia) with 50% glycerol at –85 °C.

2.4 Isolation and identification of *B. cereus* group from raw milk

Isolation of the *B.cereus* group from 97 samples of raw and pasteurised milks was carried out according to the method of Te Giffel et al. (1997). After heat treatment (85 °C for 5 minutes) to kill all vegetative cells, 1 mL of the raw milk was spread over three Mannitol Egg Yolk Polymyxin (MEYP) agar plates (MicroMedia, Australia). The MEYP plates were incubated at 30 °C for 24 hours and examined for typical pink colonies surrounded by a zone of precipitation (Te Giffel, Beumer, Granum, & Rombouts, 1997b). Colonies of the *B. cereus* group appear pink on MEYP agar due to the absence of mannitol fermentation and are usually surrounded by a zone of opaqueness due to the precipitation of hydrolysed lecithin (Figure 2.1). Other

members of the *Bacillus* group are mannitol positive, appear as green or yellow colonies and do not produce lecithinase (Greenwood, Roberts, & Wiley, 2002).

Figure 2.1 Colonies of *B. cereus* on MEYP agar surrounded by a zone of opaqueness

Gram stains were performed and the isolates were examined microscopically. The identity of colonies was confirmed by testing them for glucose fermentation, Voges-Proskauer (VP) reaction and nitrate reduction. *B. cereus* group strains are positive for these three reactions (Te Giffel et al., 1997b). All confirmed *B. cereus* group isolates were stored in cryovials containing BHI broth (Oxoid, Australia) with 50% glycerol at –80 °C.

2.5 Antimicrobial susceptibility testing

All *E. coli* and *B. cereus* group isolates were subjected to antimicrobial susceptibility tests based on the Clinical and Laboratory Standards Institute (CLSI) criteria using the disc diffusion method (CLSI, 2017; Iñiguez-Moreno et al., 2017). In this method, agar plates were inoculated with a standardised inoculum (equal to 0.5 McFarland Standard) of the *E. coli* and *B. cereus* group isolates. Then, filter paper discs containing an antimicrobial at a standard concentration were placed on the agar surface. The Petri dishes were incubated for 16–17 hours at 35 °C. The antimicrobial agent diffused into the agar and inhibited the growth of the test microorganism, following which the diameters of inhibition growth zones were measured (Figure 2.2; Balouiri et al., 2016; CLSI, 2017; Enriquez-Gómez et al., 2017).

The applied antimicrobial agents (Oxoid, UK) for *E. coli* were the following: amikacin (30 µg), ampicillin (AMP, 10µg), ceftriaxone (CRO, 30 µg), chloramphenicol (CHL, 30 µg), ciprofloxacin (CIP, 5 µg), gentamycin (GEN, 10 µg), erythromycin (ERY, 15 µg), kanamycin

(30 µg), nalidixic acid (30 µg), streptomycin (STR, 10 µg), tetracycline (TET, 30 µg) and trimethoprim-sulfamethoxazole (SXT. 1.245/23.75 µg).

The applied antimicrobial disks for *B. cereus* group were the following: AMP (10 µg), CRO (30 µg), CIP (5µg), CHL (30 µg), GEN (10 µg), clindamycin (CLI, 10µg), ERY (15 µg), penicillin (PEN, 10 U), STR (10 µg), TET (30 µg), SXT (1.245/23.75 µg) and vancomycin (VAN, 5 µg).

These antimicrobial agents were selected as they are the most common agents used in medical practice as well as veterinary medicine (Arslan, Eyi, & Küçüksarı, 2014; Enriquez-Gómez et al., 2017a), given that the isolates in this study were likely to have originated from bovine sources. The inhibition zone diameters (mm) for *E. coli* and *B. cereus* tested on Mueller-Hinton agar are presented in Tables 2.5 and 2.6, repsectively.

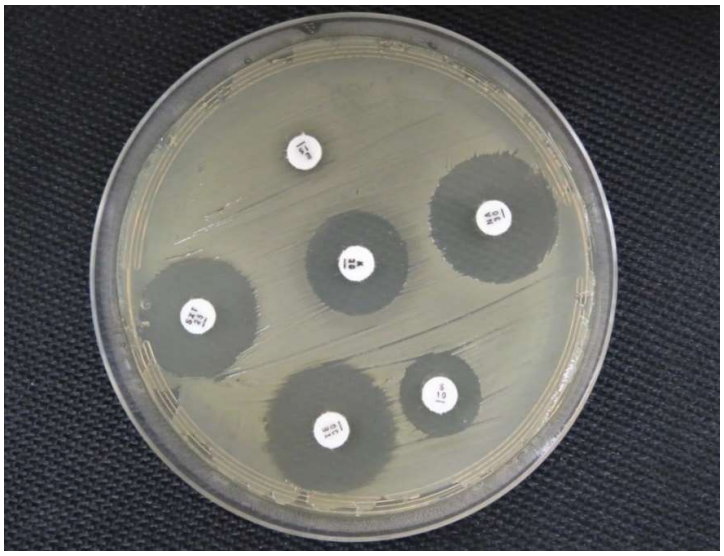

Figure 2.2 Antimicrobial susceptibility test using the disk diffusion method.

Table 2.5 Inhibition zone diameters (mm) for *E. coli* tested on Mueller-Hinton agar.

E. coli	**Sensitive**	**Intermediate**	**Resistant**
Amikacin (30 µg)	≥ 17	15–16	≤ 14
Ampicillin (10 µg)	≥ 17	14–16	≤ 13
Ceftriaxone (30 µg)	≥ 23	20–22	≤ 19
Chloramphenicol (30 µg)	≥ 18	13–17	≤ 12
Ciprofloxacin (5 µg)	≥ 21	16–20	≤ 15
Gentamycin (10 µg)	≥ 15	13–14	≤ 12
Erythromycin (15 µg)	≥ 23	14–22	≤ 13
Kanamycin (30 µg)	≥ 18	14–17	≤ 13
Nalidixic acid (30 µg)	≥ 19	14–18	≤ 13
Streptomycin (10 µg)	≥ 15	12–14	≤ 11
Tetracycline (30 µg)	≥ 15	12–14	≤ 11
Trimethoprim-sulfamethoxazole (25 µg)	≥ 16	14–15	≤ 10

Table 2.6 Inhibition zone diameters (mm) for *B. cereus* tested on Mueller-Hinton agar

B. cereus	**Sensitive**	**Intermediate**	**Resistant**
Ampicillin (10)	≥ 17	14–16	≤ 13
Ceftriaxone (30 µg)	≥ 27	25–26	≤ 24
Chloramphenicol (30 µg)	≥ 18	13–17	≤ 12
Ciprofloxacin (5 µg)	≥ 21	16–20	≤ 15
Clindamycin (10 µg)	≥ 19	16–18	≤ 15
Gentamycin (10 µg)	≥ 15	13–14	≤ 12
Erythromycin (15)	≥ 23	14–22	≤ 13

Penicillin (10 U)	≥ 28	20–27	≤ 19
Streptomycin (10 µg),	≥ 21	15–20	≤ 14
Tetracycline (30 µg),	≥ 19	15–18	≤ 14
Trimethoprim-sulfamethoxazole (25 µg)	≥ 16	11–15	≤ 10
Vancomycin (5 µg)	≥ 12	10–11	≤ 9

2.6 Biofilm formation assay

Biofilm formation was assessed using the colourimetric microplate method with modifications (Hussain & Oh, 2017; Kadam et al., 2013). This method is based on the measurement of the optical density of a biofilm after crystal violet staining. Bacterial cultures were incubated overnight in BHI broth (Oxoid, UK), which was then used to prepare 1% bacterial suspensions. Aliquots (200 µL) of this suspension were transferred to flat-bottom 96-well polystyrene microtitre plates (NUNC®, Denmark), which were then incubated at 37 ºC for 24 hours. Following this, the wells were gently washed three times with sterile water (250 µL) to remove all non-adhered cells. The biofilm was stained with crystal violet (1%, 210 µL) for 30 minutes and rewashed with sterile water (250 µL). After air drying, ethanol (96%, 225 µL) was used to dissolve the crystal violet. The absorbance was measured at 595 nm (FLUOstar Omega, BMG LABTECH, Germany). Each assay was performed in triplicate (Milanov et al., 2015; Stepanovic et al., 2004; Stepanovic et al., 2000).

Based on the absorbance, the strains were classified into four categories: non-biofilm producers and weak, moderate or strong biofilm producers. The cut-off OD (ODc) was defined as three standard deviations above the mean OD of the negative control. Biofilm-producing ability was classified as follows: $OD \leq ODc$ = non-biofilm producers, $ODc < OD \leq (2 \times ODc)$ = weak biofilm producer, $ODc < OD \leq (4 \times ODc)$ = moderate biofilm producer and $(4 \times ODc) < OD$ = strong biofilm producer (Stepanovic et al., 2004; Stepanovic et al., 2000.

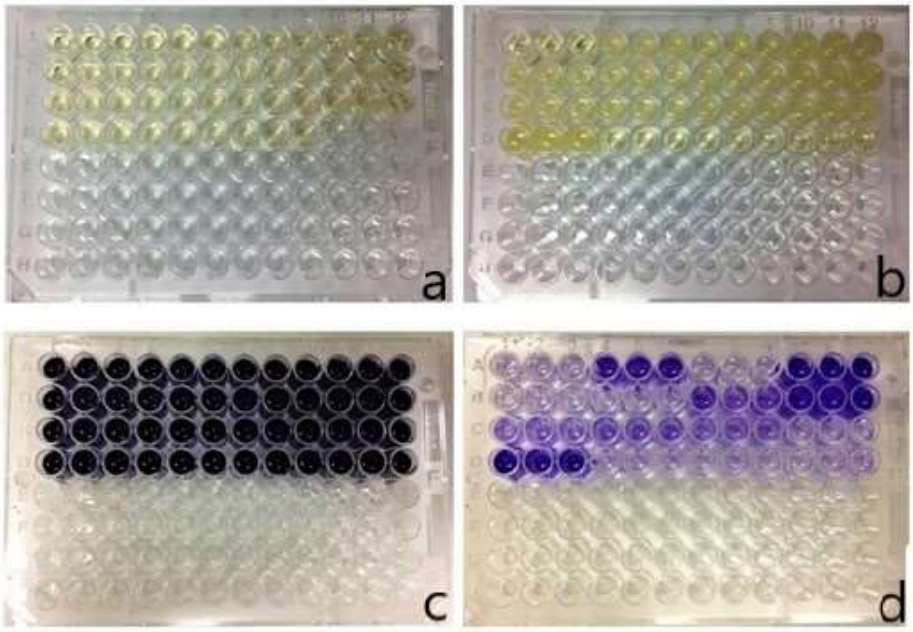

Figure 2.3 Different stages of the colourimetric microplate biofilm formation assay:
Stage a: bacterial suspensions before incubation; Stage b: bacterial suspensions after incubation; Stage c: crystal violet staining; Stage d: dissolving the crystal violet and absorbance measurement

2.7 Bacterial DNA extraction

DNA extraction from isolates was carried out according to the method of Mohammadi et al. (2012) with some modifications. Before bacterial DNA extraction, all isolates were subcultured in BHI broth for 18–24 hours. Then, overnight bacterial culture (1.8 mL) was transferred to a 2 mL collection tube and centrifuged at 14100 x g for 1 minute. After discarding the supernatant, the pellet was resuspended in extraction buffer (500 µL, containing Tris-HCL, EDTA and SDS). Lysozyme (10 $mg \cdot mL^{-1}$, 20 µL) was added to each tube and the mixture was vortexed, following which the tube was incubated for 15 minutes on ice and then bacterial cells were disrupted using a bead beater (FastPrep, MP Biomedicals) for 20 seconds at 4.0 $m \cdot s^{-1}$. The tube was then incubated for 5 minutes at 80 °C and then cooled for 5 minutes on ice. Cold ammonium acetate (250 µL) was added to the tube, which was vortexed and then kept on ice for 10 minutes. The tube was then centrifuged at 14100 x g for 5 minutes to collect precipitated proteins and bacterial tissue. Supernatant (600 µL) was pipetted into a new centrifuge tube containing isopropanol (360 µL), mixed and allowed to stand for 5 minutes at room temperature to allow the DNA to precipitate. DNA was pelleted by centrifugation at 14100 x

g for 5 minutes. The supernatant was then removed and the remaining fluid was drained by inverting the tube onto a piece of paper for 1 minute. The pellet was washed gently by adding ethanol (70%, 500 µL). The tube was then centrifuged at 14100 x g for 5 minutes and again the supernatant was discarded. The tube was left open for 10 minutes to dry out at room temperature. The pellet was resuspended in MilliQ water (100 µL) and RNase (10 $mg \cdot mL^{-1}$, 2 µL of) was added (Mohammadi, 2012; Wilson, 2001).

Isolated DNA was quantified using the Nanodrop® 2000 spectrophotometer (ThermoScientific-USA) and assessed using agarose electrophoresis before being stored at –20 °C (Atashpaz et al., 2010; Mohammadi, 2012).

2.8 Virulence gene detection

Multiplex PCR was been used to detect the virulence genes of *E. coli* isolates. The virulence genes that were investigated in this study and relevant primers are shown in Table 2.7 (Forghani et al., 2015; Huasai et al., 2012; Li et al., 2016; Rey et al., 2006).

Table 2.7 Primer sequences and predicted lengths of PCR amplicons for *E. coli* virulence gene detection

Gene	Oligonucleotide sequence	Amplicon size (bp)	Reaction conditions	References
Sxt1	CGCTGAATGTCATTCGCTCTGC CGTGGTATAGCTACTGTCACC	302	94 °C, 2 min → (94 °C, 60 s → 55 °C, 60 s → 72 °C, 60 s) 35 cycles → 72 °C, 5 min	Enriquez-Gómez et al., 2017a; Rey, Sánchez, et al., 2006
Sxt2	CTTCGGTATCCTATTCCCGG CTGCTGTGACAGTGACAAAACGC	516	94 °C, 2 min → (94 °C, 60 s → 55 °C, 60 s → 72 °C, 60 s) 35 cycles → 72 °C, 5 min	Enriquez-Gómez et al., 2017a; Rey, Sánchez, et al., 2006
eae	GAGAATGAAATAGAAGTCGT GCGGTATCTTTCGCGTAATCGCC	755	94 °C, 2 min → (94 °C, 60 s → 55 °C, 60 s → 72 °C, 60 s) 35 cycles → 72 °C, 5 min	Enriquez-Gómez et al., 2017a; Rey, Sánchez, et al., 2006

Monoplex PCR was used to detect the virulence genes of *B. cereus* group isolates. PCR screening detected the presence of seven genes, including enterotoxin (*hblA*, *hblB*, *nheA*, *nheB*, *nheC*, *cytK*) and cereulide synthesis (*ces*) genes. The primer sequences and PCR conditions are shown in Table 2.8.

Table 2.8 Primer sequences and predicted lengths of PCR amplicons for *B. cereus* group virulence gene detection

Gene	Oligonucleotide sequence	Amplicon size (bp)	Reaction condition	Reference
hblA,	AAGCAATGGAATACAATGGG AGAATCTAAATCATGCCACTGC	1154	94 °C, 2 min → (94 °C, 60 s → 56 °C, 60 s → 72 °C, 120 s) 35 cycles → 72 °C, 5 min	Kim et al., 2011
hblB,	AAGCAATGGAATACAATGGG AATATGTCCCAGTACACCCG	2684	94 °C, 2 min → (94 °C, 60 s → 58 °C, 60 s → 72 °C, 120 s) 35 cycles → 72 °C, 5 min	Guinebretiere, Broussolle V Fau - Nguyen-The, & and Nguyen-The, 2002
nheA,	GTTAGGATCACAATCACCGC ACGAATGTAATTTGAGTCGC	755	94 °C, 2 min → (94 °C, 60 s → 56 °C, 60 s → 72 °C, 120 s) 35 cycles → 72 °C, 5 min	Kim et al., 2011
nheB,	TTTAGTAGTGGATCTGTACGC TTAATGTTCGTTAATCCTGC	743	94 °C, 2 min → (94 °C, 60 s→ 54 °C, 60 s → 72 °C, 120 s) 35 cycles → 72 °C, 5 min	Kim et al., 2011
nheC,	TGGATTCCAAGATGTAACG ATTACGACTTCTGCTTGTGC	683	94 °C, 2 min → (94 °C, 60 s→ 58 °C, 60 s → 72 °C, 120 s) 35 cycles → 72 °C, 5 min	Kim et al., 2011
cytK	GTAACTTTCATTGATGATCC GAATACTAAATAATTGGTTTCC	505	95 °C, 1 min → (95 °C, 60 s → 48◦C, 60 s → 72 °C, 60s) 30	Kim et al., 2011

			cycles → 72 °C, 5 min	
ces	GGTGACACATTATCATATAAGGTG GTAAGCGAACCTGTCTGTAACAACA	1271	95 °C, 15 min → (95 °C, 60 s→ 58 °C, 75 s → 72 °C, 50 s) 25 cycles → 72 °C, 5 min	Kim et al., 2011

2.9 Fingerprinting of isolated bacteria using ERIC-PCR

After the extraction of genomic DNA, PCR using enterobacterial repetitive intergenic consensus (ERIC) primers was used to identify the strain diversity of *E. coli* and the *B. cereus* group. Two ERIC-PCR specific primer sets were applied to amplify repetitive sequences contained in the chromosomal DNA:

(ERIC-1, 5'-ATG TAA GCT CCT GGG GAT TCA C-3' and ERIC-2, 5'-AAG TAA GTG ACT GGG GTG AGC G-3') (Gillings & Holley, 1997; Versalovic, Koeuth, & Lupski, 1991; Vivek Prabhu, 2010; Wilson & Sharp, 2006).

ERIC-PCR was performed using PCR mixture (25 µL), which contained DNA (300 ng), ERIC-1 and ERIC-2 primers (1 µL of each), dNTPs (0.5 µL of each), 10x PCR assay buffer (2.5 µL) and Taq DNA polymerase (1 µL; 3 U). Deionised water was added to this mixture to make the volume up to 25 µL. The reactions were performed in 0.2 mL microcentrifuge tubes using a programmable thermocycler according to the following cycles: denaturation at 94 ºC for 5 minutes, 35 denaturation cycles at 94 ºC for 1 minute, annealing at 42 ºC for 1 minute and extension at 65 ºC for 8 minutes. One additional cycle with an extension step of 16 minutes at 65 ºC was included to complete the synthesis of unfinished products. The amplicons were electrophoresed in 1.5% agarose gel along with 1000 and 100 bp DNA ladders (Gillings & Holley, 1997; Rugeles et al., 2010; Vivek Prabhu, 2010). Illustrative agarose gel electrophoresis profiles of the ERIC-PCR fingerprints of some *E. coli* and *B. cereus* group isolates are shown in Figure 2.4.

The ERIC products were also analysed with an automated electrophoresis system (Agilent 2100 Bioanalyzer, Agilent Technology®) that provides sizing, quantitation and purity assessments for DNA samples. This method has many benefits, including ready-to-use assays and pre-packaged reagent kits, minimal sample consumption, easy-to-load chips and digital

data for convenient analysis (Gillings & Holley, 1997; Hathaway, 2007; Merzougui et al., 2013; Vivek Prabhu, 2010). Illustrative ERIC-PCR fingerprints for some *E. coli* and *B. cereus* group isolates obtained using this method are shown in Figure 2.5. A comparison of Figures 2.4 and 2.5 shows that the quality of the ERIC-PCR fingerprint images obtained using the Bioanalyzer was superior to those from agarose gel electrophoresis. Therefore, Bioanalyzer results were used for collecting ERIC-PCR fingerprint data for further analysis.

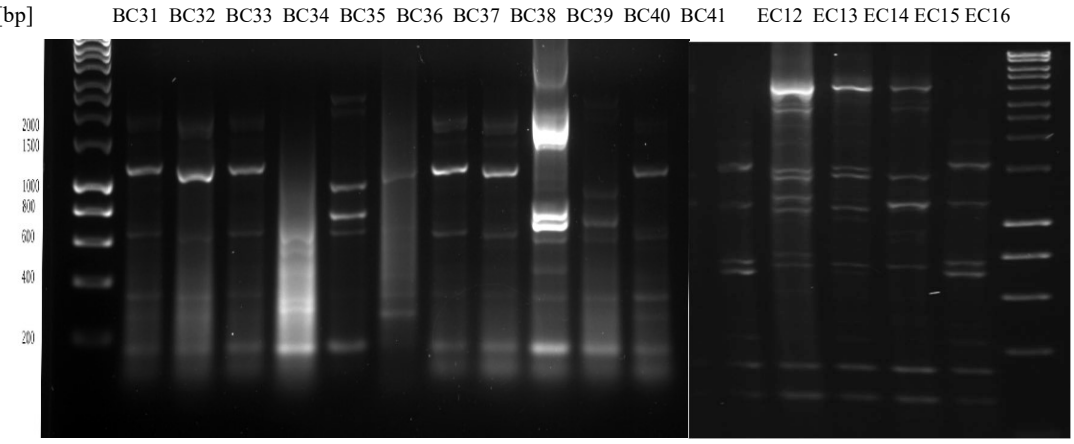

Figure 2.4 Agarose gel electrophoresis of ERIC-PCR fingerprints for some *E. coli* (EC) and *B. cereus* group (BC) isolates. Lanes1: 1 Kbp DNA ladder; 2–12: *B. cereus* group isolates BC31 to BC41; 13–17: *E. coli* isolates EC12 to EC16; 18: 1 Kbp DNA ladder.

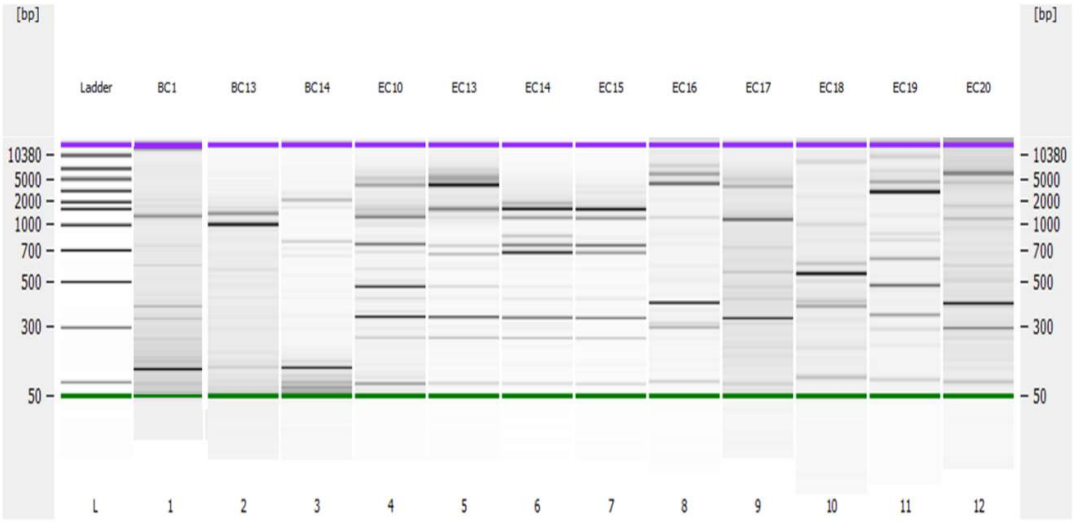

Figure 2.5 ERIC-PCR fingerprints for some *E. coli* (EC) and *B. cereus* group (BC) isolates using the Agilent 2100 Bioanalyzer system. Lane 1: Agilent DNA 12000 bp ladder; 1–3: *B. cereus* group isolates BC1, BC13 and BC14; 4 –12: *E. coli* isolates EC10, EC13 to EC20.

The ERIC-PCR fingerprinting data were transformed into a binary code depending on the presence or absence of each band. Dendrograms were generated by the unweighted pair group method with arithmetic mean (UPGMA) and sequential hierarchical and nested clustering routine. The discriminatory power of the ERIC-PCR data was measured by Simpson's index of diversity (D), which shows the average probability that a typing system gives a different type to distinct strains randomly sampled from a population (Awadallah et al., 2016; Gdoura-Ben Amor et al., 2018; Leung, Mackereth & Tien, et al., 2004).

2.10 Identification of *E. coli* and *B. cereus* group using MALDI-TOF MS

For a comparative evaluation of the performance of two commercial MALDI TOF MS systems (bioMérieux VITEK® MS versus Bruker MALDI Biotyper®), all *E. coli* and *B. cereus* group isolates were subjected to identification. Overnight bacterial cultures on BHI agar were prepared for each isolate and the direct smear method of spotting samples for MALDI-TOF MS was used for preparing VITEK® MS and Bruker MALDI Biotyper® target plates (Veloo, et al., 2011; Vithanage et al., 2014).

2.10.1 Sample preparation for MALDI-TOF MS

2.10.1.1 Preparation of matrix solution and calibrator

Before sample preparation, matrix solutions (CHCA for VITEK® MS and HCCA for Bruker MALDI Biotyper®) were prepared according to the manufacturers' instructions. To prepare the matrix, 250 µL of standard solvent was added to a tube of portioned matrix and dissolved by vortexing at room temperature until the matrix has dissolved and the solution was clear. The matrix was kept at room temperature for one week.

For VITEK® MS, *E. coli* ATCC 8739 was used as the calibration strain whereas, for the Bruker MALDI Biotyper®, the manufacturer's Bacterial Test Standard (BTS) was used for calibration. A tube containing BTS was removed from the freezer and maintained for 5 minutes at room temperature. Then, the BTS pellet was dissolved in standard solvent (50 µL) by pipetting up and down at least twenty times at room temperature. The tube was closed tightly and the BTS solution was incubated for at least 5 minutes at room temperature. Then the solution was mixed again by pipetting up and down at least 20 times. The tube was tightly closed and centrifuged briefly for 10 s at 13000–15000 rpm. The BTS tube was then stored at or below –18 °C for up to five months. To prepare BTS on a MALDI target plate, BTS solution (1.0 µL) was pipetted onto at least one unoccupied MALDI target plate position. The BTS spot was allowed to dry at room temperature and then overlayed with HCCA solution (1.0 µL) within 30 min after drying. The resulting spot was dried at room temperature (Bruker, 2020; Levesque et al., 2015).

2.10.1.2 Preparation of target plate

Overnight bacterial cultures on BHI agar were prepared for each isolate and the direct transfer procedure was used for preparing the target plate. As per the manufacturer's instructions, a small amount of bacterial colony was taken from the solid culture medium and spotted onto a disposable target plate using sterile toothpicks. The following steps were performed for the direct transfer procedure. First, an isolated colony was spread as a thin film directly onto an empty position on the MALDI target plate. Second, the material was overlaid with matrix solution (1.0 µL) within 30 minutes and dried at room temperature. A new pipette tip was used to add the matrix solution to each sample position to avoid any cross-contamination. The extraction procedure is recommended for microorganisms with a rigid cell wall (Bruker, 2020; Levesque et al., 2015; Vithanage et al., 2014). Each isolate was spotted in triplicate on the same target slide.

2.10.1.3 Cleaning procedures for steel MALDI target plates

The cleaning procedure was based on the exposure of the surface of the MALDI target plates to aqueous guanidine hydrochloride (GdnHCl; 4 M). This thorough cleaning protocol ensured the prevention of memory effects on the surface of the reusable steel MALDI target plates. This procedure was not needed when using the disposable Biotarget plates.

The GdnHCl cleaning steps are listed below (Bruker, 2020):

1. Place the MALDI target plate into a petri dish.
2. Cover the surface of the MALDI target plate with 70% ethanol.
3. Incubate for 5 min at room temperature.
4. Remove the MALDI target plate and rinse thoroughly under running tap water.
5. Using lint-free tissue, clean the MALDI target plate intensively with 70% ethanol.
6. Rinse the MALDI target plate with tap water and wipe with a lint-free tissue.
7. Cover the MALDI target plate with 4 M aqueous GdnHCl and incubate at room temperature for 10 min.
8. Rinse with plenty of tap water and wipe carefully with a lint-free tissue.
9. Intensively wipe all target positions with 4 M aqueous GdnHCl.
10. Rinse the MALDI target plate with plenty of tap water and wipe carefully with a lint-free tissue.
11. Repeat steps 9 and 10 twice.
12. Rinse the MALDI target plate with HPLC-grade water and wipe dry with a lint-free tissue.
13. Allow the MALDI target plate to dry at room temperature for at least 15 min.
14. Place the clean target plate in the plastic storage box, ready for use.

2.10.2 Bacterial identification using MALDI-TOF MS

For the Bruker MALDI Biotyper®, all measurements were performed using the manufacturer's recommended settings for automated spectrum collection. The extracted spectra were analysed using MALDI Biotyper® automation control and Bruker MALDI Biotyper® software. All identification criteria were used as recommended by the manufacturer. Data acquisition was controlled using the MALDI Biotyper® Compass software. First, the spectrum of the sample was transformed into a peak list. Utilising a biostatistical algorithm, this peak list was compared

to reference bacterial peak lists of organisms in the reference library database, generating scores of 0.00–3.00. A higher score is indicative of a higher degree of similarity between the pattern of the unknown bacteria peak list and the peak list of the database entry in the reference library. A score greater than or equal to 2.00 was considered a satisfactory probability for sample identification at the species level. The score ranges defined in MALDI Biotyper® Compass reflect the probability of organism identification (Bruker, 2020).

Bacterial identification using VITEK® MS was performed using the Axima Performance mass spectrometer (Shimadzu Scientific Instruments, USA) and Spectral Archiving and Microbial Identification System (SARAMIS v4.16; bioMérieux, France). The target plate was loaded into the Axima Performance instrument and the target spots were fired using a 337 nm nitrogen laser (50 Hz). Mass spectra were generated in the positive linear mode in the mass range of 2–20 kDa by accumulating 100 profiles for each sample using Launchpad software (V2.9, Shimadzu). The spectra were exported to SARAMIS for identification. A mean value of 75% confidence values for at least three spots on the target plate was considered a reliable identification for all samples (Alm et al., 2006; S. Jadhav et al., 2015; Welker & Moore, 2011).

2.10.3 Fingerprinting of isolated bacteria using MALDI-TOF MS

All bacterial masses extracted from VITEK® MS were extracted and subjected to protein profile fingerprinting using SPECLUST mass analyser software (v1.4). SPECLUST is a web tool for clustering protein mass spectra obtained from different instruments. The hierarchical clustering of mass spectra with SPECLUST can be useful for the screening of large MS proteomic datasets. Therefore, SPECLUST is a flexible tool for analysis of MS spectra (Alm et al., 2006; Granlund et al., 2011)

2.11 Diversity assessment using FT-IR spectroscopy

A diversity assessment of *E. coli* and *B. cereus* group isolates based on spectroscopic fingerprints was performed using the Bruker IR Biotyper® system. This uses FT-IR spectroscopy and is designed explicitly for microorganism subtyping. Sample preparation, data collection and data analysis were performed according to the manufacturer's guidelines.

2.11.1 Sample preparation for FT-IR spectroscopy

E. coli and *B. cereus* group isolates were grown at 37 °C on TSA Agar. Ethanol (70% v/v, 50 µL) was added to dedicated 1.5 mL Bruker suspension vials with inert metal cylinders for better homogenisation. One loopful of the pure overnight culture was emulsified into each tube. The sample material was taken directly from the confluent growth regions of the colonies using a 1 µL disposable inoculation loop and without transferring any agar into the sample, which required special care. The sample amount depended on the organism but typically corresponded to an overloaded loop. The biomass was submerged into the ethanol in the suspension vial and was removed from the plastic inoculation loop by flipping, with the metal cylinders facilitating biomass removal from the loop. The suspension vial was closed and vortexed to form a homogeneous suspension. The ethanol kills the microorganisms and stops further metabolic reactions. Then, deionised water (50 µL) was added to each vial to increase the surface tension of the suspension.

Each isolate suspension was pipetted on at least three spots of the IR-Biotyper silicon plate, with 15 µL per spot. Samples were applied as evenly as possible on the imprinted positions. The sample covered each imprinted position completely and formed a homogeneous layer, which was important to obtain optimal and reproducible results. In addition to loading the samples, Bruker Infrared test standards—IRTS 1 and IRTS 2 (12 µL of each), part of the Bruker IR Biotyper Kit—were spotted on the plate. Successful measurement of the test standards was mandatory for further spectra acquisition. After the spots were pipetted, the microtitre plate was incubated at 37 °C for approximately 10–30 minutes depending on factors such as the amount of material on the spots and the relative humidity with or without ventilation in the incubator. Over-drying of the samples was avoided to prevent biomass cracking and peeling off the microtitre plate.

2.11.2 FT-IR data acquisition

The prepared microtitre plate was loaded into the Bruker IR Biotyper® instrument. Technical replicates of all isolates were analysed in each independent experiment using IR Biotyper® software with the default analysis settings (Dinkelacker et al., 2018; Martak et al., 2019).

The IR Biotyper software checked different spectral properties during spectra acquisition, including absorbance intensity, signal-to-noise ratio and water vapour disturbance. This is called the spectral quality test. Spots with spectra that passed this quality test were displayed in green. If a spectrum failed the test, the corresponding spot was displayed in red. The status

of the run changed to COMPLETE after all positions were measured. Then, the final neighbour ID and the distance values for each sample were calculated and output as a table.

2.11.3 Interpretation of FT-IR results

Data analysis was performed using the IR Biotyper built-in exploration methods. Within a species, each spectrum was compared with all other spectra that were acquired via the same protocols and methods. The result of a comparison between two spectra resulted in a spectral distance value. The better the match between two spectra, the smaller the spectral distance value. Two spectra with a spectral distance of zero were considered to be identical; the greater the difference between two spectra, the higher their spectral distance value.

There are several ways to evaluate the distance between or similarity of two spectra. Mathematically speaking, each spectrum is a multidimensional vector. Therefore, the most straightforward approach is to calculate the Euclidean distance between those vectors, which is equivalent to measuring the distance between two objects in the real world. Other methods like ‘correlation’ and ‘cosine’ calculate the Pearson correlation coefficient and the angle between the vectors, respectively.

The two most useful linkage types are the ‘average linkage’ and Ward’s algorithm; therefore, these are recommended as a first approach. Although Ward’s method often results in very good and biologically meaningful clustering, one of its disadvantages is the distance scale, which varies with the number of items analysed. On the other hand, ‘average linkage’ shows a scale that is independent of the number of items. It allows the comparison of very different data sets and defines cut-off values for the distance to decide whether isolates are indistinguishable, closely related or unrelated. Linkage types define in which way the distance between two clusters is determined: the single linkage distance is the smallest distance between the members of the two clusters, the complete linkage takes the largest distance instead and the average linkage is based on the distance between the averages of the clusters.

Therefore, in the current study, relationships between the measured isolates were determined by hierarchical clustering analysis using Euclidean distance, average linkage and principal component analysis. The resulting dendrogram and scatter plot generated by the IR Biotyper® software were managed in BioNumerics, V7.6 (Campos et al., 2018; Cordovana et al., 2021; Martak et al., 2019).

2.12 Evaluation of the effectiveness of peracetic acid (PAA) against *E.coli* and *B. cereus* group isolates under different environmental conditions

2.12.1 Bacterial strain and inoculum preparation

E. coli and *B. cereus* group isolate inocula were prepared by transferring a single colony from an overnight BHI agar plate to BHI broth (10 mL). *E. coli* was incubated at 37 °C and *B. cereus* at 30 °C for 24–28 hours, then the broth was transferred to a centrifuge tube and centrifuged for 10 minutes at 2000 rpm and the supernatant was discarded. The suspended cells were washed by inverting the tube with physiological saline (0.9% w/v NaCl, 10 mL), centrifuged for another 10 minutes (2000 rpm) and the supernatant discarded. The wash procedure was repeated twice, after which the cell suspension was prepared by adding 0.9% w/v NaCl to the sedimented cells (10 mL) and vortexing. A certain volume (dependent on the growth) of this suspension was pipetted into a tube containing 0.9% w/v NaCl (10 mL) and the tube was mixed to achieve turbidity equal to 0.5 McFarland standard (approximate bacterial suspension 1.5×10^8 CFU·mL^{-1}; Baker et al., 1983; Reller et al., 2009). The exact count of each inoculum was obtained using the standard plate count method (Adams & Moss, 2008; Jay, 2005).

2.12.2 Disk diffusion assay

The antibacterial activity of peracetic acid (PAA) was evaluated using the agar disk diffusion assay, performed according to the method of Moradi et al. (2009) with some modifications. Three commonly used levels for the disk containing PAA (50, 100 and 200 ppm) were prepared by soaking the blank disks in different PAA concentrations followed by drying at 37 °C for 24 hours (Moradi et al., 2009). Then, bacterial culture inoculum (100 µL, as described in 2.11.1) was uniformly spread on the surface of each BHI agar plate and PAA disks were placed on the surface of the plates. All plates were incubated at 37 °C for 24 hours before measuring the zone of inhibition. Each combination of test conditions was performed in duplicate (Moradi et al., 2009; Sudhaus et al., 2014).

2.12.3 Statistical analysis

The statistical comparison of results was performed via two-way analysis of variance (ANOVA) using SPSS v18. Differences with P-values of < 0.05 were considered statistically significant (Banach et al., 2020).

Chapter 3

Prevalence, Characterisation and Diversity of *Escherichia coli* Isolated from Raw Milk

Abstract

Escherichia coli is an important indicator of faecal contamination. It is also a measure of the effectiveness of hygiene procedures in the food industry; that is, as an indicator of good hygienic practices, post-process contamination or inadequate processing of heat-treated foods. This study aimed to evaluate the prevalence of *E. coli* in raw milk samples collected in Victoria, Australia, and investigate the characteristics and diversity of these isolates. *E. coli* was recovered from 90% of raw milk samples, with no significant seasonal differences in raw milk contamination. However, only 4.2% of isolates carried the *sxt1* gene and could be considered as Shiga toxin–producing *E. coli* (STEC). The antibiotic susceptibility test results revealed that all raw milk *E. coli* isolates were resistant to at least one antibiotic, and 16.7% of isolates were multi-drug resistant. A high prevalence of antibiotic resistance was observed towards erythromycin, streptomycin, kanamycin and ampicillin. However, most *E. coli* isolates showed susceptibility to ciprofloxacin, chloramphenicol, gentamicin, trimethoprim-sulfamethoxazole and nalidixic acid. Most of the isolates (63.9%) could form a biofilm, although most had weak biofilm formation ability. *E. coli* that were isolated in autumn and winter had stronger biofilm formation ability than isolates collected in spring and summer. Genetic profiling using ERIC-PCR showed *E. coli* isolates to have bands ranging from 100 to 10000 bp, corresponding to a maximum band pattern of 14 and a minimum of five. According to the generated ERIC-PCR profiles, *E. coli* isolates could be divided into five major clusters and all three STEC isolates belonged to the same ERIC cluster. A strong correlation was observed between clusters, antimicrobial resistance and biofilm formation ability. ERIC-PCR also showed a correlation between clusters and the place of sampling. Overlay results showed that ERIC-PCR was able to discriminate *E. coli* isolates based on their characteristics and origin with an acceptable discriminatory power, especially to track the source of isolates. A comparison of FT-IR clusters with ERIC-PCR and MALDI-TOF MS clusters showed high agreement between some FT-IR and ERIC-PCR clusters but no significant correlation between FT-IR and MALDI-TOF MS clusters. The present study also showed that FT-IR typing has greater capability than MALDI-TOF MS for the typing and source-tracking of *E. coli* isolates, but genomics methods like ERIC-PCR still can provide more information about bacterial characteristics.

3.1 Introduction

E. coli is a common inhabitant of the intestines of warm-blooded animals and humans. Some *E. coli* strains can cause a wide variety of foodborne diseases, such as diarrhoea (Bintsis, 2017; Clermont, Bonacorsi, & Bingen, 2000). Milk and dairy products can be contaminated with *E. coli* from different sources. The main source of *E. coli* in raw milk and milk products is faecal contamination during the milking process. Therefore, *E. coli* is commonly used as an indicator of faecal contamination and the possible presence of enteric pathogens in raw milk (Ntuli et al., 2016; Ombarak et al., 2016; Smith & Fratamico, 2017).

There is an increasing number of people consuming raw (unpasteurised) milk. Enhanced nutritional qualities, taste and health benefits are reasons given for increased interest in raw milk consumption; however, scientific information to support these supposed properties of raw milk is inadequate (Artursson et al., 2018; Samet-Bali et al., 2013). However, epidemiological studies have provided conclusive evidence that a variety of pathogens can contaminate raw milk, some of which are associated with human disease (Artursson et al., 2018; Ombarak et al., 2016).

Several outbreaks and sporadic illness cases associated with enteropathogenic *E. coli* and enterohaemorrhagic *E. coli* (EHEC) have been reported (Kintz, et al., 2017). Pathogenic *E. coli* can cause disease in humans due to a collection of virulence factors. One of the most important *E. coli* pathotypes that has been reported in milk outbreaks is the Shiga toxin–producing *E. coli* (STEC), which can cause haemorrhagic colitis and haemolytic uraemic syndrome (Kintz et al., 2017; Ntuli, Njage, & Buys, 2016). STEC, also called verotoxin-producing *E. coli*, is the most important recently-emerged group of foodborne pathogens, especially the serotype O157:H7. Important STEC virulence-encoding genes associated with *E. coli* Shiga toxin-producing strains are *stx1*, *stx2* and the intimin gene *eae* (Muhlemann, 2014; Rey et al., 2006).

In Australia, outbreaks of disease by pathogenic *E. coli*-contaminated dairy products have not been reported. This is indicative of effective risk management in local dairy production. However, the safety of dairy products can only be guaranteed by a continuous awareness of the hazards and management of the risks throughout dairy production (Fegan & Desmarchelier, 2010).

The wide application of antimicrobial agents has led to an increase in bacterial antimicrobial resistance. Infection caused by resistant strains is usually more problematic for health authorities. Therefore, the identification of resistant organisms is crucial for the surveillance,

prevention and control of foodborne diseases. Accordingly, identifying antimicrobial resistance in humans and animals is important so that control measures via the use of antimicrobial agents can be effectively implemented (Harakeh et al., 2005; Ventola, 2015).

Biofilm formation ability is another important characteristic of pathogenic bacteria in the dairy industry. The development of biofilms assists *E. coli* with resistance to environmental stressors, such as disinfectants and sanitisers. Biofilm formation, especially production of the matrix components, protects *E. coli* from being killed by sanitisers (Beloin et al., 2008; DePas et al., 2014)

Additionally, describing the diversity and evolutionary relationships between pathogenic bacteria is key to understanding their sources, prevalence and distribution. Therefore, rapid and reliable typing methods for bacterial isolates are important tools for defining sources of pathogens. Available bacterial characterisation methods, like biochemical profiles, toxigenic profiles, antimicrobial susceptibility and biofilm formation ability, usually lack the discriminatory power to identify the relationships between closely-related isolates.

Several genotypic and alternative methods have been proposed to characterise bacteria and detect the differences present within different groups of microorganisms. These can subsequently be used to identify the sources from which the organisms were derived (Michelacci et al., 2016; Scott et al., 2002). Genomics techniques, like enterobacterial repetitive intergenic consensus (ERIC) PCR, have been successfully used for this purpose (Li et al., 2009; Merzougui et al., 2013). Profiling based on proteomic data obtained by MALDI-TOF MS has also gained in popularity in clinical laboratories for bacterial identification and typing, as it does not involve complicated sample preparation (Jadhav et al., 2015; Lasch et al., 2016; Welker & Moore, 2011). Fourier-Transform Infrared (FT-IR) spectroscopy is another recently developed method for rapid bacterial typing. It is a spectral-based technique that quantifies the absorption of infrared light by molecules present in the sample, such as carbohydrates, lipopolysaccharides, lipids, nucleic acids and proteins, resulting in the generation of a specific FT-IR spectrum that reflects the overall chemical composition of the isolates. This technique can be used for bacterial species identification by comparing the spectrum to a reference database. FT-IR has also been used for the typing of bacterial isolates (Campos et al., 2018; Dinkelacker et al., 2018). In the last few years, FT-IR has been used in many studies for the discrimination of bacteria at different taxonomic levels. It has been recognized as a simple, rapid, cost-effective and reliable bacterial typing technique (Alvarez-

Ordonez & Prieto, 2012; Cordovana et al., 2021a; Martak et al., 2019, Wang -Wang et al., 2022).

The main objective of this chapter is to report on the study of the prevalence of *E. coli* in raw milk in Victoria, Australia. The characteristics of isolated *E. coli,* including their toxigenic profile, antimicrobial resistance and biofilm formation ability, were explored. Additionally, the diversity of the *E. coli* isolates was investigated using genomic fingerprinting (ERIC-PCR), proteomic fingerprinting (MALDI-TOF MS) and FTIR spectroscopy to evaluate which of these provided the fastest and most reliable method to determine the diversity of the *E. coli* strains.

3.2 Results

3.2.1 Prevalence of *E. coli* in raw milk

From a total of 80 raw milk samples collected from three different sampling points (SP1, SP2 and SP3) in Victoria, *E. coli* was isolated from 72 samples (90% contamination level). The highest percentage of *E. coli*–contaminated samples was recorded at SP2 (21 out of 22 raw milk samples, 95.4%), followed by SP3 (14 out of 16 raw milk samples, 87.5%). The least number of contaminated samples was detected in raw milk samples obtained from SP1 (29 out of 34 raw milk samples, 85.3%). According to these results, no significant seasonal differences were found in raw milk contamination ($P \leq 0.05$).

All *E. coli* isolates were analysed using MALDI-TOF MS. The Vitek MS produced 97.2% identification accuracy at the genus level and 88.9% accuracy at the species level for the *E. coli* isolates. However, the Bruker Biotyper® produced 98.6% identification accuracy at the genus level and 95.8% at the species level for the *E. coli* isolates. Therefore, results from the present study and similar research showed both the Vitek MS and Bruker Biotyper® MALDI-TOF MS systems to be accurate tools for identifying *E. coli* isolated from raw milk.

3.2.2 Virulence gene distribution

Of the three virulence genes that were tested for in the raw milk isolates, only three *E. coli* isolates contained *sxt1* and *eae* (4.2%) and could be considered STEC. All these isolates were from SP2 and all were placed in the A2 ERIC cluster (see section 3.2.5). So, 13.6% of the SP2

isolates could be considered as STEC, and all originated from the same source. The results indicated that the rest of the samples did not carry any STEC genes.

3.2.3 Antimicrobial susceptibility testing

The antibiotic susceptibility test results revealed that all raw milk *E. coli* isolates were resistant to at least one out of 12 tested antibiotics, and 16.7% (n = 12) of isolates were multi-drug resistant (Table 3.1). A high prevalence of antibiotic resistance was observed towards erythromycin, streptomycin, kanamycin and ampicillin. However, most of the *E. coli* isolates showed susceptibility to ciprofloxacin, chloramphenicol, gentamicin, trimethoprim-sulfamethoxazole and nalidixic acid (Figure 3.1).

The results also showed higher antimicrobial resistance for SP3 isolates, followed by SP2 isolates, for most of the antimicrobial agents. The lowest antimicrobial resistance was found for SP1 isolates. No significant seasonal differences were found in antimicrobial resistance in the raw milk isolates ($P \leq 0.05$).

Table 3.1 Antimicrobial resistance profiles by place of sampling of *E. coli* isolated from raw milk samples.

	Total	**SP1**	**SP2**	**SP3**
Antimicrobial agent	**(*n* = 72)**	**(*n* = 34)**	**(*n* = 22)**	**(*n* = 16)**
Amikacin	11 (15.3%)	3 (8.8%)	6 (27.3%)	2 (12.5%)
Ampicillin	30 (41.7%)	12 (35.3%)	10 (45.4%)	9 (56.2%)
Ceftriaxone	9 (12.5%)	2 (5.9%)	3 (13.6%)	4 (25%)
Chloramphenicol	2 (2.7%)	1 (2.9%)	1 (4.5%)	0
Ciprofloxacin	1 (1.4%)	0	1 (4.5%)	0
Gentamycin	3 (4.2%)	1 (2.9%)	1 (4.5)	1 (6.2%)
Erythromycin	72 (100%)	34 (100%)	22 (100%)	16 (100%)
Kanamycin	32 (44.4 %)	12 (35.3%)	12 (54.5%)	11 (68.7%)
Nalidixic acid	5 (6.9%)	2 (5.9%)	2 (9.1%)	1 (6.2%)
Streptomycin	57 (64.7%)	22 (64.7%)	16 (72.7%)	16 (100%)
Tetracycline	8 (11.1%)	4 (11.8%)	3 (13.6%)	1 (6.2%)
Trimethoprim/sulfamethoxazol	4 (5.6%)	1 (2.9%)	2 (9.1%)	1 (6.2%)
Resistance to 2 antimicrobials	31 (43.1%)	12 (35.3%)	10 (45.4%)	9 (56.2%)
Resistance to 3 antimicrobials	22 (30.6%)	8 (23.5%)	8 (36.4%)	6 (37.5%)
Resistance to > 3 antimicrobials	12 (16.7%)	5 (14.7%)	4 (18.2%)	3 (18.7%)

Note: The absolute number of resistant isolates is reported, followed by the proportion.

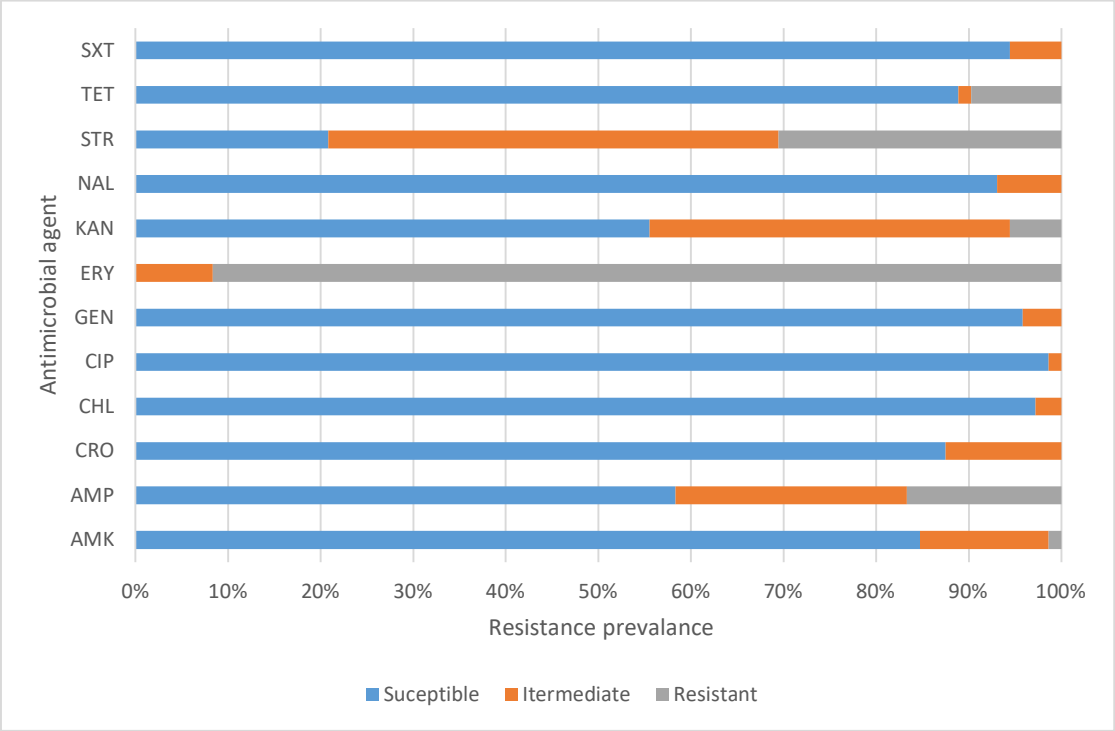

Figure 3.1 Distribution of antimicrobial resistance in the *E. coli* isolates from raw milk

3.2.4 Biofilm formation assay results

Table 3.2 show the biofilm formation ability of the tested *E.coli* strains. Most of the isolates (63.9%) could form a biofilm, although most had weak biofilm formation ability and only 9.7% showed strong biofilm formation. The highest level of biofilm formation ability was observed in SP1 isolates and the lowest in SP3 isolates. *E. coli* that were isolated in autumn and winter had greater biofilm formation ability than isolates collected in spring and summer.

Table 3.2 Biofilm formation ability of *E. coli* isolated from raw milk samples.

	Total	**SP1**	**SP2**	**SP3**
Biofilm formation ability	(*n* = 72)	(*n* = 34)	(*n* = 22)	(*n* = 16)
Non biofilm producer	26 (36.1%)	10 (29.4%)	7 (31.8%)	9 (56.2%)
Weak biofilm producer	30 (41.7%)	14 (41.2%)	10 (45.5%)	6(37.5%)
Moderate biofilm producer	9 (12.5%)	5 (14.7%)	3 (13.6%)	1 (6.3%)
Strong biofilm producer	7 (9.7%)	5 (14.7 %)	2 (9.1%)	0

Note: The absolute number of resistant isolates is reported, followed by the proportion.

As shown in Table 3.2, the *E.coli* strain isolates from different sources had different biofilm formation abilities. Most of the isolates from SP1 were weak biofilm producers and none were strong biofilm producers.

3.2.5 ERIC-PCR fingerprinting and diversity assays results for *E.coli*

The development and distribution of copies of the ERIC sequence were investigated in the genome sequences of all 72 isolates of *E. coli*. PCR with ERIC primers generated profiles with bands ranging from 100 to 10000 bp, which corresponded to a maximum band pattern of 14 and a minimum of five [Appendix: Figures A1, A2, A3, A4]. ERIC-PCR genotyping results showed that the size, number and characteristics of the bands, and thus the overall DNA fingerprints, were similar to those observed in previous research (Gao et al., 2018; Wilson & Sharp, 2006).

The dendrograms derived from ERIC-PCR are shown in Figure 3.3. According to the generated ERIC profiles, the *E. coli* isolates could be divided into five major clusters: A, B, C, D and E; clusters A and B had two sub-clusters (A1 and A2) and (B1 and B2) and the rest C, D and E had no sub-clusters. The dendrograms also showed that most of the isolates from SP1 were placed in clusters A2 and B1. Most of the isolates from SP2 were placed in clusters B2 and D, while isolates from SP3 were divided between the other clusters but not in clusters C and E (Figures 3.3 and 3.4).

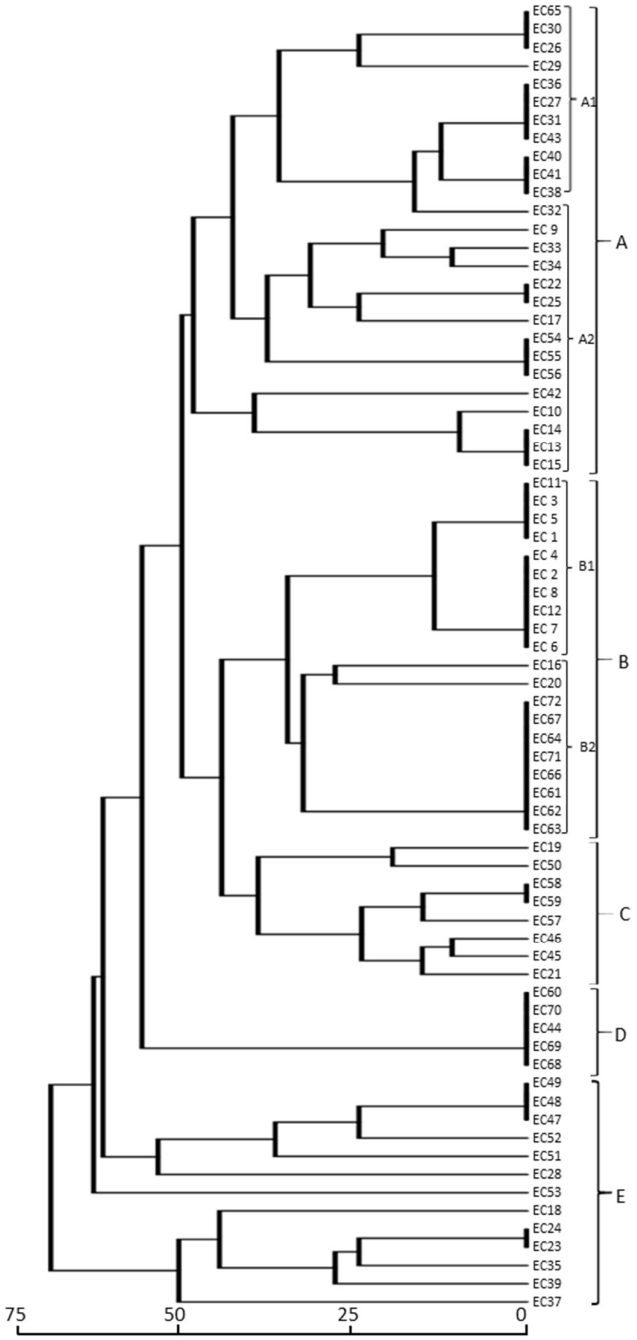

Figure 3.2 Dendrogram based on ERIC-PCR profiles of *E.coli* isolates.

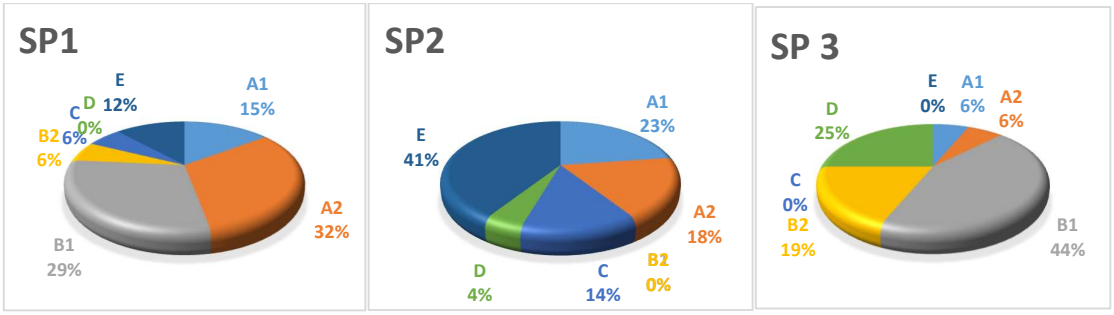

Figure 3.3 Distribution of *E. coli* isolates from different sampling points based on their ERIC-PCR profiles. SP1: sampling point 1; SP2: sampling point 2; SP3: sampling point 3.

The results showed that all three STEC isolates belonged to the A2 ERIC cluster. A strong correlation was observed between clusters and antimicrobial resistance, with most of the resistant isolates placed in cluster B2 and most of the sensitive isolates placed in clusters A2 and B1. Additionally, most of the strong biofilm producers were placed in cluster A1 and most of the weak and non-biofilm producer isolates were placed in clusters A2 and B, with the lowest observed in B2 and D.

Overall, these results showed that ERIC-PCR was able to discriminate *E. coli* isolates based on their characteristics and origin with acceptable discriminatory power, especially to track the isolate sources.

3.2.6 MALDI-TOF MS diversity assay results

After acquiring the protein profile masses of all *E. coli* isolates (Figure 3.5), the extracted masses from the VITEK® MS were subjected to fingerprinting of the protein profiles using the SPECLUST mass analyser software.

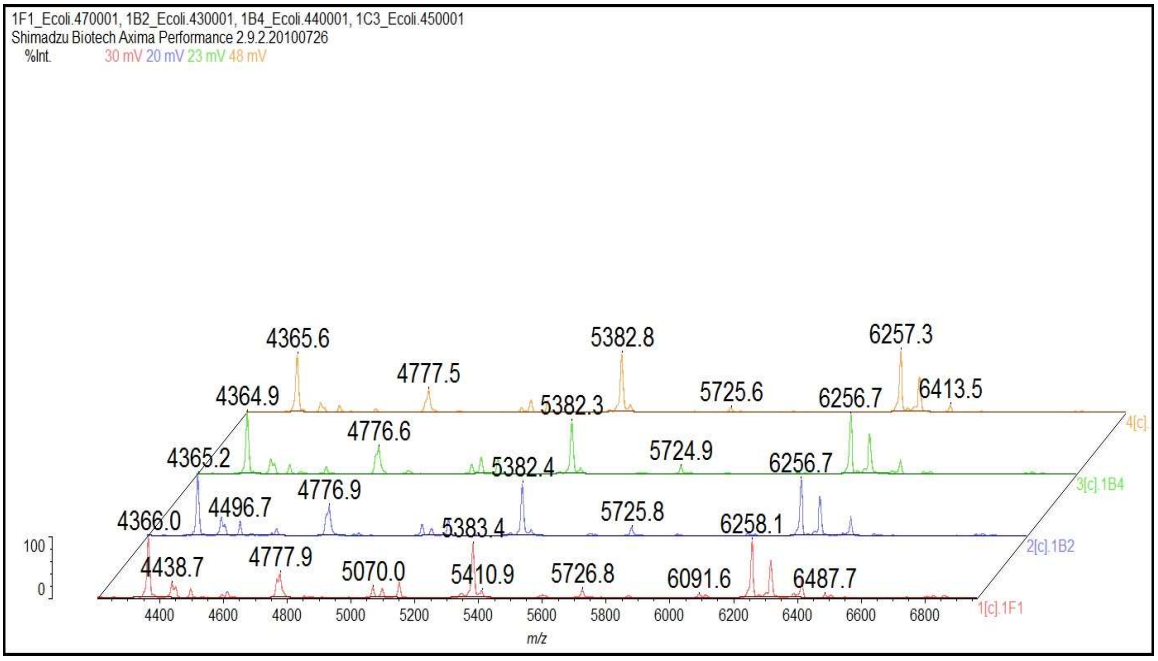

Figure 3.4 MALDI-TOF MS profiles of four *E. coli* isolates (EC43, EC44, EC45 and EC47) from raw milk samples.

The dendrogram derived from the MALDI-TOF mass spectra is shown in Figure 3.6. *E. coli* isolates were divided into five clusters (A, B, C, D and E). Although most isolates from cluster B belonged to SP1 and cluster C belonged to SP3, the rest of the isolates are distributed into different clusters (Figure 3.7). Additionally, all three STEC isolates belonged to cluster A. No correlation was observed between MALDI clusters and antimicrobial resistance. The results showed that most of the strong biofilm producers were placed in clusters B and C but the rest were placed in different clusters.

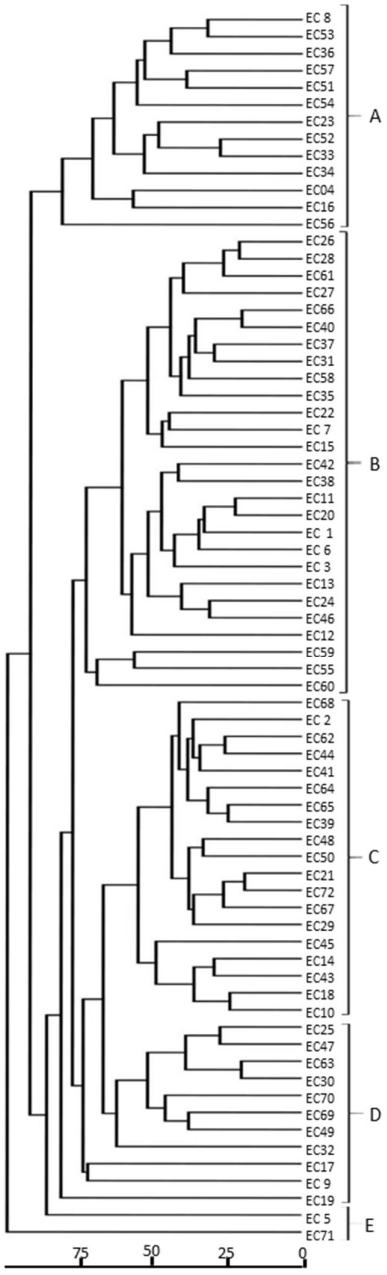

Figure 3.5 Dendrogram based on MALDI-TOF MS profiles of *E. coli* isolates.

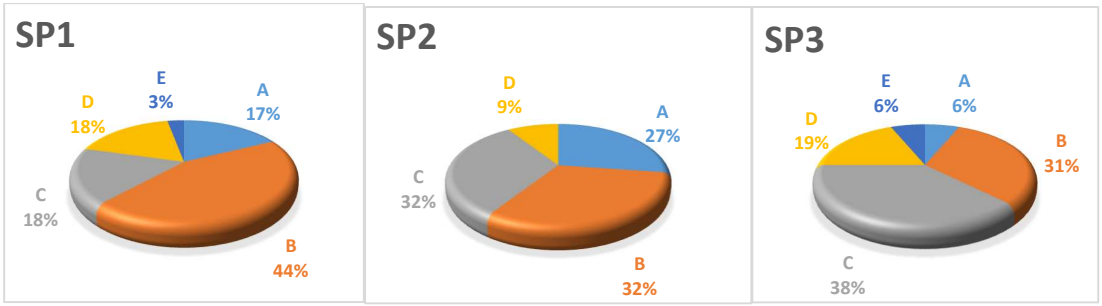

Figure 3.6 Distribution of *E. coli* isolates from different sampling points based on their MALDI-TOF profiles. SP1: sampling point 1; SP2: sampling point 2; SP3 sampling point 3.

Overall, the results showed that MALDI-TOF MS could discriminate between *E. coli* isolates based on their characteristics and origin, but its discriminatory power was considerably lower than that of ERIC-PCR.

3.2.7 FT-IR stereoscopy diversity assay results

The assessment of diversity using FT-IR spectroscopy showed that *E. coli* isolates could be divided into four major clusters (A, B, C and D; Figure 3.8). Isolates in clusters B and C were closer in their hierarchical position. FT-IR showed that most of the isolates from SP1 were placed in clusters B and D. Most of the isolates from SP3 were placed in cluster C but isolates from SP2 were divided between clusters D, B and C (Figure 3.9).

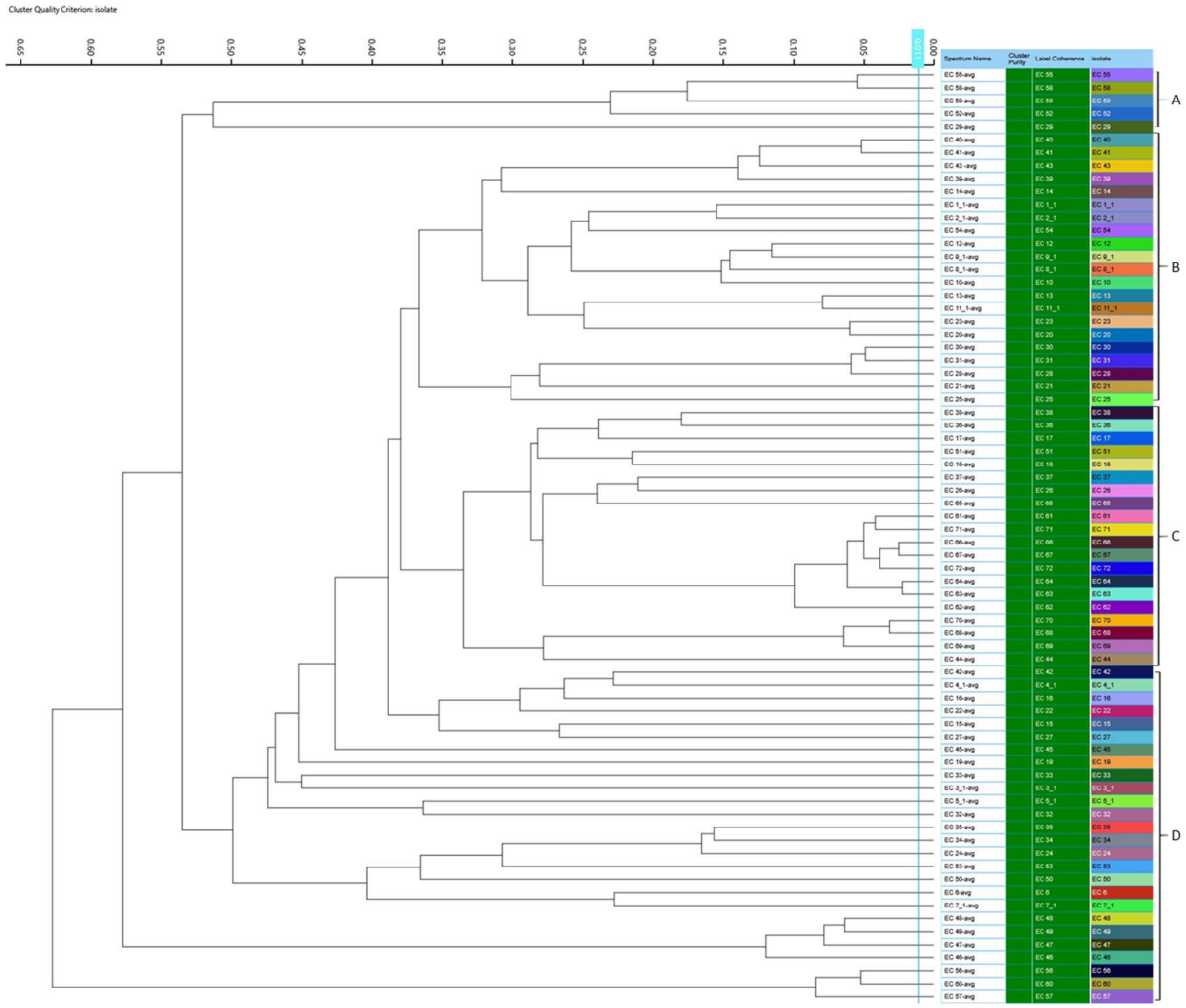

Figure 3.7 Dendrogram based on FT-IR spectroscopy profiles of *E.coli* isolates.

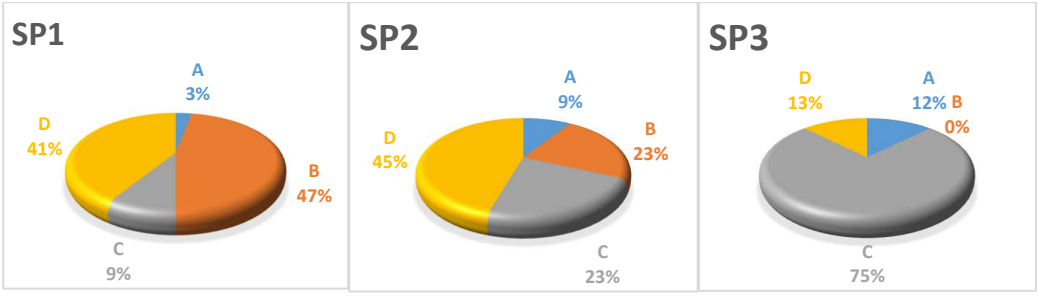

Figure 3.8 Distribution of *E. coli* isolates from different sampling points based on their FT-IR profiles.

SP1: sampling point 1; SP2: sampling point 2; SP3: sampling point 3.

The results showed that all STEC isolates belonged to cluster D. A correlation was observed between clusters and antimicrobial resistance, with most of the sensitive isolates placed in cluster B. No correlation was observed between clusters and the biofilm formation ability of isolates.

A comparison of FT-IR, ERIC-PCR and MALDI-TOF MS clusters showed high similarity between FT-IR cluster B and ERIC cluster A. There was also a correlation between FT-IR cluster C isolates and ERIC-PCR cluster B isolates. There was no similarity between the FT-IR and MALDI-TOF MS clusters.

3.3 Discussion

Escherichia coli are widespread in the environment and some can cause human infections (Gundogan & Avci, 2014; Wang et al., 2017). *E. coli* is one of the indicators of faecal contamination as it is rarely pathogenic to humans. The presence of *E. coli* in food is indicative of the possibility of faecal contamination and indicates that other pathogenic microorganisms of a faecal source may be present. To date, *E. coli* is recognised as the best indicator of faecal contamination of the commonly used faecal-indicator organisms (Bessalah et al., 2016; Scott et al., 2003; Smith & Fratamico, 2016).

Milk and dairy products can contain a variety of microorganisms and can also be a source of foodborne bacterial pathogens. The presence of bacterial pathogens in milk is due to direct contact with contaminated sources at the dairy farm. Although pasteurisation is an effective way to ensure that milk is safe for consumption, post-pasteurisation contamination or consumption of raw milk can lead to outbreaks of foodborne illness (Claeys et al., 2013; Oliver et al., 2005). Additionally, raw and unpasteurised milk is still consumed by some people in rural areas and indirectly by a much larger population through the consumption of some types of raw milk cheeses (Artursson et al., 2018; Samet-Bali et al., 2013). Based on studies of Australian dairy farms, *E. coli* is transmitted in healthy animals and is an important hazard in the food safety risk profile for the dairy industry (Fegan & Desmarchelier, 2010). Although dairy products have not been associated with reported pathogenic *E. coli* outbreaks in Australia, continual awareness of the hazards and management of the risks in the Australian dairy industry is very important (Berger, 2014; Fegan & Desmarchelier, 2010). Accordingly, the current study aimed to determine the occurrence, characteristics and diversity of *E. coli* in raw milk to evaluate the risk associated with the contamination of milk in the Victorian dairy industry.

E. coli was isolated from 72 samples; 90% of the raw milk samples had *E. coli* contamination. The highest percentage of *E. coli*–contaminated samples was recorded in SP2 at 95.4%, followed by SP3 at 87.5%. The least number of contaminated raw milk samples was from SP1 (85.3%). According to the results, no significant seasonal differences in raw milk contamination were found ($P \leq 0.05$). However, the results showed a higher level of *E. coli* contamination in the samples collected in the present study than in similar research in other countries (Gundogan & Avci, 2014; Ntuli et al., 2016; Oliver et al., 2005; Ombarak et al., 2016).

The presence of *E. coli* in raw milk products is an important indicator of faecal contamination and hygienic practices. It has been shown that *E. coli* outbreaks could be linked to the consumption of milk and dairy products, so a high level of *E. coli* contamination is considered a food safety concern (Lambertini et al., 2015; Ombarak et al., 2016). So, good dairy farming practices can help to reduce raw milk contamination levels.

In the present study, after subjecting all *E. coli* isolates to the two MALDI-TOF MS systems, VITEK® MS gave 97.2% identification accuracy at the genus level and 88.9% at the species level. The Bruker Biotyper® gave 98.6 % identification accuracy at the genus level and 95.8% at the species level. Several studies have evaluated the bacterial identification proficiency of these two market-leading instruments (Bruker Biotyper® and VITEK® MS) with their respective software and databases. In the most comprehensive study, Cherkaoui et al. (2010) conducted a comparative study with 720 bacterial isolates under laboratory conditions. According to the manufacturer's default recommendations, the isolates were analysed in parallel on both devices and the MALDI-TOF MS results were also compared with conventional biochemical identification tests. The Bruker MS system gave high-confidence identification for 680 of the 720 isolates (94.4%), whereas the VITEK® MS gave high-confidence identification for 639 isolates (88.8%). The results also showed that only 0.9% of the Bruker Biotyper® and 0.5% of the VITEK® identifications were incorrectly designated as high-confidence at the species level. All the high-confidence MS identifications were accurate at the genus level. Additionally, the Bruker Biotyper® and the VITEK® MS identified 69% and 38% of 13 isolates that were not identified by conventional phenotyping methods, respectively (Cherkaoui et al., 2010; Croxatto et al., 2012; Veen et al., 2010). Results from the present study and other similar research showed that both the VITEK® MS and Bruker Biotyper® MALDI-TOF MS systems were accurate tools for identifying *E. coli* isolated from raw milk.

In the present study, three *E. coli* isolates contained the *sxt1* gene (4.2%) and were considered as STEC. All these isolates were from SP2. So, 13.6% of the SP2 isolates were considered as STEC and all STEC isolates were from the same source. STEC are some of the most important *E. coli* strains because they may cause a life-threatening problem (Hosseini et al., 2013).

Dairy farms across the world are known to be reservoirs of STEC and other pathogenic *E. coli*, including in the United States, Europe, Oceania and Japan (Lambertini et al., 2015). The detection of STEC in dairy products has been reported at different levels in different countries, ranging from 0.45% in Spain to 13% in France (Ombarak et al., 2016; Paneto et al., 2007; Rugeles et al., 2010). Therefore, the present study showed a similar prevalence of STEC contamination to most of the similar research in other countries. However, whereas other studies have indicated more contamination in warm weather (Lambertini et al., 2015; Monaghan et al., 2011), no seasonal correlation was observed in the present study.

At present, antibiotic resistance is a major public health concern. In both developed and developing countries, antibiotics are generally used as growth supplements in livestock; these can affect the environmental microbiome and lead to an increase in the proportion of resistant microorganisms (Jamali et al., 2015; Ventola, 2015). Studies have shown that the antibiotic-resistant bacteria that exist in milk could be transferred to humans by the ingestion of milk (Gundogan & Avci, 2014; Ntuli et al., 2016). Therefore, studying the antimicrobial resistance of animal-originating pathogens is a major part of the characterisation in bacterial research (Jamali et al., 2015; Ntuli et al., 2016).

The results of the antibiotic susceptibility test revealed that all raw milk *E. coli* isolates were resistant to at least one antibiotic and 16.7% (n = 12) of isolates were multi-drug resistant. A high prevalence of antibiotic resistance was observed towards erythromycin, streptomycin, kanamycin and ampicillin. However, most of the *E. coli* isolates showed susceptibility to ciprofloxacin, chloramphenicol, gentamicin, trimethoprim-sulfamethoxazole and nalidixic acid. A high level of agreement was found between the findings of this study and other research on antibiotic resistance from around the world (Dolejska et al., 2008; Gundogan & Avci, 2014; Ntuli et al., 2016).

Additionally, the results showed that there was higher antimicrobial resistance in isolates from SP3, followed by SP2, for most of the antimicrobial agents. The lowest level of antimicrobial resistance was found in isolates from SP1. No significant seasonal differences were found in the antimicrobial resistance of raw milk isolates.

Biofilms are an important source of microbial contamination that have received little attention in the dairy industry. They are one of the main recontamination sources for milk (Beloin et al., 2008; Marchand et al., 2012; Teh et al., 2014a). Furthermore, it has been shown that the biofilm formation ability of *E. coli* increases its antibiotic resistance (Marchand et al., 2012).

In the present study, most of the isolates (63.9%) could form a biofilm. However, most had weak biofilm formation ability and only 9.7% of isolates showed strong biofilm formation ability. Picoli et al. (2017) indicated that *E. coli* was the most resistant bacteria and 56.3% produced biofilm, while Milanov et al. (2015) showed that 64% of *E. coli* isolates had biofilm formation ability, which was similar to the findings of this study.

In the present study, the highest level of biofilm formation ability was observed in isolates from SP1 and the lowest was observed in isolates from SP3. *E. coli* isolated in autumn and winter had higher biofilm formation ability than isolates collected in spring and summer. Also, although it has been shown that biofilm formation ability can increase the antibiotic resistance of *E. coli* (Marchand et al., 2012), no correlation was found between biofilm formation ability and antimicrobial resistance in the present study.

Several microbial source–tracking methods have shown some success in differentiating and predicting the host sources of *E. coli*. The ERIC-PCR genomic fingerprinting method has been used extensively to classify various bacterial species at the subspecies and strain levels (Leung et al., 2004; Vivek Prabhu, 2010; Wilson & Sharp, 2006). As previously described, ERIC-PCR is a PCR-based typing method and therefore has the advantages of accuracy, ease of use and low cost (Vivek Prabhu, 2010; Otokunefor, Ogugbue & Fajoyomi, 2020). Interestingly, ERIC copy numbers are significantly diverse among different strains of *E. coli.* This diversity suggests the evolution processes among bacterial strains within a particular species like *E. coli.* The clonal variability in bacterial species, such as *E. coli*, is performed by homolog primers to ERIC sequences, in which the emerged patterns are valuable for evaluating the phylogenetic closeness (Wilson & Sharp, 2006Ranjbar et al., 2017).

In this study*,* the ERIC-PCR profiles of *E. coli* isolates had bands ranging from 100 to 10000 bp, which corresponded to a maximum band pattern of 14 and a minimum of five. ERIC-PCR genotyping results showed that the size, number and characteristics of the bands were similar to those observed in other research (Gao et al., 2018; Li et al., 2016; Rugeles et al., 2010; Vivek Prabhu, 2010).

The dendrograms derived from ERIC-PCR showed that the *E. coli* isolates could be divided into five major clusters, A, B, C, D and E. Clusters A and B contained two sub-clusters (A1

and A2) and (B1 and B2) and there rest contained no sub-clusters. The ERIC-PCR results also showed that most of the isolates from SP1 were placed in clusters A2 and B1, which showed that these isolates originated from two separate sources. Most of the isolates from SP3 were placed in clusters B2 and D, and isolates from SP2 were divided between the other clusters. Additionally, all three STEC isolates belonged to the A2 cluster. Furthermore, a strong correlation was observed between clusters and antimicrobial resistance. Most of the resistant isolates were placed in cluster B2 and most of the sensitive isolates were placed in clusters A2 and B1. Other similar research has shown that isolates of the same *E. coli* strain can express the same resistance patterns; however, resistance patterns cannot be explained only by strain prevalence (Chen et al., 2010; Sabate et al., 2008; Smith et al., 2007). Finally, the highest levels of biofilm formation ability were observed in clusters A2 and B1, and the lowest levels were observed in clusters B2 and D. Significant correlation between biofilm formation ability and ERIC-PCR genotype was also observed in some similar studies (Chen et al., 2010).

These findings, in combination with other studies, showed that ERIC-PCR can be used to predict some bacterial characteristics of *E. coli* isolates based on the similarity of their origin (Chen et al., 2010; Leung, Mackereth & Tien, 2004; Radmehr et al., 2020).

In the past decade, several studies have suggested that MALDI-TOF MS is capable of tracing *E. coli* strains (Clark et al., 2013; Santos et al., 2015; Weiss et al., 2019). Based on the MALDI-TOF dendrograms obtained in the present study, the *E. coli* isolates were divided into five clusters. Although most isolates from clusters B and C were from SP1 and SP3, respectively, the rest of the isolates were distributed into different clusters. All three STEC isolates belonged to cluster A and no correlation was observed between MALDI clusters and antimicrobial resistance. Finally, most of the strong biofilm producers were placed in clusters B and C but the rest were placed in different clusters.

Despite there being many studies on the identification of microorganisms by MALDI-TOF MS, few studies have tried to discriminate between strains of the same species using this technique (Giebel, Fredenberg, & Sandrin, 2008; Santos et al., 2015). To the best of our knowledge, this study is the first investigation comparing MALDI-TOF MS clusters with those obtained by ERIC-PCR for *E. coli* isolated from raw milk.

Similar studies have suggested an acceptable correlation between phenotypic characteristics and phylogenetic relationships as delineated by MALDI-TOF and MLST, but isolate clustering did not correspond to the clusters obtained by PFGE. This was probably because PFGE profiles

result from the accumulation of multiple mutational and horizontal gene transfer events that are not revealed in the expression of the bulk of proteins screened in MALDI-TOF MS via established methods (Clark et al., 2013; S. Jadhav et al., 2015; Novais et al., 2014). However, some research has even shown good correlation between phenotypic characteristics obtained by MALDI-TOF and PFGE (Jadhav et al., 2012; Jadhav et al., 2015; Novais et al., 2014).

Despite the accepted advantages of MALDI-TOF MS compared to genomics methods, such as rapidity, accuracy, sensitivity and specificity, this study showed that using MALDI-TOF MS and clustering data analysis could not discriminate between *E. coli* isolates from raw milk with as much success as ERIC-PCR. Thus, the findings of this study were in accordance with those of other studies; for example, Feng et al. (2020) reported that MALDI-TOF MS gave poor bacterial classification and identification accuracy for *E. coli* and *Shigella*. Other similar studies have also suggested that, despite the advantages of MALDI-TOF MS, other traditional and molecular typing methods provide more complementary information (Feng et al., 2020; Novais et al., 2014; Santos et al., 2015; Wenning et al., 2014).

FT-IR spectroscopy is a phenotypic method that has been conventionally applied for determining the molecular composition of samples. FT-IR can also discriminate bacterial strains by quantifying the absorption of IR light according to the variety of molecules present in the bacteria, particularly the component carbohydrates and lipopolysaccharides in the bacterial cell wall (Dinkelacker et al., 2018; Hu et al., 2021; Martak et al., 2019; Passaris et al., 2022). Recently, several research groups have assessed the discriminatory power of FT-IR as a rapid and high-throughput technique for bacterial typing. In most of these studies, the discriminatory power of FT-IR was compared with routine genotyping methods such as MLST and PFGE (Cordovana et al., 2021b; Dinkelacker et al., 2018; Martak et al., 2019; Radmehr et al., 2020).

Accordingly, in the current study, the application of FT-IR for the assessment of the diversity of *E. coli* isolates from raw milk was explored. The results showed that the *E. coli* isolates could be divided into four major clusters, A, B, C and D. Isolates in clusters B and C were closer in their hierarchical position. Most of the isolates from SP1 were placed in clusters B and D. Most of the isolates from SP3 were placed in cluster C but isolates from SP2 were divided between clusters D, B and C. Additionally, all STEC isolates belonged to cluster D. Significant correlation was observed between clusters and antimicrobial resistance, with most

of the sensitive isolates being placed in cluster B. No correlation was observed between clusters and the biofilm formation ability of the isolates.

A comparison of the FT-IR clusters with the ERIC-PCR and MALDI-TOF clusters showed high similarity between FT-IR cluster B and ERIC-PCR cluster A. There was also a correlation between FT-IR cluster C isolates and ERIC-PCR cluster B isolates. There was no significant similarity between any of the FT-IR and MALDI-TOF clusters.

This finding was in agreement with similar studies which indicated that, despite the suitability and applicability of MALDI-TOF MS for routine microbial identification of foodborne microorganisms, FT-IR is a better method for typing *E. coli* isolates than MALDI-TOF MS (Dinkelacker et al., 2018; Feng et al., 2020; Wenning et al., 2014).

3.4. Conclusion

The present study showed that *E. coli* contamination of raw milk samples in the Victorian dairy industry is high but varies in different regions. Despite the high-level contamination, very few isolates carried STEC virulence genes. The antibiotic susceptibility test results showed that all raw milk *E. coli* isolates were resistant to at least one antibiotic. However, most of the isolates were susceptible to three or more antibiotics. Additionally, a high number of isolates could form a biofilm, although most of them had weak biofilm formation ability. However, pasteurisation can easily eliminate *E. coli*. These findings indicated that the Australian dairy industry should be vigilant in preventing any possible cross-contamination by applying food hygienic practices. The present study also showed that FT-IR has greater capability than MALDI-TOF MS for the typing and source-tracking of *E. coli* isolates. However, genomics methods like ERIC-PCR provide more information about bacterial characteristics.

Chapter 4

Prevalence, Characterisation and Diversity of *Bacillus cereus* group Isolates from Raw and Pasteurised Milk

Abstract

Bacillus cereus sensu lato (the *B. cereus* group) is a problematic foodborne pathogen and food spoilage organism that is present in many environmental sources. Unfortunately, the elimination of *B. cereus* group contamination is not guaranteed by current pasteurisation and sanitation processes for milk and dairy products. This study aimed to evaluate the prevalence of the *B. cereus* group in raw and pasteurised milk samples collected in Victoria, Australia, and to investigate the characteristics and diversity of these isolates. *B. cereus* group was recovered from 42.3% of 97 raw and pasteurised milk samples, with higher recovery from pasteurised milk samples. Virulence studies identified genes *nheA*, *nheB*, *hblA* and *nheC* in most isolates and the *cyk* gene in 46% of all isolates, indicating that many potentially diarrhoeal strains were present in the collected samples. Antimicrobial susceptibility testing showed a high prevalence of antibiotic resistance towards ampicillin, ceftriaxone and penicillin, with all isolates resistant to at least two antimicrobials. Antimicrobial resistance was considerably higher in pasteurised milk samples than in raw milk samples. The biofilm formation capacity within the *B. cereus* group isolates showed that most (53.7%) could form a biofilm, with a higher prevalence of biofilm producers observed in pasteurised milk isolates (57%). Genetic profiling of isolates using ERIC-PCR placed most *B. cereus* group isolates from pasteurised milk in the same cluster, demonstrating that they probably originated from a similar source, whereas the raw milk isolates showed greater diversity, indicating various sources. Chemical profiling with the new IR Biotyper (Bruker Daltonics GmbH), which uses Fourier-transform infrared (FT-IR) spectroscopy, showed high agreement with genetic (ERIC-PCR) profiling. In contrast, a low level of agreement between proteomic (MALDI-TOF MS) and genetic typing was observed. The present study showed that the IR Biotyper could be adopted as a rapid and low-cost tool for the typing of *B. cereus* group isolates. Overall, the virulence and antimicrobial resistance characteristics, together with the capability of isolates to produce biofilm, clearly indicated the importance of the *B. cereus* group in the Australian dairy industry.

4.1 Introduction

Bacillus cereus sensu lato, commonly known as the *Bacillus cereus* group, contains at least eight species: *Bacillus cereus* (*sensu strico*)*, Bacillus anthracis*, *Bacillus cytotoxicus, Bacillus mycoides, Bacillus pseudomycoides, Bacillus thuringiensis, Bacillus toyonensis* and *Bacillus weihenstephanensis* (Gdoura-Ben Amor et al., 2018; Porcellato et al., 2019; Muggi et al., 2022). The *B. cereus* group is recognised as a major foodborne pathogen. It is also an important spoilage organism in milk and dairy products because its elimination is not guaranteed by current pasteurisation and sanitation processes (Kumari & Sarkar, 2016; Mugadza & Buys, 2017). *B. cereus* group toxins can cause two different forms of foodborne diseases that induce diarrhoea or vomiting (Granum et al., 2014; Griffiths & Schraft, 2017; Merzougui et al., 2013). The diarrhoeal syndrome is caused by the production of the enterotoxins haemolysin BL (HBL), non-haemolytic enterotoxin (NHE) and cytotoxin K (CytK). Emetic food poisoning is caused by cereulide, a heat-stable dodecadepsipeptide toxin (Merzougui et al., 2013; Yu et al., 2020).

The heat-resistant spores of the *B. cereus* group survive pasteurisation and can cause problems in a wide range of dairy products. Some *B. cereus* group strains can adhere to solid surfaces in the dairy processing environment and produce multicellular biofilm communities. These biofilms are protected from chemicals and antimicrobial agents during plant cleaning because extracellular polymeric substances limit the penetration of disinfectants into the bulk of the biofilm structure (Hussain & Oh, 2017; Montville, 2012; Organji et al., 2015).

Recently, the high levels of antimicrobial resistance among bacteria isolated from food—including the *B. cereus* group—have become a public health concern. In Australia, antibiotic-resistant *B. cereus* group bacteria were the most prevalent bacteria isolated from dairy farm environments (Eglezos et al., 2010; Fernandes & Ackerman, 1989; McAuley et al., 2014).

Describing the diversity and evolutionary relationships between pathogenic bacteria is important to understand their origins, prevalence and distribution. Thus, rapid and reliable typing of bacterial isolates is an essential tool for determining the sources and transmission of pathogens more broadly. Commonly available bacterial characterisation methods, such as biochemical tests, toxigenic profiles, biofilm formation ability and antimicrobial susceptibility, often lack the discriminatory power to make conclusions about the relationships between closely related isolates. Genomics techniques, like Enterobacterial Repetitive Intergenic

Consensus (ERIC) PCR, have been successfully used for this purpose (Wenjun Li et al., 2009; Merzougui et al., 2013). Profiling based on proteomic data obtained by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) has also gained popularity in clinical laboratories for bacterial identification and typing as it does not involve complicated sample preparation (Jadhav et al., 2015; Lasch et al., 2016; Welker & Moore, 2011).

Additionally, Fourier-transform infrared (FT-IR) spectroscopy has emerged as a potentially rapid, cost-effective and straightforward method for bacterial typing. It is a spectral-based technique that quantifies the absorption of IR light by molecules present in the sample, such as carbohydrates, lipopolysaccharides, lipids, nucleic acids and proteins, resulting in the generation of a specific FT-IR spectrum that reflects the overall chemical composition of the isolate. This technique can be applied for bacterial species identification by comparing the spectrum to a reference database. FT-IR has also been used for the typing of bacterial isolates (Campos et al., 2018; Dinkelacker et al., 2018; Passaris et al., 2022).

The main objective of this research was to study the prevalence of *B. cereus* group isolates in raw and pasteurised milk in Victoria, Australia, and investigate their characteristics, including toxigenic profile, antimicrobial resistance and biofilm formation ability. Additionally, genomic fingerprinting (ERIC-PCR), proteomic fingerprinting (MALDI-TOF MS) and spectroscopic fingerprinting (FT-IR spectroscopy) methods for determining the diversity of *B. cereus* group isolates were compared.

4.2 Results

4.2.1 Prevalence of *B. cereus* group in raw and pasteurised milk

A total of 41 isolates of the *B. cereus* group were recovered from 97 analysed samples, with 42.3% of samples containing *B. cereus*. The contamination by the *B. cereus* group was considerably higher in pasteurised milk samples than in raw milk samples: *B. cereus* group was recovered from 20 (33.3%) of 60 raw milk samples and 21 (56.8%) of 37 pasteurised milk samples. No significant seasonal differences in *B. cereus* group isolation rates were found in raw milk or pasteurised milk from different manufacturers ($P \leq 0.05$). The isolates recovered from raw milk and pasteurised milk were denoted BC1-20 and BC21-41, respectively (Table 4.2).

After subjecting all *B. cereus* group isolates to MALDI-TOF MS analysis, VITEK® MS identified 95.2% of the confirmed isolates as belonging to the *B. cereus* group. In contrast, the MALDI Biotyper® correctly identified all tested isolates as *B. cereus* group members, which is essential for detection and confirmation purposes and shows MALDI Biotyper® to be the more accurate system for *B. cereus* group identification.

4.2.2 Virulence genes distribution

The distribution of virulence genes is summarised in Figure 4.1. In raw milk isolates, the most frequent genes were *nheA*, *nheB*, *nheC* and *hblA*, whereas, in pasteurised isolates, the most frequent genes were *nheB*, *nheA*, *hblB* and *hblA*. Forty-six per cent of isolates had the *cyk* gene but none contained *ces*, indicating that only potentially diarrhoeal strains were present in the collected samples. Pasteurised milk isolates contained significantly more virulence genes than raw milk isolates ($P < 0.05$).

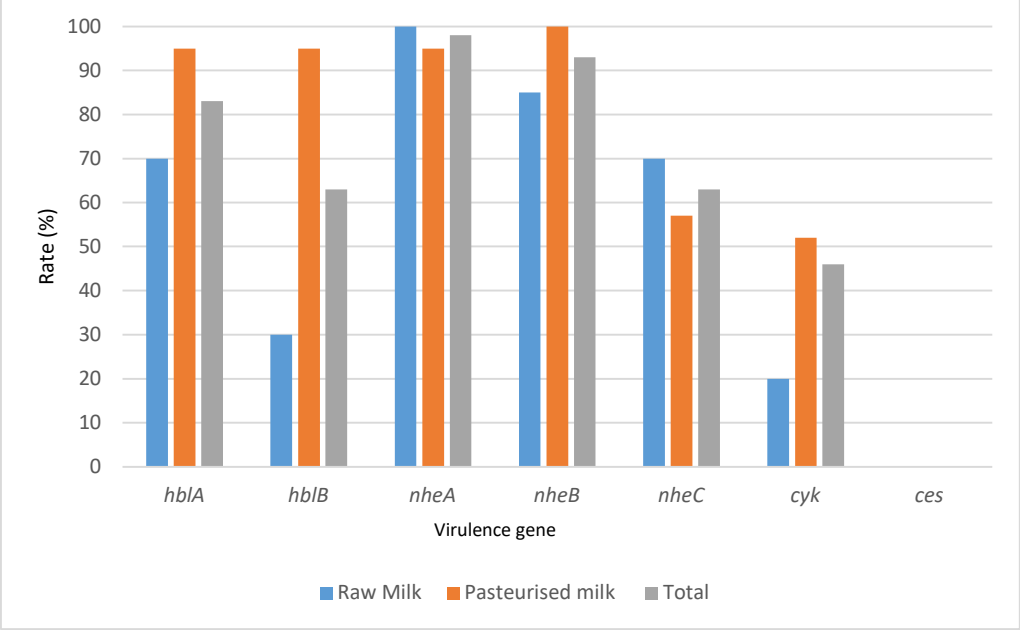

Figure 4.1 Distribution of virulence genes in the *B. cereus* group isolates from raw and pasteurised milk.

4.2.3 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing for the *B. cereus* group showed a high prevalence of antibiotic resistance toward ampicillin, ceftriaxone and penicillin. In contrast, all isolates were sensitive to chloramphenicol, ciprofloxacin, clindamycin and gentamycin (Figure 4.2). All isolates were resistant to at least two antimicrobials and 97.6% of the isolates were resistant to three antimicrobials.

Antimicrobial resistance was considerably higher in isolates from pasteurised samples than those from raw milk samples (Table 4.2). All of the pasteurised milk isolates were resistant to at least three antimicrobials. For example, 100% of pasteurised milk isolates showed intermediate or resistance towards streptomycin but only 25% of raw milk isolates showed intermediate or resistance towards this antibiotic.

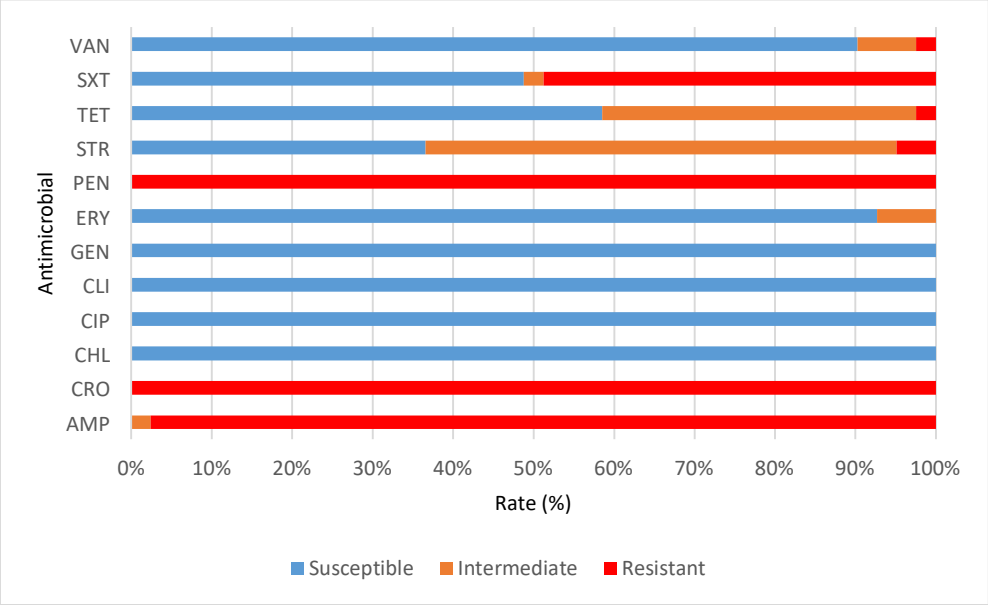

Figure 4.2 Distribution of antimicrobial resistance in the *B. cereus* group isolates from raw and pasteurised milk.

4.2.4 Biofilm formation assay results

The biofilm formation ability of the *B. cereus* group isolates from raw and pasteurised milk samples is summarised in Table 4.1. Most of the isolates (53.7%) could form a biofilm, although most had weak biofilm formation ability and only 4.9% showed strong biofilm formation ability. A higher number of biofilm producers (57.1%) was observed in pasteurised

milk isolates than in raw milk isolates. Figure 4.3 shows the biofilm formation ability of the *B. cereus* group strains.

Table 4.1 Biofilm formation capacity of *B.cereus* group isolates from raw and pasteurised milk samples.

	Total	**Raw milk**	**Pasteurised**
Biofilm formation ability	(*n* = 41)	(*n* =20)	(*n* = 21)
Non biofilm producer	19 (46.3%)	10 (50.0%)	9 (42.9%)
Weak biofilm producer	18 (43.9%)	8 (40.0%)	10 (47.7%)
Moderate biofilm producer	2 (4.9%)	1 (5.0%)	1 (4.7%)
Strong biofilm producer	2 (4.9%)	1 (5.0%)	1 (4.7%)

Note: the absolute number of resistant isolates is reported, followed by the proportion.

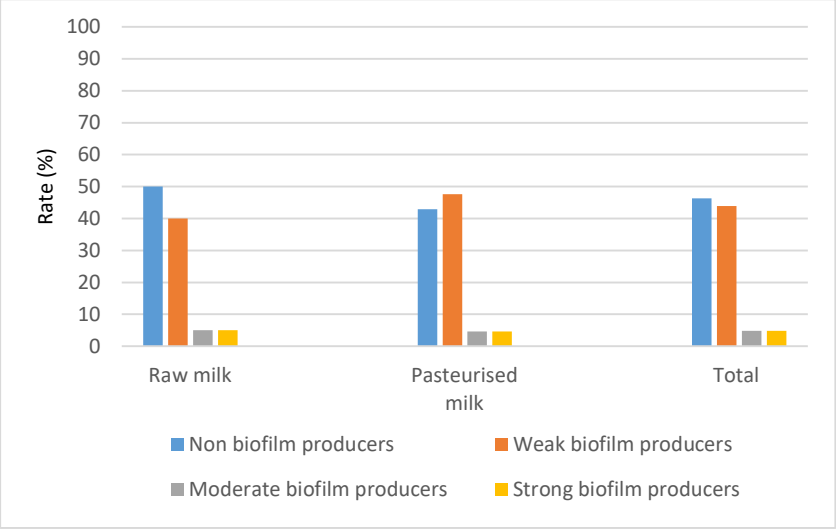

Figure 4.3 Biofilm formation capacity of the *B. cereus* group isolates from raw and pasteurised milk.

4.2.5 ERIC-PCR fingerprinting and diversity assay results

ERIC-PCR generated profiles with 3–11 distinct bands ranging from 150 to 4300 bp. According to these profiles, *B. cereus* group isolates could be divided into three major clusters, A, B and C. Cluster A contained two sub-clusters (A1 and A2) and cluster B contained three sub-clusters (B1, B2 and B3; Figure 4.4). The ERIC-PCR profiles for *the B. cereus* group showed that all of the isolates from pasteurised milk (isolates BC21 to BC41) were placed in clusters B3 and A1, indicating that they probably had few sources of origin, unlike the raw milk isolates. The ERIC-PCR results also showed that most of the isolates from one of the pasteurised milk producers were placed in cluster A1 and the rest of the samples from the three other producers were placed in cluster B3.

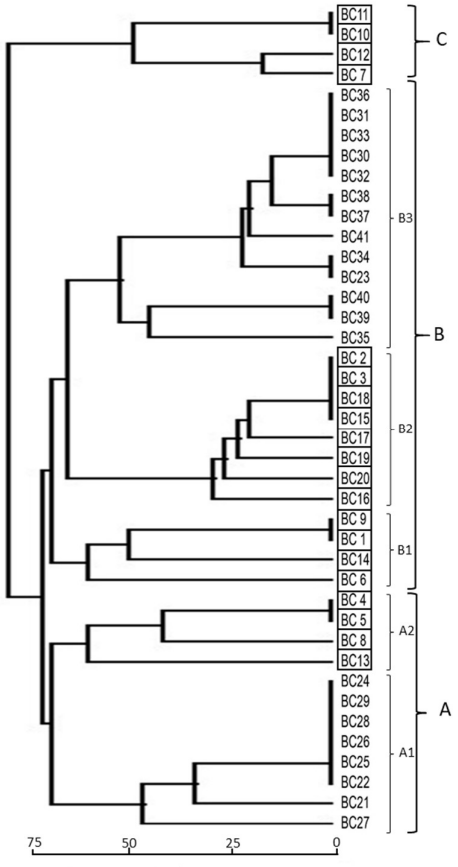

Figure 4.4 Dendrogram based on the ERIC-PCR profiles of *B. cereus* group isolates. The raw milk isolates are within boxes.

4.2.6 MALDI-TOF MS diversity assay results

Examples of MALDI-TOF MS profiles are shown in Figure 4.5. The protein profile masses of all *B. cereus* group isolates, extracted from VITEK® MS, were subjected to fingerprinting using the SPECLUST mass analyser software.

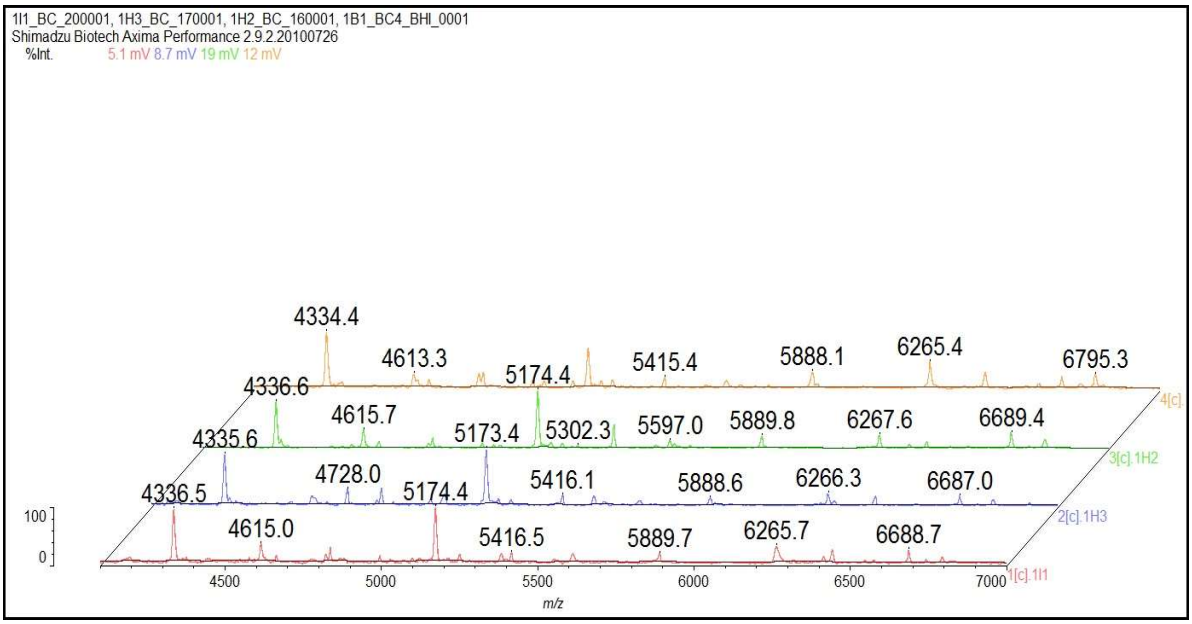

Figure 4.5 MALDI-TOF MS profiles of four *B. cereus* group isolates (BC4, BC16, BC17 and BC20) from milk samples.

The dendrograms derived from the MALDI-TOF mass spectra divided the isolates into four primary clusters (A, B, C, and D) and two sub-clusters (A1 and A2; Figure 4.5). Most pasteurised milk isolates (71%) were placed in cluster A but no relationship was found between milk producers and clusters. Raw milk isolates were distributed among all four primary clusters.

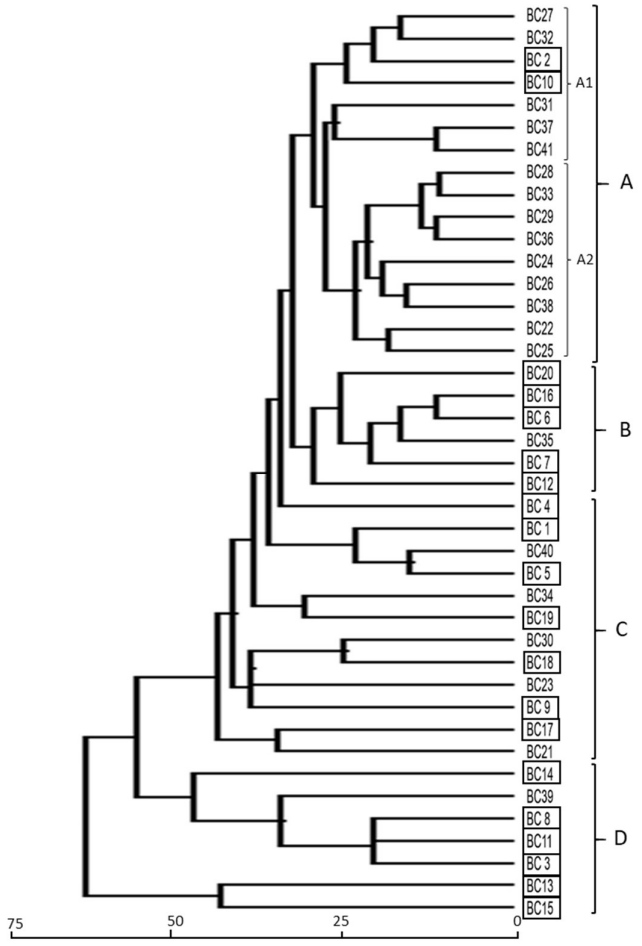

Figure 4.6 Dendrogram based on MALDI-TOF MS profiles of *B. cereus* group isolates. The raw milk isolates are within boxes.

4.2.7 FT-IR spectroscopy diversity assay results

The assessment of diversity using FT-IR spectroscopy showed that the *B. cereus* group isolates could be divided into two major clusters (A and B), both with two sub-clusters (A1 and A2, B1 and B2; Figure 4.7). Almost all of the isolates from raw milk (BC21 to BC41) were placed in cluster A and those isolated from pasteurised milk were placed in cluster B, indicating that the raw and pasteurised milk isolates originated from different sources. Principal component analysis (PCA) based on FT-IR spectroscopy also showed a stronger correlation between pasteurised milk isolates and more diversity amongst raw milk isolates (Figure 4.8).

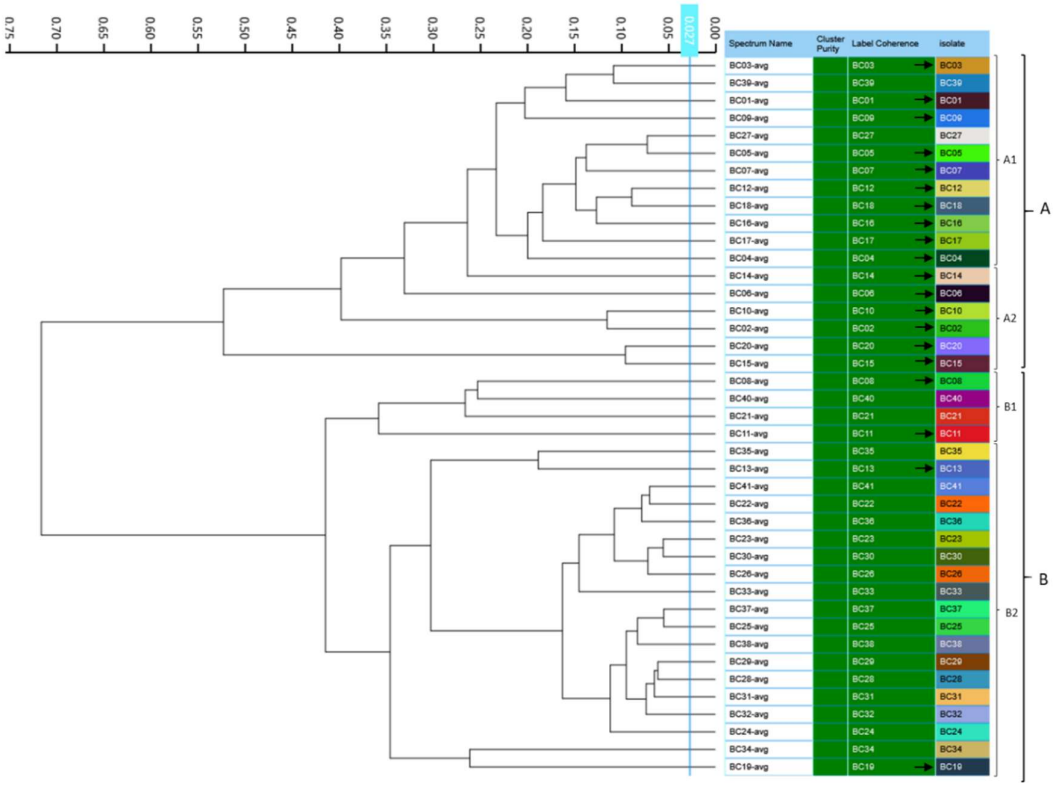

Figure 4.7 Dendrogram based on FT-IR spectroscopy profiles of *B. cereus* group isolates. Arrows indicate raw milk isolates.

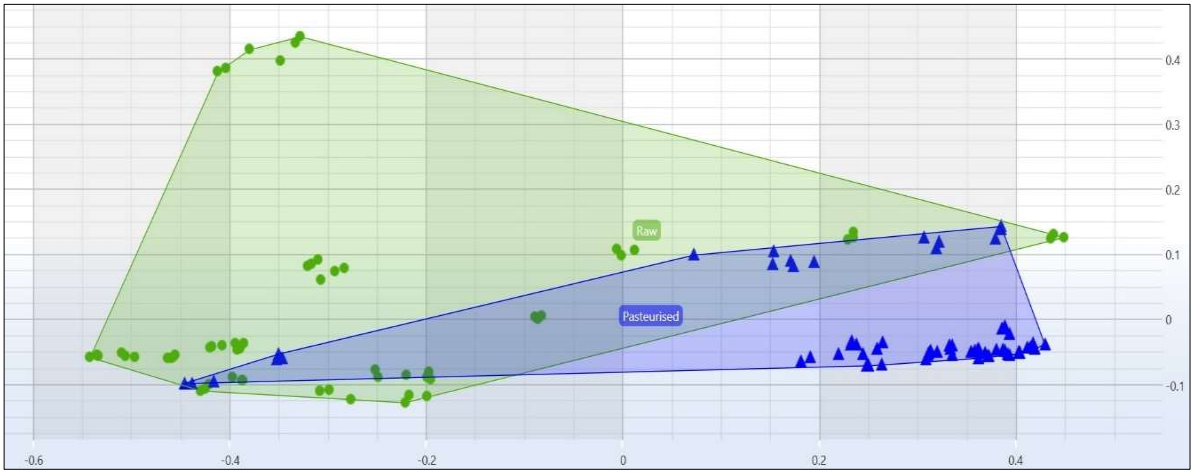

Figure 4.8 PCA plot based on FT-IR spectroscopy profiles of raw and pasteurised isolates of the *B. cereus* group. Raw milk and pasteurised milk isolates are indicated by green circles and blue triangles, respectively.

Information on the origin, characteristics and diversity of all *B. cereus* group isolates is summarised in Table 4.2.

Table 4.2 Diversity grouping of *B. cereus* group isolates based on ERIC-PCR, MALDI-TOF MS and FT-IR spectroscopy and characteristics of the isolates.

Isolate	**Origin**	**ERIC cluster**	**MALDI cluster**	**IR-Biotyper cluster**	**Antimicrobial resistance profile**	**Biofilm production**	**Virulence gene profile**
BC1	Raw milk	B1	C	A1	AMP, CRO, PEN	Non-biofilm producer	*hblB, nheA, nheB, nheC*
BC2	Raw milk	B2	A1	A2	AMP, CRO, PEN, ERY	Non-biofilm producer	*nheA, nheB*
BC3	Raw milk	B2	D	A1	AMP, CRO, PEN	Non-biofilm producer	*hblB, nheA, nheB, cyK*
BC4	Raw milk	A2	C	A1	AMP, CRO, PEN	Non-biofilm producer	*nheA, nheB*
BC5	Raw milk	A2	C	A1	AMP, CRO, PEN	Weak biofilm producer	*hblB, nheA, nheB, nheC, cytK*
BC6	Raw milk	B1	B	A2	AMP, CRO, PEN, STR	Non-biofilm producer	*hblA, nheA, nheB, nheC*
BC7	Raw milk	C	B	A1	AMP, CRO, PEN	Weak biofilm producer	*hblA, nheA, nheB*
BC8	Raw milk	A2	D	B1	AMP, CRO, PEN, STR	Non-biofilm producer	*hblA, nheA, nheB, nheC*
BC9	Raw milk	B1	C	A1	AMP, CRO, PEN, ERY	Non-biofilm producer	*hblA, nheA, nheB, nheC, cytK*
BC10	Raw milk	C	A1	A2	AMP, CRO, PEN, ERY	Weak biofilm producer	*nheA, nheC*
BC11	Raw milk	C	D	B1	AMP, CRO, PEN, STR	Intermediate biofilm producer	*hblA, nheA, nheB, nheC, cytK*
BC12	Raw milk	C	B	A1	AMP, CRO, PEN	Non-biofilm producer	*hblA, nheA, nheB, nheC*
BC13	Raw milk	A2	D	B2	AMP, CRO, PEN, STR	Weak biofilm producer	*hblA, nheA, nheC*
BC14	Raw milk	B1	D	A2	AMP, CRO, PEN	Weak biofilm producer	*hblA, hblB, nheA, nheB, nheC*
BC15	Raw milk	B2	D	A2	AMP, CRO, PEN	Non-biofilm producer	*hblA, hblB, nheA, nheB, nheC*
BC16	Raw milk	B2	B	A1	AMP, CRO, PEN	Strong biofilm producer	*hblA, nheA, nheB, nheC*
BC17	Raw milk	B2	C	A1	AMP, CRO, PEN	Weak biofilm producer	*hblA, nheA, nheB, nheC*
BC18	Raw milk	B2	C	A1	AMP, CRO, PEN, STR, SXT	Non-biofilm producer	*hblA, hblB, nheA, nheB, nheC*
BC19	Raw milk	B2	C	B2	AMP, CRO, PEN	Weak biofilm producer	*hblA, nheA*

BC20	Raw milk	B2	B	A2	AMP, CRO, PEN, STR	Weak biofilm producer	*hblA, nheA, nheB*
BC21	Pasteurised milk	A1	C	B1	AMP, CRO, PEN, STR, SXT, VAN	Weak biofilm producer	*nheB, nheC, cytK*
BC22	Pasteurised milk	A1	A2	B2	AMP, CRO, PEN, STR, TET, SXT, VAN	Non-biofilm producer	*hblA, hblB, nheA, nheB, cytK*
BC23	Pasteurised milk	B3	C	B2	AMP, CRO, PEN, STR, TET, SXT	Non-biofilm producer	*hblA, hblB, nheA, nheB, cytK*
BC24	Pasteurised milk	A1	A2	B2	AMP, CRO, PEN, TET, SXT	Non-biofilm producer	*hblA, hblB, nheA, nheB, cytK*
BC25	Pasteurised milk	A1	A2	B2	AMP, CRO, PEN, STR, TET, SXT	Non-biofilm producer	*hblA, hblB, nheA, nheB, cytK*
BC26	Pasteurised milk	A1	A2	B2	AMP, CRO, PEN, STR, TET, SXT	Non-biofilm producer	*hblA, hblB, nheA, nheB, cytK*
BC27	Pasteurized milk	A1	A1	A1	AMP, CRO, PEN, STR TET, SXT	Non-biofilm producer	*hblA, hblB, nheA, nheB, nheC*
BC28	Pasteurised milk	A1	A2	B2	AMP, CRO, PEN, STR, TET, SXT	Non-biofilm producer	*hblA, hblB, nheA, nheB, cytK*
BC29	Pasteurised milk	A1	A2	B2	AMP, CRO, PEN, STR, TET, SXT	Weak biofilm producer	*hblA, hblB, nheA, nheB, nheC, cytK*
BC30	Pasteurised milk	B3	C	B2	AMP, CRO, PEN, STR, TET, SXT	Weak biofilm producer	*hblA, hblB, nheA, nheB, cytK*
BC31	Pasteurised milk	B3	A1	B2	AMP, CRO, PEN, STR, SXT	Strong biofilm producer	*hblA, hblB, nheA, nheB, cytK*
BC32	Pasteurised milk	B3	A1	B2	AMP, CRO, PEN, STR, SXT	Weak biofilm producer	*hblA, hblB, nheA, nheB, nheC, cytK*
BC33	Pasteurised milk	B3	A2	B2	AMP, CRO, PEN, STR, TET, SXT	Weak biofilm producer	*hblA, hblB, nheA, nheB, nheC, cytK*
BC34	Pasteurised milk	B3	C	B2	AMP, CRO, PEN, STR, TET, SXT, VAN	Weak biofilm producer	*hblA, hblB, nheA, nheB, nheC*
BC35	Pasteurised milk	B3	B	B2	AMP, CRO, PEN, STR, TET, SXT	Non-biofilm producer	*hblA, hblB, nheA, nheB, nheC*
BC36	Pasteurised milk	B3	A2	B2	AMP, CRO, PEN, STR, TET, SXT	Weak biofilm producer	*hblA, hblB, nheA, nheB, nheC*
BC37	Pasteurised milk	B3	A1	B2	AMP, CRO, PEN, STR, TET, SXT	Weak biofilm producer	*hblA, hblB, nheA, nheB, nheC, cytK*
BC38	Pasteurised milk	B3	A2	B2	AMP, CRO, PEN, STR, TET, SXT	Weak biofilm producer	*hblA, hblB, nheA, nheB, nheC, cytK*
BC39	Pasteurised milk	B3	D	A1	AMP, CRO, PEN, STR	Intermediate biofilm producer	*hblA, hblB, nheA, nheB, nheC*
BC40	Pasteurised milk	B3	C	B1	AMP, CRO, PEN, STR, TET, SXT, VAN	Weak biofilm producer	*hblA, hblB, nheA, nheB, nheC*
BC41	Pasteurised milk	B3	A1	B2	AMP, CRO, PEN, STR, TET, SXT	Non-biofilm producer	*hblA, hblB, nheA, nheB, cytK*

4.3 Discussion

The *B. cereus* group is one of the most important groups of spoilage microorganisms in the dairy industry and may result in several problems. It is also a great safety concern for dairy production as it is associated with food poisoning (Tiantian Gao et al., 2018; Gdoura-Ben Amor et al., 2018; Kumari & Sarkar, 2016). Since the *B. cereus* group is well known to be potentially pathogenic and several incidents of food and dairy contamination have been reported from different parts of the world, understanding the characteristics and diversity of this foodborne pathogen is of great interest (Chaves, Pires & Vivoni 2011; Helgason et al., 1998; Kumari & Sarkar, 2016). Milk and dairy products can be easily contaminated by the *B. cereus* group during transportation and at processing facilities. *B. cereus* strains can survive pasteurisation processes because of the production of spores, and they can affect the shelflife of milk and dairy products (Kumari & Sarkar, 2016; Larsen & Jorgensen, 1997; Porcellato et al., 2019). To the best of our knowledge, no comprehensive study has determined the characteristics and diversity of *B. cereus* group isolates from raw and pasteurised milk in Australia.

In this study, *B. cereus* group isolates were obtained from 42.2% of milk samples, with a higher level of recovery from pasteurised milk (56.7%) compared to raw milk (33.3%). This level of contamination is higher than in other countries (Banyko & Vyletelova, 2009; Gao et al., 2018; Granum et al., 2014; Larsen & Jorgensen, 1997; Zhou et al., 2008), although no significant differences were found between the time of sampling for raw milk and different producers for pasteurised milk. A higher prevalence of *B. cereus* group contamination in pasteurised milk can be a result of the season, processing conditions and storage temperature (Artursson et al., 2018; Porcellato et al., 2018).

Two MALDI-TOF MS systems were evaluated as part of the bacterial identification aspect of this study. VITEK® MS identified fewer isolates than the MALDI Biotyper® (95.2% vs. 100%). The difference between the identification levels of these instruments may have been due to the different reference spectral profiles of food bacteria in their respective databases (Vithanage et al., 2014; Muggi et al., 2022). Previous studies have shown that the VITEK® MS provides better species-level identification for Gram-positive bacteria (Levesque et al., 2015; Veloo et al., 2011).

Several studies have confirmed the presence of the enterotoxigenic *B. cereus* group in dairy food products (Arslan et al., 2014; Li et al., 2016; Owusu-Kwarteng et al., 2017). In the present

study, various enterotoxin genes related to the virulence of the *B. cereus* group were found among the isolates. The most prevalent genes in the raw milk isolates were *nheA*, *nheB*, *nheC* and *hblA*, whereas, in pasteurised milk isolates, the most prevalent genes were *nheB*, *nheA*, *hblB* and *hblA*. The presence of the *cyk* gene in many isolates (46%) revealed that a large number of potentially diarrhoeal strains were present in the collected samples. No isolates contained the *ces* gene. Other similar studies have reported almost the same prevalence of detected virulence genes (Arslan et al., 2014; Owusu-Kwarteng et al., 2017). The pasteurised milk isolates showed significantly more virulence genes than raw milk isolates, indicating the necessity of the implementation of good hygienic and manufacturing practices by dairy producers to prevent post-pasteurisation contamination and subsequent potential food poisoning incidents by the *B. cereus* group.

Antimicrobial susceptibility testing of the isolates showed a high prevalence of antibiotic resistance towards ampicillin, ceftriaxone and penicillin. All isolates were resistant to at least two antimicrobials and 97.6% of the isolates were resistant to three antimicrobials. In agreement with previous reports, the *B. cereus* group isolates showed susceptibility to chloramphenicol, ciprofloxacin, clindamycin and gentamycin (Owusu-Kwarteng et al., 2017; Yu et al., 2020). The results of the present study showed that antimicrobial resistance was considerably higher in pasteurised milk samples than in raw milk samples and all the pasteurised milk isolates were resistant to at least three antimicrobials. In Australia, the prevalence of antibiotic-resistant bacteria is on the rise. Resistance varies from state to state, being highest in the eastern states (New South Wales, Victoria and Queensland) and lowest in Tasmania and Western Australia (Fernandes & Ackerman, 1989; Hardy-Holbrook et al., 2013). There is no comprehensive information available on the antimicrobial properties of *B. cereus* group in dairy products in Australia. Therefore, the findings of this study can provide useful information for public health authorities.

Biofilms are a threat to the dairy and food industries as they can cause safety problems, leading to health and economic consequences. Many studies have focused on the role of bacterial biofilm formation in the dairy industry to increase understanding of biofilms and to identify a solution to avoid contamination of dairy products (Oosthuizen et al., 2001; Shemesh & Ostrov, 2020; Tang et al., 2010; Teh et al., 2014). The ability to form a biofilm varies between species and within species, with different strains and serovars showing variability in this phenotype (Oosthuizen et al., 2001; Srey et al., 2013). Thus, investigation of the biofilm formation ability of important dairy pathogens is an essential part of their characterisation and behaviour.

The results of the present study showed that most of the *B. cereus* isolates (53.7%) could form a biofilm, albeit weakly, and only 4.9% displayed strong biofilm formation ability. A greater prevalence of biofilm producers was observed in pasteurised milk isolates (57%) compared to raw milk isolates. To the best of our knowledge, there is no other research available to compare the level of biofilm formation ability of milk-derived *B. cereus* group isolates in Australia. However, since biofilm elimination is challenging for the dairy industry, this high level of biofilm production by the *B. cereus* group, especially in pasteurised milk, needs to be considered by dairy producers.

Microbiological detection and identification methods in food microbiology have focused on identifying, subtyping, and source-tracking food bacterial pathogens. These methods have changed significantly in recent years, developing more discriminatory, faster, and less expensive techniques. However, the same methodologies and techniques that have been used for food safety are progressively being adopted to address contamination due to spoilage microorganisms (Foleya, Lynne & Nayak, 2009; Martin, Torres-Frenzel & Wiedmann, 2021). In the present study, the performance of MALDI-TOF MS and FT-IR for typing of *B. cereus* group isolates from raw and pasteurised milk was evaluated to determine the discriminatory power of these rapid analysis methods. ERIC-PCR was employed as a genomic reference method. ERIC-PCR profiling placed most *B. cereus* group isolates from pasteurised milk in the same cluster, indicating that they probably originated from the same source. In contrast, the raw milk isolates showed greater diversity, indicating various sources. This finding was in contrast to that of Banyko & Vyletelova (2009). However, very few studies are available on the genetic typing of the *B. cereus* group in dairy products (Banyko & Vyletelova, 2009). Of particular interest in the present study was the high level of agreement between the ERIC-PCR and FT-IR clustering results. In contrast, a low level of agreement between MALDI-TOF MS and ERIC-PCR typing was observed, which was consistent with other studies (Dinkelacker et al., 2018; Feng et al., 2020). Therefore, the present study showed FT-IR to be a powerful tool for the typing of *B. cereus* group isolates. Moreover, its major advantage over genomic methods that is, fast turnaround time encourages the use of this technology for typing and source-tracking of the *B. cereus* group and possibly other relevant foodborne pathogens.

4.4 Conclusions

This study showed a high level of *B. cereus* group contamination of milk samples, especially pasteurised milk, collected from dairy producers in Victoria, Australia. All isolates carried at least one virulence gene, with pasteurised milk isolates having more virulence genes than raw milk isolates. Moreover, the prevalence of antibiotic resistance in *B. cereus* group isolates was very high. These characteristics, and the ability of isolates to produce biofilm, clearly indicated the importance of the *B. cereus* group in the Australian dairy industry. The present study also showed that FT-IR typing by IR Biotyper® could be adopted as a rapid and low-cost tool for the typing of *B. cereus* group isolates as it performed as well as an established genomic typing method.

Chapter 5

Effectiveness of Peracetic Acid (PAA) Disinfectant Against *Escherichia coli* and *Bacillus cereus* group

Abstract

Cleaning and sanitation are essential aspects of dairy processing operations to control bacterial contamination. At present, peracetic acid (PAA) is emerging as a superior alternative to other sanitisers in dairy plants. Accordingly, this study investigated the effect of PAA on *E. coli* and *B. cereus* group isolates from raw and pasteurised milk to evaluate its efficacy as a potential method of controlling these bacteria in the dairy industry.

The antimicrobial effect of PAA against *E. coli* isolates was significantly increased as the PAA concentration increased from 50 to 200 ppm across all isolates and at each sampling point. Therefore, PAA effectively controlled *E. coli* growth even at a concentration as low as 50 ppm. There was no significant difference in the effect of 50 or 100 ppm PAA based on the biofilm formation ability of the *E. coli* isolates, and only isolates with strong biofilm formation ability were significantly more sensitive to 200 ppm PAA.

The antimicrobial effect of PAA on *B. cereus* group isolates also increased as the PAA concentration increased, both for raw milk and pasteurised milk isolates. Additionally, PAA was significantly more effective on raw milk isolates than pasteurised milk isolates at 100 and 200 ppm concentrations of PAA. Furthermore, the biofilm production ability of *B. cereus* group isolates could affect the effectiveness of PAA, with the antimicrobial effect of PAA on non-biofilm producing *B. cereus* isolates being significantly stronger than that on biofilm-producing isolates. Therefore, biofilm producers were more resistant to PAA.

PAA was significantly more effective against *E. coli* isolates than *B. cereus* group isolates. The growth of *E. coli* isolates was controlled to a greater extent by 50 ppm PAA than was the growth of *B. cereus* group isolates by 200 ppm PAA.

Therefore, the present study showed PAA to be an effective sanitiser against *E. coli* and *B. cereus* group isolates from raw and pasteurised milk. However, it was effective against *B. cereus* group isolates at higher concentrations than required for *E. coli* and this resistance increased with increasing biofilm formation ability of *B. cereus*, potentially constituting a hygiene concern for the dairy industry.

5.1 Introduction

Adequate disinfection is a part of good hygienic practices in the food industry, including against bacteria and their spores. Only a few commonly applied chemical disinfectants are appropriate for use in the food and dairy industry (Iniguez-Moreno et al., 2017; Orth, 1998).

As explained in Chapter 2, bacterial cells tend to attach to surfaces in dairy production areas and form a complex structure called a biofilm. Once biofilms are formed on a contact surface, they develop resistance to antimicrobial agents because of the layers of extracellular material produced by the bacteria. The persistence of biofilms on milk contact surfaces may constitute a continuous source of contamination. Furthermore, several reports have investigated the ability of bacterial species to produce biofilms on materials generally used in the dairy industry, such as stainless steel and rubber. Bacterial cells in biofilms are considered more difficult to eradicate than their planktonic counterparts. Additionally, biofilm formation on dairy equipment can lead to economic losses due to the deterioration of equipment (Lee et al., 2016; Teh et al., 2014).

Pathogenic microorganisms, including *E. coli* and the *B. cereus* group, are of great concern in the dairy industry and have demonstrated a high capacity to produce biofilms on surfaces, particularly in the dairy plant environment. In the dairy industry, the development of biofilms depends on sanitation regimens. The efficacy of disinfectants against biofilms improves with increasing biocide concentration and treatment duration. However, the resistance of pathogenic bacteria to sanitisers that are broadly used in the dairy industry can be one key factor contributing to the association of specific pathogens in foodborne outbreaks (Lee et al., 2016; Mogha et al., 2014).

Peracetic acid (PAA), also known as peroxyacetic acid, is an organic chemical compound used in several applications. Because of the germicidal properties of PAA, it has been used for a long time and has recently found application in the food industry, being used as a potent chlorine replacement in the food industry as a sanitiser (Banach et al., 2020; Cheng et al., 2020). Indeed, PAA is now one of the most commonly used sanitisers in the dairy industry. It is applied to control microbes, odours and biofilms on food contact surfaces and as a microbial control agent for food contact surfaces. In comparison to other frequently used sanitisers in the food industry, PAA may be more compatible with organic handling than halogen-based sanitisers and disinfectants such as chlorine (bleach), iodine/phosphorus or quaternary

ammonium compounds. PAA is an oxidising agent that can dissolve in water. In comparison to other disinfectants, PAA decomposes into safe and environmentally friendly byproducts (acetic acid and hydrogen peroxide). Other advantages of PAA include its efficacy at low concentrations, broad spectrum of antimicrobial activity, performance at low temperatures, relative insensitivity to organic material, including protein residues, ease of application, rapid degradation in the environment, minimal residue and low residual aquatic toxicity (Cheng et al., 2020; Lee et al., 2016). Therefore, it can be applied without the risk of contaminating food with toxic residues (Lee et al., 2016; Ribeiro et al.,2019). PAA can be used at concentrations of up to 200 ppm without requiring a rinse but standard usage rates are 80 ppm with contact times of 1 minute to achieve 99.9% microbial reduction (Cheng et al., 2020; Gawande et al., 2013; Micciche et al., 2019).

However, little information is available on the efficiency and effectiveness of PAA on pathogens, especially biofilm-producing strains in the dairy industry (Lee et al., 2016; Sudhaus et al., 2014). Therefore, this study investigated the efficacy of PAA as a routine sanitiser in the dairy industry on *E. coli* and *B. cereus* group isolates from raw and pasteurised milk. The effect of PAA on different groups of bacteria with different biofilm formation abilities was also compared to form e a comprehensive picture of the potential resistance of *E. coli* and the *B. cereus* group dairy isolates.

5.2 Results

5.2.1 Inhibitory effect of PAA on *E. coli* isolates

The anti–*E. coli* effects of PAA were assessed using the disk diffusion assay using the dairy *E. coli* isolates characatreised in this study. Figures 5.1 and 5.2 summarise the results of the disk diffusion assay after an overnight incubation period.

The results showed that the antimicrobial effect of PAA significantly increased as the PAA concentration increased from 50 to 200 ppm across all isolates and also within isolates from each sampling point ($P < 0.05$). No significant difference was observed in the effect of 50 and 100 ppm PAA at different sampling points but isolates from SP3 showed more sensitivity to the effect of 200 ppm PAA.

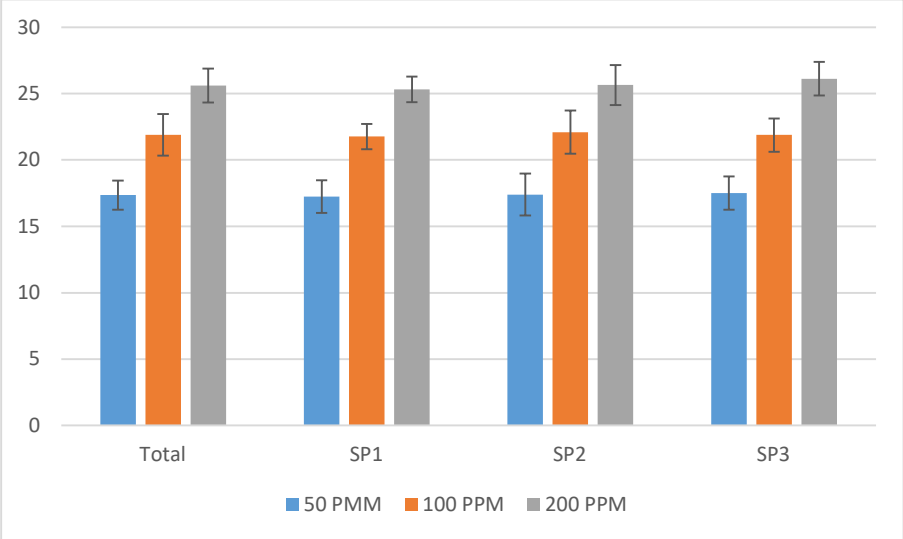

Figure 5.1 Effects of different concentrations of PAA on the growth of *E. co*li isolates from different sampling points.

No significant difference was observed in the effect of 50 and 100 ppm PAA on the biofilm formation ability of the *E. coli* isolates. Only isolates with strong biofilm formation ability were significantly more sensitive to 200 ppm PAA ($P < 0.05$).

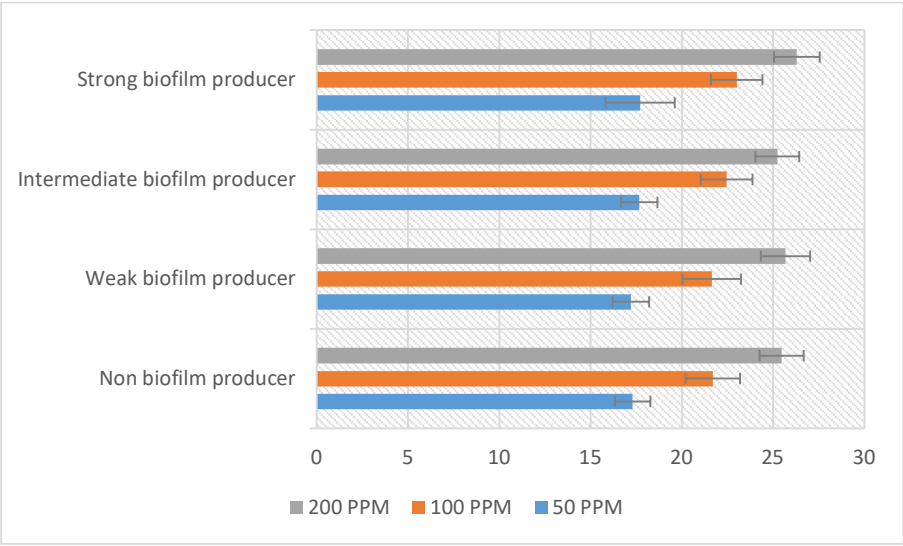

Figure 5.2 Effects of different concentrations of PAA on the biofilm formation ability and growth of *E. coli* isolates.

5.2.2 Inhibitory effect of PAA on *B. cereus* group isolates

Results of the anti–*B. cereus* effects of PAA within the *B. cereus* group dairy isolates after an overnight incubation period are summarised in Figures 5.3 and 5.4. The antimicrobial effect of PAA significantly increased as the PAA concentration increased from 50 to 200 ppm across all isolates and within raw and pasteurised isolates ($P < 0.05$). Furthermore, PAA was significantly more effective on raw milk isolates than on pasteurised milk isolates at concentrations of 100 and 200 ppm, but the difference in the efficacy of 50 ppm PAA between raw and pasteurised isolates was not significant ($P > 0.05$).

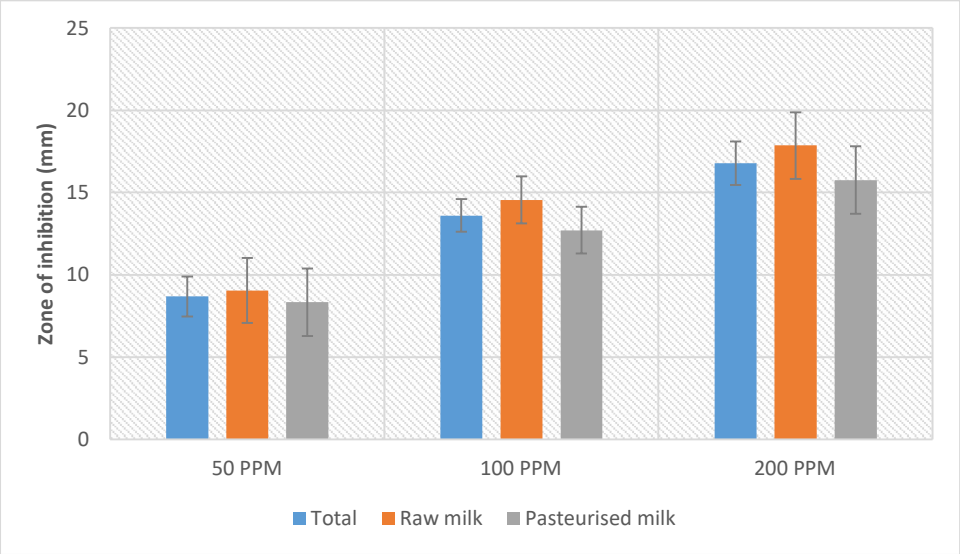

Figure 5.3 Effects of different concentrations of PAA on the growth of *B. cereus* group isolates from raw and pasteurised milk samples.

The results also indicated that the biofilm formation ability of *B. cereus* group isolates could change the effectiveness of PAA. The effect of PAA was significantly different between non-biofilm producers and biofilm producers of both the raw and pasteurised milk isolates ($P < 0.05$). Although the antimicrobial effect of PAA on biofilm producer isolates showed a significant difference between weak and strong biofilm producers ($P < 0.05$), no significant difference was found between weak and intermediate and intermediate and strong biofilm producer isolates.

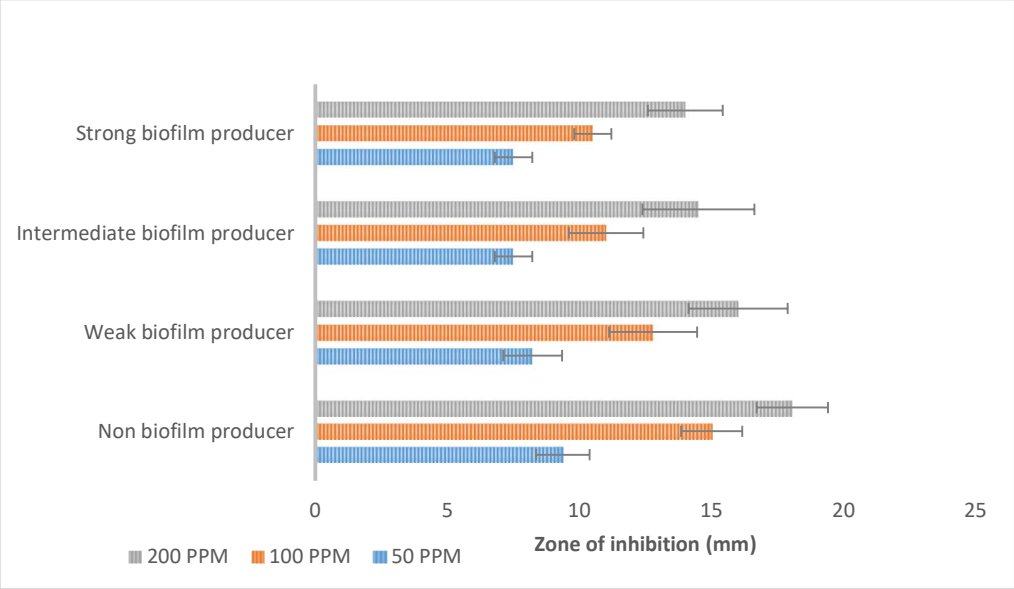

Figure 5.4 Effect of biofilm formation ability on the impact of different concentrations of PAA on the growth of *B. cereus* group isolates from raw and pasteurised milk samples.

5.2.3 Comparision of the effect of PAA on *E. coli* and *B. cereus* group isolates

The minimum inhibition zone around the PAA disks was determined for both the *E. coli* and *B. cereus* group isolates. For *E. coli*, the inhibition zone around the PAA disk at 50 ppm PAA was 17.3 mm and the maximum inhibition zone of 25.6 mm was at 200 ppm PAA (Figure 5.5). Thus, the effect of PAA on the growth of *E. coli* significantly increased with sanitiser concentration.

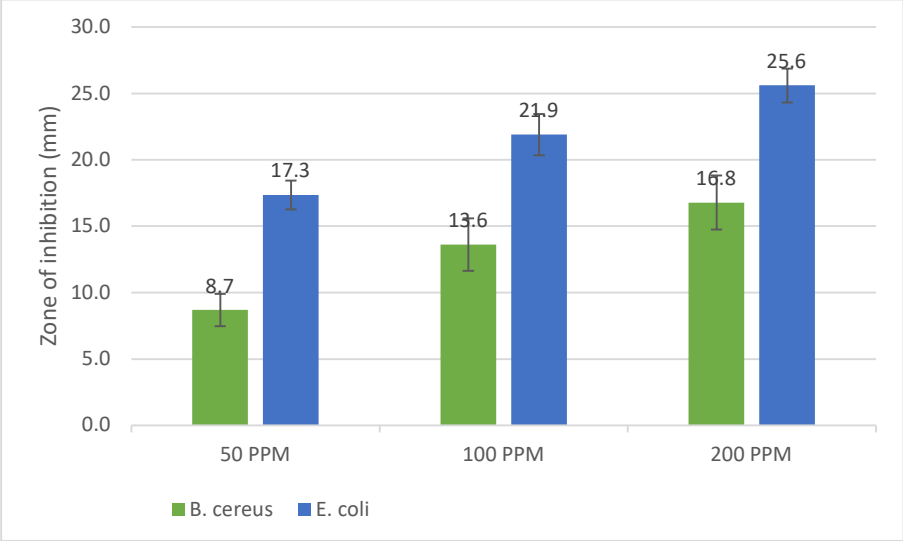

Figure 5.5 Effects of different concentrations of PAA on the growth of *E. coli* and *B. cereus* group isolates from milk samples.

Similar results were observed for the *B. cereus* group. As the PAA concentration increased, the inhibitory effect increased accordingly, with a minimum inhibition zone of 8.7 mm at 50 ppm PAA and a maximum of 16.8 mm at 200 ppm PAA.

These results also showed that PAA was significantly more effective on *E. coli* isolates than on *B. cereus* group isolates at all concentrations. The most significant difference was observed at 50 ppm PAA (8.7 mm of inhibition vs. 17.3 mm of inhibition) and the least significant difference was at 200 ppm PAA. This indicated that 50 ppm PAA had a greater effect on the growth of *E. coli* isolates than that of 200 ppm PAA on *B. cereus* group isolates.

5.3 Discussion

The use of chemicals is the most common method for controlling pathogenic bacteria in food. However, food businesses must ensure that any sanitiser used is safe for food contact surfaces (Lee et al., 2016; Tang et al., 2010). Currently, one of the most commonly used sanitisers in dairy rocessing environments is PAA, which has broad spectrum of antimicrobial activity and

other favourable characteristics that make it a perfect choice for sanitation. For example, PAA is an oxidising agent that can dissolve in water to produce hydrogen peroxide and acetic dioxide so, in comparison to other disinfectants, PAA byproducts are not toxic (Cheng et al., 2020; Lee, Cappato et al.,2016).

To gain more information about the characteristics and behaviour of the *E. coli* and *B. cereus* group isolates, the efficacy of PAA disinfectant in controlling their growth was explored. The correlation of these results with other factors considered in this study was also explored. The results showed that the antimicrobial effect of PAA against *E. coli* isolates increased significantly as the PAA concentration increased from 50 to 200 ppm across all isolates and within the isolates from each sampling point. Banach et al. (2020) showed that 75 ppm PAA could effectively control *E. coli* strains, while Neo et al. (2013) showed that 70 ppm PAA was effective against *E. coli* and significantly better than other sanitisers such as chlorine (Banach et al., 2020; Neo et al., 2013).

Also, the results of the present study showed no difference in the effect of 50 and 100 ppm PAA on *E. coli* isolates with different biofilm formation abilities. Additionally, only the isolates with strong biofilm formation ability were significantly more sensitive to PAA at 200 ppm than other isolates. However, the ability of PAA to control biofilm formation by *E. coli* has already been verified (Barbosa et al., 2016; Pironti et al., 2021). So, in agreement with similar studies, it could be concluded that PAA at concentrations of even as low as 50 ppm is effective in controlling dairy *E. coli* isolates (Banach et al., 2020; Singh, Hung, & Qi, 2018).

It was also observed that the antimicrobial effect of PAA increased as the disinfectant concentration increased for the raw and pasteurised milk *B. cereus* group isolates. PAA was significantly more effective on raw milk isolates than pasteurised milk isolates at 100 and 200 ppm concentrations, but the difference in efficacy between raw and pasteurised isolates was not significant at 50 ppm PAA. Many studies have assessed the effect of PAA-based disinfectants against *B. cereus* in the food and dairy industries (Ribeiro et al., 2019; Sudhaus et al., 2012; Te Giffel et al., 1997; Te Giffel et al.,1995). In some other studies, PAA was effective against *B. cereus* group at 100 to 200 ppm. Higher concentrations of PAA are more effective against this group of bacteria; however, using high concentrations of sanitisers in industrial environments may cause corrosion (Ceragioli et al., 2010; Silva et al., 2018). To the best of our knowledge, no published study has compared the efficacy of PAA on *B. cereus* group isolates from raw and pasteurised milks.

This study also showed that the biofilm production ability of *B. cereus* group isolates could change the effectiveness of PAA. The effect of PAA was significantly different between non-

biofilm producers and biofilm producers isolated from both raw and pasteurised milks. The antimicrobial effect of PAA was significantly stronger on non-biofilm producing isolates than on biofilm producers. Therefore, biofilm producers were more resistant to PAA. Also, although several studies have focused on the effect of PAA on biofilm formation by foodborne bacteria, none were found that have compared the effect of PAA on biofilm and non-biofilm–producing bacteria (Barbosa et al., 2016; Lee et al., 2016; Visvalingam et al., 2019).

In the present study, PAA was significantly more effective on *E. coli* isolates than on *B. cereus* group isolates. The results indicated that 50 ppm PAA could control the growth of *E. coli* isolates to a greater extent than 200 ppm PAA could control the growth of *B. cereus* group isolates. Despite there being several studies on bacterial resistance to sanitisers, the results are difficult to compare because of differences in the test methods or definitions of bacterial susceptibility. Bridier et al. (2011) showed Gram-positive and Gram-negative bacteria to have similar susceptibility to PAA, which was probably due to the non-specific mode of action of this highly reactive oxidising antimicrobial agent. Other similar research has indicated that PAA has a significantly stronger effect on Gram-negative bacteria than Gram-positive bacteria, especially in the *B. cereus* group (Bridier et al., 2011; Lee et al., 2016; Moradi et al., 2009; Neo et al., 2013). Some studies have also suggested that higher concentrations of PAA are more effective against the *B. cereus* group; however, using high concentrations of sanitisers in industrial environments may cause corrosion. To improve the ability of PAA to destroy and remove biofilms from food processing facilities, the use of combined sanitising treatments may be more effective than the use of any individual treatment (Ceragioli et al., 2010; Park & Kang, 2015; Silva et al., 2018; Tang et al., 2010).

The present study showed PAA to be an effective sanitiser against *E. coli* and *B. cereus* group isolates from raw and pasteurised milks. However, it was only effective against *B. cereus* group isolates at higher concentrations. This resistance increased with increasing biofilm formation ability and could be a hygiene concern for the dairy industry. Future studies could address possible reasons for the greater resistance of the strong biofilm producers and conduct the test on biofilm cells.

5.4 Conclusion

This study showed that PAA could be used as a potent sanitiser to control *E. coli* and *B. cereus* group dairy isolates. *E. coli* isolates were sensitive to PAA at lower concentrations, but *B. cereus* group isolates—especially biofilm producers—showed reduced sensitivity to PAA.

Therefore, control of biofilm-producing *B. cereus* group isolates is an essential task in the dairy industry. Furthermore, tracking the source of contamination and the use of alternative sanitising methods is necessary for safety assurance across the dairy industry. Planned and regular cleaning and sanitising to minimise the risk of possible contaminations is suggested. Further studies to find critical control points of bacterial contamination with an assessment of effect of PAA in a manufacturing plant are also recommended.

Chapter 6

Conclusions and Future Directions

6.1 Summary of conclusions

Foodborne diseases are a substantial concern for public health and the food industry. Despite the considerable effort of the food industry and researchers, foodborne diseases are still not under control and foodborne outbreaks continue to cause health and economic losses. The financial costs associated with foodborne disease can affect people's health, and the reputations of food companies and countries (Bintsis, 2017; Morris, 2013; Oliver et al., 2005). The food industry, including food authorities and food producers, is the main party concerned with controlling pathogenic microorganisms in food, where failure to detect a pathogen may lead to undesirable consequences.

Pathogenic bacteria are a significant cause of foodborne diseases globally. They place a major burden on global healthcare systems and negatively impact economic growth and social stability. Therefore, there is great effort globally in trying to control and minimise the occurrence of food pathogens in the food production and distribution chains. So, the continuous monitoring of food contaminants and identification of risk factors to ensure food safety is an everyday task for the food industry (Fung et al., 2018; Rohde et al., 2017)

Considerable research worldwide has been conducted to determine the characteristics of pathogenic bacteria, including features such as prevalence, antibiotic resistance, transmission vectors, biofilm formation ability and genetic diversity (McAuley et al.,2014; Stenfors Arnesen et al., 2007). However, information about some bacterial food contaminants, their characteristics and diversity— especially in the dairy industry—is lacking. Therefore, to fill this gap, the present study aimed to provide substantial information about two important bacterial groups that are not frequently studied: *E. coli* and *B. cereus*. This study also aimed to compare rapid, simple and novel methods of bacterial source-tracking to identify the most practical methods and techniques for detection and typing of these bacteria.

In the first experimental chapter (Chapter 3), the prevalence, characterisation and diversity of *E. coli*—as a representative of a Gram-negative and non-spore–forming dairy contaminant found in raw milk—was reported. *E. coli* was isolated from 90% of raw milk samples, ranging from 95.4% to 85.3% in the different sampling sites. *E. coli* contamination in the samples collected in the present study was considerably higher than that reported in similar research in other countries. Pasteurisation can eliminate *E. coli* from milk and milk products, but it has

been shown that *E. coli* outbreaks could be linked to the consumption of milk and dairy products. So, a high level of *E. coli* contamination still can be a concern for dairy producers (Oliver et al., 2005; Rabee A. Ombarak et al., 2016). However, only 4.2% of *E. coli* isolates had the *sxt1* gene and were considered as STEC, with the rest of the samples not carrying any STEC virulence genes. The level of STEC contamination in this study was similar to that observed in other countries (Lambertini et al., 2015; R. A. Ombarak et al., 2016; Paneto et al., 2007). Despite other studies that indicated greater levels of contamination in warm weather, no seasonal correlation was observed in the present study.

All raw milk *E. coli* isolates in this study were resistant to at least one antibiotic and 16.7% of isolates were multi-drug resistant. A high prevalence of antibiotic resistance was observed toward erythromycin, streptomycin, kanamycin and ampicillin. However, most *E. coli* isolates showed susceptibility to ciprofloxacin, chloramphenicol, gentamicin, trimethoprim-sulfamethoxazole and nalidixic acid. There was a high level of agreement between the findings of the present study and other studies on antibiotic resistance from around the world (Gundogan & Avci, 2014; Ntuli et al., 2016b).

In the present study, most of the *E. coli* isolates could form a biofilm. However, most had weak biofilm formation ability and less than 10% of isolates showed strong biofilm formation ability. Also, although it has been shown that biofilm formation ability can increase the antibiotic resistance of *E. coli*, the present study did not show any correlation between biofilm formation ability and antimicrobial resistance. The current study's findings compared to the other similar studies showed that *E. coli* contamination and characteristics in the Victorian dairy industry could be a concern for dairy producers. The high level of contamination, regardless of the potential antimicrobial resistance and biofilm formation ability of isolates, showed that regular monitoring, control of critical points and source-tracking of *E. coli* contamination could help to assure the safety and quality of dairy products.

In the second experimental chapter (Chapter 4), the prevalence, characterisation and diversity of the *B. cereus* group—as a representative of a Gram-positive and important spore-forming dairy contaminant found in raw and pasteurised milk—was reported. The *B. cereus* group was recovered from 42.2% of milk samples, with higher levels of recovery from pasteurised milk. The level of contamination detected in the current study was higher than that reported in other similar studies in other countries. A higher prevalence of *B. cereus* group contamination in pasteurised milk could be due to the processing conditions, post-pasteurisation contamination likely from biofilms and incorrect storage temperatures (Banyko & Vyletelova, 2009; D.

Porcellato et al., 2018). Various enterotoxin genes related to the virulence of the *B. cereus* group were found among the *B. cereus* group isolates. The most frequent virulence genes in the raw milk isolates were *nheA*, *nheB*, *nheC* and *hblA*, whereas, in pasteurised milk isolates, the most frequent virulence genes were *nheB*, *nheA*, *hblB* and *hblA*. The identification of the *cyk* gene in many isolates revealed that a large number of potentially diarrhoeal strains were present in the collected samples. No isolates contained the cereulide synthetase (*ces*) gene. Other similar studies have reported similar prevalence of detected virulence genes (Arslan et al., 2014; Owusu-Kwarteng et al., 2017). The pasteurised milk isolates contained significantly more virulence genes than raw milk isolates, indicating the necessity to implement good hygienic and manufacturing practices by dairy producers to prevent post-pasteurisation contamination and subsequent potential food poisoning incidents by the *B. cereus* group.

Antimicrobial susceptibility testing of the isolates showed a high prevalence of antibiotic resistance towards ampicillin, ceftriaxone and penicillin. All isolates were resistant to at least two antimicrobials, and 97.6% of the isolates were resistant to three antimicrobials. In agreement with previous reports, the *B. cereus* group isolates showed susceptibility to chloramphenicol, ciprofloxacin, clindamycin and gentamycin. (Owusu-Kwarteng et al., 2017; Yu et al., 2020). Antimicrobial resistance was considerably higher in pasteurised milk samples than in raw milk samples.

Most of the *B. cereus* group isolates could form a biofilm but only 4.9% displayed strong biofilm formation ability. A greater prevalence of biofilm producers was observed in pasteurised isolates compared to raw milk isolates.

The present study provided some remarkable information about *B. cereus* group isolates from milk samples in Victoria, especially by comparing the characteristics of raw and pasteurised milk samples. The higher levels of *B. cereus* group isolates from pasteurised milk samples than in raw milk samples may be a cause of concern regarding the safety and quality of dairy products. Pasteurised milk isolates also contained more virulence genes than raw milk isolates. Furthermore, antimicrobial resistance was considerably higher in pasteurised milk samples than in raw milk samples, with all the pasteurised milk isolates resistant to at least three antimicrobials. It is known that, in Australia, the prevalence of antibiotic-resistant bacteria is on the rise. There is no comprehensive information available on the antimicrobial properties of the *B. cereus* group in dairy products in Australia. Therefore, the findings of this study can provide useful information for public health authorities.

A greater prevalence of biofilm producers was also observed in pasteurised isolates compared to raw milk isolates. To the best of our knowledge, there is no research available to compare

the level of biofilm formation ability in milk-derived *B. cereus* group isolates in Australia. However, since biofilm elimination is challenging for the dairy industry, this high level of biofilm production by the *B. cereus* group, especially in pasteurised milk, needs to be considered by dairy producers. All of the above results indicate the necessity that dairy producers implement good hygienic and manufacturing practices to prevent post-pasteurisation contamination and subsequent potential food poisoning incidents by the *B. cereus* group.

In Chapters 3 and 4, the evaluation of the bacterial identification proficiency of two market-leading instruments—the Bruker Biotyper® and VITEK® MS, with their respective software and databases—was reported. For the *E. coli* isolates, VITEK® MS accurately identified 97.2% at the genus level and 88.9% at the species level, while the Bruker Biotyper® accurately identified 98.6% at the genus level and 95.8% at the species levels. As for *E. coli*, the VITEK® MS identified fewer *B. cereus* group isolates than the Bruker Biotyper® (95.2% vs. 100%). In most similar studies, the Bruker Biotyper® system gave higher identification levels than the VITEK® MS. The difference between the identification levels of these instruments could be due to their different spectral profiles of food bacteria in their reference databases and the quality of the created spectra (Cherkaoui et al., 2010; Croxatto et al., 2012; Veen et al., 2010). However, results from the present study and similar research showed that both the VITEK® MS and Bruker Biotyper® are accurate tools for identifying *E. coli* and *B. cereus* group isolates.

Employing a comprehensive approach for relevant and cost-effective hygiene control throughout dairy production lines requires the combination of several systems, from surface sampling to molecular analyses. At present, the grouping and classifying of microorganisms based on their phenotypic, genetic and phylogenetic characteristics is a tool for determining bacterial diversity and source tracking. Routine fingerprinting of the collected microbial isolates enables proper monitoring of the contamination and enables practical root-cause analyses and proper corrective actions in safety management. Consequently, various phenotypic approaches have been used by researchers to classify microorganisms (Wenjun Li et al., 2009; Martak et al., 2019; Scott et al., 2002). Therefore, the other main focus of the present study was on determining the diversity of the *E. coli* and *B. cereus* group isolates by using different typing techniques.

In this study, the ERIC-PCR fingerprinting method was used as a genomic fingerprinting method. ERIC-PCR genotyping results for *E. coli* isolates showed that the size, number and characteristics of the isolate bands were similar to those observed in other studies (Rúgeles et

al., 2010; Vivek Prabhu, 2010). The dendrograms derived from ERIC-PCR showed that the *E. coli* isolates could be divided into five major clusters. ERIC-PCR also showed correlations between clusters and the place of sampling for *E. coli* isolates, as well as a strong correlation between clusters and antimicrobial resistance. Other similar research has shown that isolates of the same *E. coli* strain can express the same resistance patterns. A correlation was also observed between clusters and biofilm formation ability, which has also been observed in similar studies. These findings, in combination with those of other studies, showed that ERIC-PCR can be used to predict some bacterial characteristics of *E. coli* isolates based on the similarity of their origins.

ERIC-PCR was also employed as a genomic reference method for fingerprinting the *B. cereus* group. ERIC-PCR profiling placed most *B. cereus* group isolates from pasteurised milk in the same cluster, indicating that they probably originated from the same source. In contrast, the raw milk isolates showed greater diversity, indicating various sources. So, these results, in combination with those from other studies, indicated that ERIC-PCR is a reliable method for source tracking of the *B. cereus* group in the dairy industry.

As previously discussed, the MALDI-TOF MS technique was used in the present study for bacterial identification. At present, MALDI-TOF MS represents an advanced approach for the classification of microorganisms based on their phenotypic characteristics. As described in several studies, MALDI-TOF MS provides reproducible and accurate results at the species level that are, in most cases, accordant with genomic identification methods (Carbonnelle et al., 2011; Wieser & Schubert, 2016). Consequently, MALDI-TOF MS has the potential to be used in taxonomy. In contrast to gene sequencing, which provides taxonomical data on a single gene at a time, MALDI-TOF MS fingerprints offer information about the multiple protein components that characterise a microorganism. Furthermore, MALDI-TOF MS fingerprints provide data on both presence and intensity, creating a two-dimensional taxonomical asset that provides a better discriminative resolution for microorganism classification. The goal would be to use MALDI-TOF MS for rapid typing at the time of identification, which should significantly benefit the application of microbial epidemiology and infection control procedures that need to be applied to prevent the spreading of bacterial pathogens. This could considerably improve the methods currently used to monitor epidemiological outbreaks and pathogenic bacteria surveillance (de Koster & Brul, 2016; Sandrin et al., 2013; Santos et al., 2015).

Accordingly, in this study, MALDI-TOF MS was also used for bacterial fingerprinting and diversity determination. Based on the MALDI-TOF MS dendrograms obtained in the present study, *E. coli* isolates were divided into five clusters. No correlation was observed between the *E. coli* MALDI-TOF MS clusters and antimicrobial resistance. Most of the strong biofilm–producing *E. coli* isolates were placed in two main clusters but the rest were placed in different clusters. The dendrograms derived from the MALDI-TOF mass spectra for the *B. cereus* group divided the isolates into four primary clusters. Most pasteurised milk *B. cereus* group isolates were placed in one cluster but no relationship was found between milk producers and clusters. Raw milk isolates were distributed among all four primary clusters. Despite many studies on the identification of microorganisms by MALDI-TOF MS, few studies have tried to distinguish between strains of the same species. To the best of our knowledge, this study is the first investigation comparing MALDI-TOF MS clusters with those obtained by ERIC-PCR for *E. coli* and the *B. cereus* group isolates from raw milk.

Similar studies have suggested an acceptable correlation between phenotypic characteristics and phylogenetic relationships delineated by MALDI-TOF MS and MLST but not between MALDI-TOF MS and PFGE. However, some studies have shown some correlation between the phenotypic characteristics of MALDI-TOF MS and PFGE (Feng et al., 2020; Jadhav et al., 2012; Novais et al., 2014). Despite the accepted advantages of MALDI-TOF MS compared to genomics methods—such as rapidity, accuracy, sensitivity and specificity—this study showed that using MALDI-TOF MS and clustering data analysis could not discriminate between *E. coli* isolates or *B. cereus* group isolates based on their sources and characteristics to the same degree as ERIC-PCR. Some other similar studies have also suggested that, despite the advantages of MALDI-TOF MS, other traditional and molecular typing methods still deliver more complementary information (Novais et al., 2014; Tiago Santos et al., 2015; Wenning et al., 2014).

When time matters during a contamination emergency, rapid screening prior to genome sequencing critically helps in setting up efficient epidemiological study plans. Recently, several research groups have assessed the discriminatory power of FT-IR as a rapid and high-throughput technique for bacterial typing, in most cases in comparison with routine genotyping methods such as MLST and PFGE (Cordovana et al., 2021a; Martak et al., 2019). FT-IR spectroscopy is a phenotypic method that is conventionally applied for determining the molecular composition of samples. FT-IR can also discriminate bacterial strains by quantifying the absorption of infrared light by the variety of molecules present in the bacteria, particularly

the component carbohydrates and lipopolysaccharides in the bacterial cell wall (Dinkelacker et al., 2018; Hu et al., 2021; Martak et al., 2019).

Accordingly, in the current study, the application of FT-IR as an emerging method of bacterial typing was evaluated in assessing the diversity of *E. coli* and *B. cereus* group isolates. The results showed a significant correlation between clusters and antimicrobial resistance for *E. coli* isolates but no correlation between clusters and the biofilm formation ability of isolates. Assessment of the diversity of *B. cereus* group isolates showed that almost all raw milk isolates were placed in cluster A and isolates from pasteurised milk were placed in cluster B, indicating that the raw and pasteurised milk isolates originated from different sources. FT-IR spectroscopy also showed a stronger correlation between pasteurised milk isolates and more diversity amongst raw milk isolates. These findings were in agreement with similar studies that indicated FT-IR to be a powerful tool for the typing of *E. coli* and *B. cereus* group isolates. Furthermore, its major advantage over genomic methods—that is, rapid turnaround time—encourages the use of this technology for typing and source-tracking of *E. coli* and *B. cereus* group and possibly other relevant foodborne pathogens.

In the dairy industry, product quality and shelflife are dependent on an effective hygiene program and sanitation. In the Australian dairy industry, peracetic acid (PAA) is one of the most commonly used sanitisers with a broad spectrum of antimicrobial activity. PAA is active at low temperatures, safe and environmentally friendly, and its efficacy is not affected by organic residues; therefore, it can be applied without the risk of contaminating dairy products with toxic residues. However, little information is available on the effectiveness of PAA on biofilm-forming bacterial strains in the Australian dairy industry (Cheng et al., 2020; Lee et al., 2016; Lowry, 2010).

Accordingly, the final chapter reported on the effectiveness of different concentrations of PAA on biofilm-forming *E. coli* and *B. cereus* group dairy isolates. This aimed to evaluate and ensure the effectiveness of current sanitation processes in the Australian dairy industry, despite the presence of resistant bacteria with the ability to produce biofilm. The findings of this study showed that PAA could be used as a potent sanitiser to control dairy *E. coli* and *B. cereus* group isolates. *E. coli* isolates were sensitive to PAA at lower concentrations but *B. cereus* group isolates showed reduced sensitivity to PAA, especially within biofilm producers. Therefore, control of biofilm-producing *B. cereus* group organisms is an essential task for the Australian dairy industry.

6.2 Future directions

The present study showed that, despite best efforts of good manufacturing practice (GMP) in the Victorian dairy farming and processing industries, the levels of *E. coli* contamination in raw milk samples and *B. cereus* group contamination in raw and pasteurised milk samples are considerably high. The situation seems even worse when it comes to other characteristics of these bacteria like virulence genes, antimicrobial resistance and biofilm formation ability, especially for pasteurised milk samples. These two bacteria are representative only of Gram-negative and non-spore–forming (*E. coli*) and Gram-positive and spore-forming (*B. cereus* group) bacterial species. Therefore, the findings of this research provide some significant motivation for developing an effective control program to limit the presence of all pathogenic and spoilage bacteria in dairy processing environments. The Victorian dairy industry needs to better understand the whole contamination flow from farm to product. GMP in farms during the production and storage of raw milk should be implemented, and approaches to decrease the bacterial population in raw milk should be reinforced. Better execution of standard quality systems, like HACCP, in milk processing lines should be considered. More research on the bacterial characteristics in milk processing environments is needed to develop better CCP regimes for eliminating bacteria from dairy processing lines.

Additionally, the current study also showed that different methods of bacterial fingerprinting and source tracking can provide helpful information about bacterial characteristics and can be a useful tool in bacterial contamination control management. The findings of this study indicated that some recently developed fingerprinting and source tracking approaches, like FT-IR stereoscopy, have major advantages over genomic methods including fast turnaround time and ease of use; these advantages could encourage the use of these technologies in the dairy industry. Therefore, future research could focus on applying rapid fingerprinting and source tracking methods and strategies to the source tracking of harmful bacteria, thereby expanding opportunities for contamination control in the dairy industry.

Finally, besides tracking the source of contamination, the use of alternative sanitising methods is necessary for dairy safety assurance in the Victorian dairy industry. Planned and regular cleaning and sanitising to minimise the risk of possible contamination is suggested. Further studies to identify CCPs of bacterial contamination are also recommended. Furthermore, optimisation of the existing sanitation procedures and the development of effective strategies

are of great importance to the Victorian dairy industry as these may lead to quality improvements in products and processes.

References:

Abee, T., Groot, M. N., Tempelaars, M., Zwietering, M., Moezelaar, R., & Voort, M, 2011, Germination and outgrowth of spores of *Bacillus cereus* group members: Diversity and role of germinant receptors. *Food Microbiology,* Vol. 28, no. 2, pp. 199-208.

Adams, M. R., & Moss, M. O, 2008, *Food Microbiology*, 3rd edn. Cambridge: Royal Society of Chemistry.

Alm, R., Johansson, P., Hjerno, K., Emanuelsson, C., Ringner, M., & Hakkinen, J, 2006, Detection and identification of protein isoforms using cluster analysis of MALDI-MS mass spectra. *Journal of Proteome Research,* Vol. *5,* no. 4, pp. 785-792.

Alvarez-Ordonez, A., & Prieto, M, 2012, Technical and Methodological Aspects of Fourier Transform Infrared Spectroscopy in Food Microbiology Research. *Fourier Transform Infrared Spectroscopy in Food Microbiology,* Vol. 1, pp. 1-18.

Aminov, R. I, 2010, A Brief History of the Antibiotic Era: Lessons Learned and Challenges for the Future. *Frontiers in Microbiology,* Vol. 1, pp. 134.

Arslan, S., Eyi, A., & Kucuksarı, R, 2014, Toxigenic genes, spoilage potential, and antimicrobial resistance of *Bacillus cereus* group strains from ice cream. *Anaerobe, 25*, 42-46.

Artursson, K., Schelin, J., Thisted Lambertz, S., Hansson, I., & Olsson Engvall, E, 2018, Foodborne pathogens in unpasteurized milk in Sweden. *International Journal of Food Microbiology*, Vol. 2, no. 284, pp. 120-127.

Atashpaz, S., Khani, S., Barzegari, A., Barar, J., Vahed, S. Z., Azarbaijani, R., & Omidi, Y, 2010, A robust universal method for extraction of genomic DNA from bacterial species. *Microbiology,* Vol. 79 no. 4, pp. 538-542.

Awadallah, M. A., Ahmed, H. A., Merwad, A. M., & Selim, M. A, 2016, Occurrence, genotyping, shiga toxin genes and associated risk factors of *E. coli* isolated from dairy farms, handlers and milk consumers. *The Veterinary Journal,* Vol. 217, pp. 83-88.

Bagcıoglu, M., Fricker, M., Johler, S., & Ehling-Schulz, M, 2019, Detection and Identification of *Bacillus cereus, Bacillus cytotoxicus, Bacillus thuringiensis, Bacillus mycoides* and *Bacillus weihenstephanensis* via Machine Learning Based FTIR Spectroscopy. *Frontiers in Microbiology,* Vol. 10 pp. 902.

Bagheripoor-Fallah, N., Mortazavian, A., Hosseini, H., Khoshgozaran-Abras, S., & Rad, A. H, 2015, Comparison of Molecular Techniques with other Methods for Identification and Enumeration of Probiotics in Fermented Milk Products. *Critical Reviews in Food Science and Nutrition,* Vol. 55, no. 3, pp. 396-413.

Baker, C. N., Thornsberry, C., & Hawkinson, R. W, 1983, Inoculum standardization in antimicrobial susceptibility testing: evaluation of overnight agar cultures and the Rapid Inoculum Standardization System. *Journal of Clinical Microbiology,* Vol. 17, no. 3, pp. 450-457.

Balouiri, M., Sadiki, M., & Ibnsouda, S. K, 2016, Methods for in vitro evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis,* Vol. 6, no. 2, pp. 71-79.

Banach, J. L., van Bokhorst-van de Veen, H., van Overbeek, L. S., van der Zouwen, P. S., Zwietering, M. H., & van der Fels-Klerx, H. J, 2020, Effectiveness of a peracetic acid solution on *Escherichia coli* reduction during fresh-cut lettuce processing at the laboratory and industrial scales. *International Journal of Food Microbiology,* Vol. 321, e108537.

Banyko, J., & Vyletelova, M, 2009, Determining the source of *Bacillus cereus* and *Bacillus licheniformis* isolated from raw milk, pasteurized milk and yoghurt. *Letters in Applied Microbiology,* Vol. 48 no. 3, pp. 318-323.

Barbosa, J., Cuppini, M., Flach, J., Steffens, C., Cansian, R. L., & Toniazzo, G, 2016, Removal of *Escherichia coli* in boning knives with different sanitizers. *LWT - Food Science and Technology,* Vol. 71, pp. 309-315.

Baylis, C. L, 2009, Raw milk and raw milk cheeses as vehicles for infection by Verocytotoxin-producing *Escherichia coli*. *International Journal of Dairy Technology,* Vol. 62, no. 3, pp. 293-307.

Beloin, C., Roux, A., & Ghigo, J. M, 2008, *Escherichia coli* Biofilms. *Bacterial Biofilms,* T. Romeo edn, Springer, Berlin Heidelberg.

Berger, S, 2014, *Infectious Disease of Australia*. Gideon e-book series.

Berthomieu, C., & Hienerwadel, R, 2009, Fourier transform infrared (FTIR) spectroscopy. *Photosynthesis Research,* Vol. 101, no. 2, pp. 157-170.

Bessalah, S., Fairbrother, J. M., Salhi, I., Vanier, G., Khorchani, T., Seddik, M. M., & Hammadi, M, 2016, Antimicrobial resistance and molecular characterization of virulence genes, phylogenetic groups of *Escherichia coli* isolated from diarrheic and healthy camel-calves in Tunisia. *Comparative Immunology, Microbiology*

and Infectious Diseases , Vol .49, pp. 1-7.

Bethune, M., & Armstrong, D. P, 2004, Overview of the irrigated dairy industry in Australia. *Australian Journal of Experimental Agriculture,* Vol. 44, no. 2, pp. 127-129.

Billington, G., Kingsbury, J.M., & Rivas, L, 2022, Metagenomics Approaches for Improving Food Safety: *A Review. Journal of food Protection,* Vol. 85, No. 3, pp. 448-464.

Bintsis, T, 2017, Foodborne pathogens. *AIMS microbiology,* Vol. 3 no. 3, pp. 529-563.

Bohme, K., Barros-Velazquez, J., & Calo-Mata, P, 2017, Fingerprinting for Detecting Contaminants in Food. In S. Kennedy edn, *Food Protection and Security*. Woodhead Publishing.

Bridier, A., Briandet, R., Thomas, V., & Dubois-Brissonnet, F, 2011, Comparative biocidal activity of peracetic acid, benzalkonium chloride and ortho-phthalaldehyde on 77 bacterial strains. *Journal of Hospital Infection,* Vol. 78, no. 3, pp. 208-213.

Bruker. (2020). MALDI Biotyper Protocol Guide, 6th edn, Germany, Bruker.

Campos, J., Sousa, C., Mourão, J., Lopes, J., Antunes, P., & Peixe, L, 2018, Discrimination of non-typhoid *Salmonella* serogroups and serotypes by Fourier Transform Infrared Spectroscopy: A comprehensive analysis. *International Journal of Food Microbiology,* Vol. 285, pp. 34-41.

Carbonnelle, E., Mesquita, C., Bille, E., Day, N., Dauphin, B., Beretti, J.-L., & Nassif, X, 2011, MALDI-TOF mass spectrometry tools for bacterial identification in clinical microbiology laboratory. *Clinical Biochemistry,* Vol. 44, no. 1, pp. 104-109.

Ceragioli, M., Mols, M., Moezelaar, R., Ghelardi, E., Senesi, S., & Abee, T, 2010, Comparative Transcriptomic and Phenotypic Analysis of the Responses of *Bacillus cereus* to Various Disinfectant Treatments. *Applied and Environmental Microbiology,* Vol. 76, no. 10, pp. 3352-3360.

Chandan, R. C., Kilara, A., & Shah, N. P, 2009, *Dairy processing and quality assurance*: John Wiley & Sons.

Chaves, J. Q., Pires, E. S., & Vivoni, A. M, 2011, Genetic diversity, antimicrobial resistance and toxigenic profiles of *Bacillus cereus* isolated from food in Brazil over three decades. *International Journal of Food Microbiology,* Vol. 147 no. 1, pp. 12-16.

Chen, C., Liao, X., Jiang, H., Zhu, H., Yue, L., Li, S., & Liu, Y. (2010). Characteristics of *Escherichia coli* biofilm production, genetic typing, drug resistance pattern and gene expression under aminoglycoside pressures. *Environmental Toxicology and Pharmacology,* Vol. 30, no.1, pp. 5-10.

Cheng, C., Li, H., Wang, J., Wang, H., & Yang, X, 2020, A review of measurement methods for peracetic acid (PAA). *Frontiers of Environmental Science & Engineering,* Vol. 14, no. 5, pp. 87.

Cherkaoui, A., Hibbs, J., Emonet, S., Tangomo, M., Girard, M., Francois, P., & Schrenzel, J, 2010, Comparison of Two Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry Methods with Conventional Phenotypic Identification for Routine Identification of Bacteria to the Species Level. *Journal of Clinical Microbiology,* Vol. 48, no. 4, pp. 1169-1175.

Choi, C., Cho, W.-S., Chung, H.-K., Jung, T., Kim, J., & Chae, C, 2001, Prevalence of the enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 (EAST1) gene in isolates in weaned pigs with diarrhea and/or edema disease. *Veterinary Microbiology,* Vol. 81, no. 1, pp. 65-71.

Christner, M., Dressler, D., Andrian, M., Reule, C., & Petrini, O, 2017, Identification of Shiga-Toxigenic *Escherichia coli* outbreak isolates by a novel data analysis tool after matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *PLOS ONE,* Vol. 12, no. 9, e0182962.

Claeys, W. L., Cardoen, S., Daube, G., De Block, J., Dewettinck, K., Dierick, K., & Herman, L, 2013, Raw or heated cow milk consumption: Review of risks and benefits. *Food Control,* Vol. 31, no. 1, pp. 251-262.

Clark, C. G., Kruczkiewicz, P., Guan, C., McCorrister, S. J., Chong, P., Wylie, J., & Westmacott, G. R, 2013, Evaluation of MALDI-TOF mass spectroscopy methods for determination of Escherichia coli pathotypes. *Journal of Microbiological Methods,* Vol. 94, no. 3, pp. 180-191.

Clermont, O., Bonacorsi, S., & Bingen, E, 2000, Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Applied Environmental Microbiology,* Vol. 66, no.10, pp. 4555-4558.

CLSI, 2017, Performance Standards for Antimicrobial Susceptibility Testing. In CLSI supplement M100 edn, Wayne PA: Clinical and Laboratory Standards Institute.

Cordovana, M., Mauder, N., Kostrzewa, M., Wille, A., Rojak, S., Hagen, R. M., & Dekker, D, 2021, Classification of *Salmonella enterica* of the (Para-)Typhoid Fever Group by Fourier-Transform Infrared (FTIR) Spectroscopy. *Microorganisms,* Vol. 9*,* no. 4, pp. 853.

Croxatto, A., Prodhom, G., & Greub, G, 2012, Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology. *FEMS Microbiology Reviews,* Vol. 36, no. 2, pp. 380-407.

de Koster, C. G., & Brul, S, 2016, MALDI-TOF MS identification and tracking of food spoilers and food-borne pathogens. *Current Opinion in Food Science,* Vol. 10, pp. 76-84.

DePas, W. H., Syed, A. K., Sifuentes, M., Lee, J. S., Warshaw, D., Saggar, V., & Chapman, M. R, 2014, Biofilm formation protects *Escherichia coli* against killing by *Caenorhabditis elegans* and *Myxococcus xanthus*. *Applied and Environmental Microbiology,* Vol. 80, no. 22, pp. 7079-7087.

Dinkelacker, A. G., Vogt, S., Oberhettinger, P., Mauder, N., Rau, J., Kostrzewa, M., & Liese, J, 2018, Typing and Species Identification of Clinical *Klebsiella* Isolates by Fourier Transform Infrared Spectroscopy and Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry. *Journal of Clinical Microbiology,* Vol. 56, no.11

Dolejska, M., Senk, D., Cizek, A., Rybarikova, J., Sychra, O., & Literak, I, 2008, Antimicrobial resistant *Escherichia coli* isolates in cattle and house sparrows on two Czech dairy farms. *Research in Veterinary Science,* Vol. *85*, no. 3, pp. 491-494.

Donnenberg, M, 2014, *Escherichia coli Pathotypes and Principles of Pathogenesis*, 2nd edn, Saint Louis.

Eglezos, S., Huang, B., Dykes, G. A., & Fegan, N, 2010, The prevalence and concentration of *Bacillus cereus* in retail food products in Brisbane, Australia. *Foodborne Pathogen and Disease,* Vol. *7*, no. 7, pp. 867-870.

Elmerdahl Olsen, J, 2000, DNA-based methods for detection of food-borne bacterial pathogens. *Food Research International,* Vol. *33*, no. 3, pp. 257-266.

Enriquez-Gomez, E., Talavera-Rojas, M., Soriano-Vargas, E., Navarro-Ocaña, A., Vega-Sánchez, V., Oca, S. A.-M. d., & Acosta-Dibarrat, J, 2017, Serotypes, virulence genes profiles and antimicrobial resistance patterns of *Escherichia coli* recovered from feces of healthy lambs in Mexico. *Small Ruminant Research,* Vol. 153, pp. 41-47.

Escobar-Paramo, P., Le Menach, A., Le Gall, T., Amorin, C., Gouriou, S., Picard, B.,& Denamur, E, 2006, Identification of forces shaping the commensal *Escherichia coli* genetic structure by comparing animal and human isolates. *Environmental Microbiology,* Vol. 8, no. 11, pp. 1975-1984.

Fegan, N., & Desmarchelier, P, 2010, Pathogenic *E. coli* in the dairy industry: Implications for Australia. *Australian Journal of Dairy Technology,* Vol. 65, pp. 68-73.

Feng, B., Shi, H., Xu, F., Hu, F., He, J., Yang, H., & Yu, S, 2020, FTIR-assisted MALDI-TOF MS for the identification and typing of bacteria. *Analytica Chimica Acta,* Vol. 11, pp. 75-82.

Fernandes, C. J., & Ackerman, V. P, 1989, Antibiotic resistance in Australia with special reference to sulbactam/ampicillin. *Diagnostic Microbiology and Infectious Disease,* Vol. 12, no. 4, pp. 153-157.

Fernandes, J. B. C., Zanardo, L. G., Galvao, N. N., Carvalho, I. A., Nero, L. A., & Moreira, M. A. S, 2011, *Escherichia coli* from clinical mastitis serotypes and virulence factors. *Journal of Veterinary Diagnostic Investigation,* Vol. 23, pp. 1146-1152.

Fogele, B., Granta, R., Valciņa, O., & Berzins. A, 2018, Occurrence and diversity of *Bacillus cereus* and moulds in spices and herbs. *Food Control*, Vol. 83, pp. 96 -74.

Foleya, S. L., Lynne, S. L., & Nayak R, 2009, Molecular typing methodologies for microbial source tracking and epidemiological investigations of Gram-negative bacterial foodborne pathogens. *Infection, Genetics and Evolution.* Vol. 9, no. 4, pp. 430-440.

Forghani, F., Langaee, T., Eskandari, M., Seo, K.-H., Chung, M.J., & Oh, D.H, 2015, Rapid detection of viable *Bacillus cereus* emetic and enterotoxic strains in food by coupling propidium monoazide and multiplex PCR (PMA-mPCR). *Food Control,* Vol. 55, pp. 151-157.

Fricker, M., Reissbrodt, R., & Ehling-Schulz, M, 2008, Evaluation of standard and new chromogenic selective plating media for isolation and identification of *Bacillus cereus. International Journal of Food Microbiology,* Vol. 121, no. 1, pp. 27-34.

Fung, F., Wang, H.-S., & Menon, S, 2018, Food safety in the 21st century. *Biomedical journal,* Vol. 41, no. 2, pp. 88-95.

Gao, T., Ding, Y., Wu, Q., Wang, J., Zhang, J., Yu, S., & Wu, H, 2018, Prevalence, Virulence Genes, Antimicrobial Susceptibility, and Genetic Diversity of *Bacillus cereus* Isolated From Pasteurized Milk in China. *Frontiers in Microbiology,* Vol. *9*, pp. 533.

Gawande, H., Dhotre, A., M, S., & M, K, 2013, Peroxyacetic Acid : A Potent Food Industry Sanitizer. *Indian Food Industry Magazine*, Vol. 32, no. 3, pp. 26-30.

Gdoura-Ben Amor, M., Siala, M., Zayani, M., Grosset, N., Smaoui, S., Messadi-Akrout, F., & Gdoura, R, 2018, Isolation, Identification, Prevalence, and Genetic Diversity of *Bacillus cereus* Group Bacteria From Different Foodstuffs in Tunisia. *Frontiers in Microbiology,* Vol. 9, pp. 447.

Ghyselinck, J., Van Hoorde, K., Hoste, B., Heylen, K., & De Vos, P, 2011, Evaluation of MALDI-TOF MS as a tool for high-throughput dereplication. *Journal of Microbiological Methods,* Vol. 86, no. 3, pp. 327-336.

Giebel, R. A., Fredenberg, W., & Sandrin, T. R, 2008, Characterization of environmental isolates of *Enterococcus* spp. by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Water Research,* Vol. 42, no. 4, pp. 931-940.

Gillings, M., & Holley, M, 1997, Repetitive element PCR fingerprinting (rep-PCR) using enterobacterial repetitive intergenic consensus (ERIC) primers is not necessarily directed at ERIC elements. *Letters in Applied Microbiology,* Vol. *25*, no. 1, pp. m17-21.

Gopal, N., Hill, C., Ross, P. R., Beresford, T. P., Fenelon, M. A., & Cotter, P. D, 2015, The Prevalence and Control of *Bacillus* and Related Spore-Forming Bacteria in the Dairy Industry. *Frontiers in Microbiology,* Vol. 6, pp. 1418.

Granlund, I., Kieselbach, T., Alm, R., Schroder, W. P., & Emanuelsson, C, 2011, Clustering of MS spectra for improved protein identification rate and screening for protein variants and modifications by MALDI-MS/MS. *Journal of Proteomics,* Vol. 74, no. 8, pp. 1190-1200.

Granum, P. E., Stenfors Arnesen, L., & From, C, 2014, Bacteria: *Bacillus cereus* and Other Pathogenic *Bacillus* Species. *Encyclopedia of Food Safety*. Waltham: Academic Press.

Greenwood, M., Roberts, D. P. D., & Wiley, I, 2002, *Practical food microbiology*, 3rd edn, Malden, Mass: Blackwell Publication.

Griffiths, M. W., & Schraft, H, 2017, Chapter 20 - *Bacillus cereus* Food Poisoning. In *Foodborne Diseases,* 3rd edn, Academic Press.

Grutsch, A. A., Nimmer, P. S., Pittsley, R. H., & McKillip, J. L, 2018, Chapter 7 - *Bacillus* spp. as Pathogens in the Dairy Industry. In A. M. Holban & A. M. Grumezescu edn, *Foodborne Diseases*, Academic Press.

Guembe, M., Perez-Granda, M. J., Cruces, R., Rodriguez-Sanchez, B., Ruiz, A., Alcala, L., & Bouza, E, 2016, MALDI-TOF is not useful in the diagnosis of catheter colonization based on superficial cultures: results from an in vitro study. Diagnostic Microbiology and Infectious Disease , Vol. 84, no. 1, pp. 7-11.

Guinebretiere, M. H., Broussolle V Fau - Nguyen-The, C., & and Nguyen-The, C, 2002, Enterotoxigenic profiles of food-poisoning and food-borne *Bacillus cereus* strains. *Journal of clinical Microbiology,* Vol. *40*, no. 8, pp. 4.

Gundogan, N., & Avci, E, 2014, Occurrence and antibiotic resistance of *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus* in raw milk and dairy products in Turkey. *International Journal of Dairy Technology,* Vol. *67*, no 4, pp. 562-569.

Harakeh, S., Yassine, H., Gharios, M., Barbour, E., Hajjar, S., El-Fadel, M., & Tannous, R. , 2005, Isolation, molecular characterization and antimicrobial resistance patterns of *Salmonella* and *Escherichia coli* isolates from meat-based fast food in Lebanon. *Science of The Total Environment,* Vol. *341*, no. 1, pp. 33-44.

Hardy-Holbrook, R., Aristidi, S., Chandnani, V., DeWindt, D., & Dinh, K, 2013, Antibiotic resistance and prescribing in Australia: current attitudes and practice of GPs. *Healthcare infection,* Vol. *18*, no. 4, pp. 147-151.

Hathaway LJ, B. S., Martynova A, Aebi S, Mühlemann K, 2007, Use of the Agilent 2100 bioanalyzer for rapid and reproducible molecular typing of *Streptococcus pneumoniae. Journal of Clinical Microbiology,* Vol. *45*, no. 3, pp. 803-809.

Haug, A., Hostmark, A. T., & Harstad, O. M, 2007, Bovine milk in human nutrition – a review. *Lipids in Health and Disease,* Vol. *6*, no. 1, pp. 6-25.

Helgason, E., Caugant, D. A., Lecadet, M.-M., Chen, Y., Mahillon, J., Lövgren, A., & Kolsto, A.B, 1998, Genetic Diversity of *Bacillus cereus/B. thuringiensis* Isolates from Natural Sources. *Current Microbiology,* Vol. *37*, no. 2, pp. 80-87.

Horvath, G., Jambor, N., Vegh, A., Boszormenyi, A., Lemberkovics, E., Hethelyi, E., & Kocsis, B, 2010, Antimicrobial activity of essential oils: the possibilities of TLC–bioautography. *Flavour and Fragrance Journal,* Vol. *25*, no. 3, pp. 178-182.

Hosseini, H., Jamshidi, A., Basssami, M. R., Khaksar, R., Zeynali, T., Mousavi Khaneghah, A., & Khanzadi, S, 2013, Isolation, Identification and Virulence Gene Profiling of *Escherichia coli* O157:H7 in Retail Doner Kebabs, Iran. *Journal of Food Safety,* Vol. *33*, no. 4, pp. 489-496.

Hu, Y., Zhou, H., Lu, J., Sun, Q., Liu, C., Zeng, Y., & Zhang, R, 2021, Evaluation of the IR Biotyper for *Klebsiella pneumoniae* typing and its potentials in hospital hygiene management. *Microbial Biotechnology,* Vol. *1*4, no. 4, pp. 1343-1352.

Huasai, S., Chen, A., Wang, C.-j., Li, Y., & Tongrige, B, 2012, Occurrence and characteristics of virulence genes of *Escherichia coli* strains isolated from healthy dairy cows in Inner Mongolia, China. *Brazilian Journal of Microbiology,* Vol. 43, pp. 528-534.

Hussain, M. S., & Oh, D. H, 2017, Substratum attachment location and biofilm formation by *Bacillus cereus* strains isolated from different sources: Effect on total biomass

production and sporulation in different growth conditions. *Food Control,* Vol. *77*, pp. 270-280.

Iniguez-Moreno, M., Avila-Novoa, M. G., Iniguez-Moreno, E., Guerrero-Medina, P. J., & Gutierrez-Lomeli, M, 2017, Antimicrobial activity of disinfectants commonly used in the food industry in Mexico. *Journal of Global Antimicrobial Resistance,* Vol. 10, pp. 143-147.

Innis, M. A, Gelfand, D. H., & Sinisky, J.J, 1999, *PCR Applications Protocols for Functional Genomics*. Burlington, Academic Press.

Jadhav, S., Bhave, M., & Palombo, E. A, 2012, Methods used for the detection and subtyping of *Listeria monocytogenes. Journal of Microbiological Methods,* Vol. 88, no. 3, pp. 327-341.

Jadhav, S., Gulati, V., Fox, E. M., Karpe, A., Beale, D. J., Sevior, D., & Palombo, E. A, 2015, Rapid identification and source-tracking of *Listeria monocytogenes* using MALDI-TOF mass spectrometry. *International Journal of Food Microbiology,* Vol. 202, pp. 1-9.

Jamali, H., Paydar, M., Radmehr, B., Ismail, S., & Dadrasnia, A, 2015, Prevalence and antimicrobial resistance of *Staphylococcus aureus* isolated from raw milk and dairy products. *Food Control,* Vol. *54*, pp. 383-388.

Jay, J. M, 2005, *Modern Food Microbiology*: Boston, MA : Springer.

Jenness, R.,Wong N. P., Jenness, M, & Marth E. H, 1988, Composition of Milk. *Fundamentals of Dairy Chemistry* Boston, MA: Springer.

Jonasson, E., Matuschek, E., & Kahlmeter, G, 2020, The EUCAST rapid disc diffusion method for antimicrobial susceptibility testing directly from positive blood culture bottles. *The Journal of antimicrobial chemotherapy,* Vol. 75, no. 4, pp. 968-978.

Kadam, S. R., den Besten, H. M. W., van der Veen, S., Zwietering, M. H., Moezelaar, R., & Abee, T, 2013, Diversity assessment of *Listeria monocytogenes* biofilm formation: Impact of growth condition, serotype and strain origin. *International Journal of Food Microbiology,* Vol. 165 , no.3, pp. 259-264.

Kaipainen, T., Pohjanvirta, T., Shpigel, N., Shwimmer, A., Pyörälä, S., & Pelkonen, S, 2002, Virulence factors of *Escherichia coli* isolated from bovine clinical mastitis. *Veterinary microbiology,* Vol. *85*, pp. 37-46.

Keane, O. M, 2016, Genetic diversity, the virulence gene profile and antimicrobial resistance of clinical mastitis-associated *Escherichia coli. Research in Microbiology,* Vol. *167*, no. 8, pp. 678-684.

Kim, J.B., Kim, J.M., Cho, S.H., Oh, H.S., Choi, N. J., & Oh, D.H, 2011, Toxin Genes Profiles and Toxin Production Ability of *Bacillus cereus* Isolated from Clinical and Food Samples. *Journal of Food Science,* Vol. *76*, no. 1, pp. 25-29.

Kintz, E., Brainard, J., Hooper, L., & Hunter, P, 2017, Transmission pathways for sporadic Shiga-toxin producing *E. coli* infections: A systematic review and meta-analysis. *International Journal of Hygiene and Environmental Health,* Vol. *220*, no. 1, pp. 57-67.

Krasny, L., Hynek, R., & Hochel, I, 2013, Identification of bacteria using mass spectrometry techniques. *International Journal of Mass Spectrometry,* Vol. *353*, pp. 67-79.

Kumar, C. G., & Anand, S. K, 1998, Significance of microbial biofilms in food industry: a review. *International Journal of Food Microbiology,* Vol. *42*, no. 1, pp. 9-27.

Kumari, S., & Sarkar, P. K, 2016, *Bacillus cereus* hazard and control in industrial dairy processing environment. *Food Control,* Vol. 69, pp. 20-29.

Lambertini, E., Karns, J. S., Van Kessel, J. A. S., Cao, H., Schukken, Y. H., Wolfgang, D. R., & Pradhan, A. K, 2015, Dynamics of *Escherichia coli* Virulence Factors in Dairy Herds and Farm Environments in a Longitudinal Study in the United States. *Applied and Environmental Microbiology,* Vol. 81, no. 13, pp. 4477 - 4488.

Larsen, H. D., & Jorgensen, K, 1997, The occurrence of *Bacillus cereus* in Danish pasteurized milk. *International Journal of Food Microbiology,* Vol. 34, no. 2, pp. 179-186.

Lasch, P., Jacob, D., Grunow, R., Schwecke, T., & Doellinger, J, 2016, Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) for the identification of highly pathogenic bacteria. *TrAC Trends in Analytical Chemistry,* Vol. *85*, pp. 103-111.

Lay Jr, J. O, 2001, MALDI-TOF mass spectrometry of bacteria. *Mass Spectrometry Review,* Vol. 20, no. 4, pp.

Lee, S. H. I., Cappato, L. P., Corassin, C. H., Cruz, A. G., & Oliveira, C. A. F, 2016, Effect of peracetic acid on biofilms formed by *Staphylococcus aureus* and *Listeria monocytogenes* isolated from dairy plants. *Journal of Dairy Science,* Vol. 99, no. 3, pp. 2384-2390.

Leung, K. T., Mackereth, R., Tien, Y.-C., & Topp, E, 2004, A comparison of AFLP and ERIC-PCR analyses for discriminating *Escherichia coli* from cattle, pig and human sources. *FEMS Microbiology Ecology,* Vol. 47, no. 1, pp. 111-119.

Levesque, S., Dufresne, P. J., Soualhine, H., Domingo, M. C., Bekal, S., Lefebvre, B., & Tremblay, C, 2015, A Side by Side Comparison of Bruker Biotyper and VITEK MS: Utility of MALDI-TOF MS Technology for Microorganism Identification in a Public Health Reference Laboratory. *PLOS ONE,* Vol. *1*o, no. 12, e0144878.

Li, F., Zuo, S., Yu, P., Zhou, B., Wang, L., Liu, C., & Hengyi, X, 2016, Distribution and expression of the enterotoxin genes of *Bacillus cereus* in food products from Jiangxi Province, China. *Food Control,* Vol. *67*, pp. 155-162.

Li, R., Tan, X., Xiao, J., Wang, H., Liu, Z., Zhou, M., & Miyamoto, T, 2016, Molecular screening and characterization of Shiga toxin-producing *Escherichia coli* in retail foods. *Food Control,* Vol. *60*, pp. 180-188.

Li, W., Raoult, D., & Fournier, P.E, 2009, Bacterial strain typing in the genomic era. *FEMS Microbiology Reviews,* Vol. *33*, no. 5, pp. 892-916.

Lorenzo, J. M., Munekata, P. E., Dominguez, R., Pateiro, M., Saraiva, J. A., & Franco, D, 2018, Main Groups of Microorganisms of Relevance for Food Safety and Stability: General Aspects and Overall Description. *Innovative Technologies for Food Preservation*, Vol. 1, pp. 53-107.

Lowry, D, 2010, Advances in cleaning and sanitation. *Australian Journal of Dairy Technology,* Vol. 65, no. 2, pp. 106-112.

Marchand, S., De Block, J., De Jonghe, V., Coorevits, A., Heyndrickx, M., & Herman, L, 2012, Biofilm Formation in Milk Production and Processing Environments; Influence on Milk Quality and Safety. *Comprehensive Reviews in Food Science and Food Safety,* Vol.*11*, no. 2, pp. 133-147.

Mariey, L., Signolle, J. P., Amiel, C., & Travert, J, 2001, Discrimination, classification, identification of microorganisms using FTIR spectroscopy and chemometrics. *Vibrational Spectroscopy,* Vol. *26*, no. 2, pp.151-159.

Marrollo, R, 2016, Chapter 5 - *Bacillus cereus* Food-Borne Disease. *The Diverse Faces of Bacillus cereus*, Academic Press.

Martak, D., Valot, B., Sauget, M., Cholley, P., Thouverez, M., Bertrand, X., & Hocquet, D, 2019, Fourier-Transform InfraRed Spectroscopy Can Quickly Type Gram-Negative Bacilli Responsible for Hospital Outbreaks. *Frontiers in Microbiology,* Vol. 10, pp. 1440.

Marth, E. H., & Steele, J, 2001, *Applied dairy microbiology*, CRC Press.

Martin, N.H., Torres-Frenzel, P., & Wiedmann, M, 2021, Invited review: Controlling dairy product spoilage to reduce food loss and waste. *Journal of Dairy Science.* Vol. 104, no. 2, pp. 1251-1261.

Martinovic, T., Andjelkovic, U., Gajdosik, M. S., Resetar, D., & Josic, D, 2016, Foodborne pathogens and their toxins. *Journal of Proteomics,* Vol. 147, pp. 226-235.

McAuley, C. M., McMillan, K., Moore, S. C., Fegan, N., & Fox, E. M, 2014, Prevalence and characterization of foodborne pathogens from Australian dairy farm environments. *Journal of Dairy Science,* Vol. 97, no. 12, pp. 7402-7412.

Merzougui, S., Lkhider, M., Grosset, N., Gautier, M., & Cohen, N, 2013, Differentiation by molecular typing of *Bacillus cereus* isolates from food in Morocco: PFGE-ERIC PCR. *Food and Public Health,* Vol. *3*, no. 4, pp. 223-227.

Micciche, A. C., Feye, K. M., Rubinelli, P. M., Lee, J. A., Knueven, C. J., & Ricke, S, 2019, Comparison of Acid Sanitizers on *Salmonella Typhimurium* Inoculated Commercial Poultry Processing Reuse Water. *Frontiers in Sustainable Food Systems,* Vol. *2,* pp. 90

Michelacci, V., Prosseda, G., Maugliani, A., Tozzoli, R., Sanchez, S., Herrera-Leon, S., & Morabito, S, 2016, Characterization of an emergent clone of enteroinvasive *Escherichia coli* circulating in Europe. *Clinical Microbiology and Infection,* Vol. 22, no. 3, pp. 287- 289.

Mietke, H., Beer, W., Schleif, J., Schabert, G., & Reissbrodt, R, 2010, Differentiation between probiotic and wild-type *Bacillus cereus* isolates by antibiotic susceptibility test and Fourier transform infrared spectroscopy (FT-IR). *International Journal of Food Microbiology,* Vol. 140, no. 1, pp. 57-60.

Milanov, D., Pruni , B., Velhner, M., Todorovj , D., & Pola ek, V, 2015. Investigation of Biofilm Formation and Phylogenetic Typing of *Escherichia Coli* Strains Isolated from Milk of Cows with Mastitis. *Acta Veterinaria,* Vol. 65, pp. 202 - 216.

Minnaard, J., Delfederico, L., Vasseur, V., Hollmann, A., Rolny, I., Semorile, L., & Perez, P. F, 2007, Virulence of *Bacillus cereus*: A multivariate analysis. *International Journal of Food Microbiology,* Vol. 116, no. 2, pp.197-206.

Mogha, K. V., Shah, N., Prajapati, J. B., & Chaudhari, A. R, 2014, Biofilm - A threat to dairy industry. *Indian journal of dairy science,* Vol. 67, no. 6, pp. 459- 466.

Mohammadi, M., Torkamaneh, D., Hashemi, M., Mehrabi, R. & Ebrahimi, A, 2012, Fast and inexpensive DNA isolation from wheat (Triticum aestivum) and other small grains.

Wheat Information Service. Electronic Newsletter for Wheat Researchers., Vol. 114, pp. 17-20.

Momba, M. N. B., Kfir, R., Venter, S. N., & Cloete, T. E, 2000, Overview of biofilm formation in distribution systems and its impact on the deterioration of water quality. In. *Water Research Commission* .

Monaghan, A., Byrne, B., Fanning, S., Sweeney, T., McDowell, D., & Bolton, D. J, 2011, Serotypes and Virulence Profiles of Non-O157 Shiga Toxin-Producing *Escherichia coli* Isolates from Bovine Farms. *Applied and Environmental Microbiology,* Vol. *77*, no. 24, pp. 8662.

Montville, T. J, 2012, *Food microbiology : an introduction*, 3rd edn Washington, D.C.

Moradi , A. A. V., Shahmoradi, M., Ghemi, E., Tabaraei, A. J., Sadegh sheshpoli, M., Bazori, M., & Kouhsari, H, 2009, Broad spectrum antibacterial activity of peracetic acid (Percidin). *Zahedan Journal of Research in Medical Sciences.* Vol.11, no.1.

More, S. J, 2009, Global trends in milk quality: implications for the Irish dairy industry. *Irish Veterinary Journal,* Vol. 62, no. 4.

Morris, J. J. G, 2013, *Foodborne Infections and Intoxications*, 4th edn, Burlington.

Mugadza, D. T., & Buys, E. M, 2017, Diversity of *Bacillus cereus* strains in extended shelf life. *International Dairy Journal,* Vol. *73*, pp.144-150.

Muhlemann, M, 2014, Safety of Food and Beverages: Dairy Products: Cheese. In *Encyclopedia of Food Safety* . Waltham: Academic Press.

Muigg, V., Cuenod, A., Purushothaman, V., Siegemund, M., Wittwer, M., Pfluger, V., Schmidt, K. M., Weisser, M., Ritz, N., Widmer, A., Goldenberger, D., Hinic, V., Tim Roloff, T., Kirstine K. Sogaard, K. K., Adrian Egli, A., & Helena M.B. Seth-Smith, H. M. B, 2022, Diagnostic challenges within the *Bacillus cereus* group: finding the beast without teeth. *New Microbes and New Infections,* Vol 50, e101040.

Nandanwar, N., Janssen, T., Kühl, M., Ahmed, N., Ewers, C., & Wieler, L. H, 2014, Extraintestinal pathogenic *Escherichia coli* (ExPEC) of human and avian origin belonging to sequence type complex 95 (STC95) portray indistinguishable virulence features. *International Journal of Medical Microbiology,* Vol. *304*, no. 7, pp. 835-842.

Nataro, J. P., & Steiner, T, 2002, Enteroaggregative and Diffusely Adherent *Escherichia Coli*: In *Escherichia Coli*. Academic Press.

Nataro, J. P., Steiner, T., & Guerrant, R. L. (1998). Enteroaggregative *Escherichia coli. Emerging infectious diseases,* Vol. *4*, no. 2, pp. 251-261.

Neo, S. Y., Lim, P. Y., Phua, L. K., Khoo, G. H., Kim, S. J., Lee, S. C., & Yuk, H. G, 2013, Efficacy of chlorine and peroxyacetic acid on reduction of natural microflora, *Escherichia coli* O157:H7, *Listeria monocyotgenes* and *Salmonella* spp. on mung bean sprouts. *Food Microbiology,* Vol. *36*, no. 2, pp. 475-480.

Neoh, H. M., Tan, X. E., Sapri, H. F., & Tan, T. L, 2019, Pulsed-field gel electrophoresis (PFGE): A review of the "gold standard" for bacteria typing and current alternatives. *Infection, Genetics and Evolution,* Vol.*74*, e103935.

Nickerson, S. C, 1995, Milk production: Factors affecting milk composition. *Milk Quality*, Boston, MA: Springer.

Novais, A., Freitas, A. R., Rodrigues, C., & Peixe, L, 2019, Fourier transform infrared spectroscopy: unlocking fundamentals and prospects for bacterial strain typing. *European Journal of Clinical Microbiology & Infectious Diseases,* Vol. *38*, no. 3, pp. 427-448.

Novais, A., Sousa, C., de Dios Caballero, J., Fernandez-Olmos, A., Lopes, J., Ramos, H., & Peixe, L, 2014, MALDI-TOF mass spectrometry as a tool for the discrimination of high-risk *Escherichia coli* clones from phylogenetic groups B2 (ST131) and D (ST69, ST405, ST393). *European Journal of Clinical Microbiology & Infectious Diseases,* Vol.*33*, no. 8, pp.1391-1399.

Ntuli, V., Njage, P. M. K., & Buys, E. M, 2016, Characterization of *Escherichia coli* and other *Enterobacteriaceae* in producer-distributor bulk milk. *Journal of Dairy Science,* Vol. *99*, no. 12, pp. 9534-9549.

Ojima-Kato, T., Yamamoto, N., Suzuki, M., Fukunaga, T., & Tamura, H, 2014, Discrimination of *Escherichia coli* O157, O26 and O111 from Other Serovars by MALDI-TOF MS Based on the S10-GERMS Method. *PLOS ONE,* Vol. *9*, no. 11, e113458.

Olive, D. M., & Bean, P, 1999, Principles and Applications of Methods for DNA-Based Typing of Microbial Organisms. *Journal of Clinical Microbiology,* Vol. *37*, no. 6, pp. 1661-1669.

Oliver, S. P., Jayarao, B. M., & Almeida, R. A, 2005, Foodborne pathogens in milk and the dairy farm environment: food safety and public health implications. *Foodborne Pathogen and Disease,* Vol. *2*, no. 2, pp.115-129.

Oliver, S. P., Murinda, S. E., & Jayarao, B. M, 2011, Impact of antibiotic use in adult dairy cows on antimicrobial resistance of veterinary and human pathogens: a comprehensive review. *Foodborne Pathogen and Disease,* Vol. *8*, no. 3, pp. 337-355.

Ombarak, R. A., Hinenoya, A., Awasthi, S. P., Iguchi, A., Shima, A., Elbagory, A.-R. M., & Yamasaki, S, 2016, Prevalence and pathogenic potential of *Escherichia coli* isolates from raw milk and raw milk cheese in Egypt. *International Journal of Food Microbiology,* Vol.*221*, pp. 69-76.

Oosthuizen, M. C., Steyn, B., Lindsay, D., Brözel, V. S., & von Holy, A, 2001, Novel method for the proteomic investigation of a dairy-associated *Bacillus cereus* biofilm. *FEMS Microbiology Letters,* Vol. *194*, no. 1, pp. 47-51.

Organji, S. R., Abulreesh, H. H., Elbanna, K., Osman, G. E. H., & Khider, M, 2015, Occurrence and characterization of toxigenic *Bacillus cereus* in food and infant feces. *Asian Pacific Journal of Tropical Biomedicine,* Vol. *5*, no. 7, pp. 515-520.

Orth, R, 1998, The importance of disinfection for the hygiene in the dairy and beverage production. *International Biodeterioration & Biodegradation,* Vol. *41*, no. 3, pp. 201-208.

Othman, M., Loh, H. S., Wiart, C., Khoo, T. J., Lim, K. H., & Ting, K. N, 2011, Optimal methods for evaluating antimicrobial activities from plant extracts. *Journal of Microbiological Methods,* Vol. *84*, no. 2, pp. 161-166.

Otokunefor, K., Ogugbue, C. J., & Fajoyomi, B. U, 2020, Enterobacterial Repetitive Intergenic Consensus (ERIC) as a tool for genetic characterisation of bacterial isolates in Nigeria. *Nigerian Journal of Biotechnology*. Vol. 37, no. 1, pp. 122-128.

Owusu-Kwarteng, J., Wuni, A., Akabanda, F., Tano-Debrah, K., & Jespersen, L, 2017, Prevalence, virulence factor genes and antibiotic resistance of *Bacillus cereus sensu lato* isolated from dairy farms and traditional dairy products. *BMC microbiology,* Vol. *17*, no. 1, pp. 65-65.

Paauw, A., Jonker, D., Roeselers, G., Heng, J. M. E., Mars-Groenendijk, R. H., Trip, H., & Speksnijder, A. G. C. L, 2015, Rapid and reliable discrimination between *Shigella* species and Escherichia coli using MALDI-TOF mass spectrometry. *International Journal of Medical Microbiology,* Vol. 305, no 4, pp. 446-452.

Pagotto, M., & Halog, A, 2016, Towards a Circular Economy in Australian Agri-food Industry: An Application of Input-Output Oriented Approaches for Analyzing Resource Efficiency and Competitiveness Potential. *Journal of Industrial Ecology,* Vol. 20, no. 5, pp.1176-1186.

Paneto, B., Schocken-Iturrino, R., Macedo, C., Santo, E., & Marin, J, 2007, Occurrence of toxigenic *Escherichia coli* in raw milk cheese in Brazil. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia,* Vol. *59*, pp. 508-512.

Park, S.H., & Kang, D.H, 2015, Combination treatment of chlorine dioxide gas and aerosolized sanitizer for inactivating foodborne pathogens on spinach leaves and tomatoes. *International Journal of Food Microbiology,* Vol. *207*, pp. 103-108.

Peng, X., Ed-Dra, A., & Yue, M, 2022, Whole genome sequencing for the risk assessment of probiotic lactic acid bacteria. *Critical Reviews in Food Science and Nutrition.*In press.

Parsot, C, 2005, *Shigella* spp. and enteroinvasive *Escherichia coli* pathogenicity factors. *FEMS Microbiology Letters,* Vol. *252*, no. 1, pp.11-18.

Picoli, T., Peter, C. M., Zani, J. L., Waller, S. B., Lopes, M. G., Boesche, K. N., & Fischer, G, 2017,. Melittin and its potential in the destruction and inhibition of the biofilm formation by *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* isolated from bovine milk. *Microb Pathog,* Vol. *112*, pp. 57-62.

Pironti, C., Dell'Annunziata, F., Giugliano, R., Folliero, V., Galdiero, M., Ricciardi, M., & Franci, G, 2021, Comparative analysis of peracetic acid (PAA) and permaleic acid (PMA) in disinfection processes. *Science of The Total Environment,* Vol. 797, e149206.

Porcellato, D., Aspholm, M., Skeie, S. B., & Mellegard, H, 2019, Application of a novel amplicon-based sequencing approach reveals the diversity of the Bacillus cereus group in stored raw and pasteurized milk. Food Microbiology, Vol. 81, pp. 32-39.

Porcellato, D., Aspholm, M., Skeie, S. B., Monshaugen, M., Brendehaug, J., & Mellegard, H, 2018, Microbial diversity of consumption milk during processing and storage. *International Journal of Food Microbiology,* Vol. 266, pp. 21-30.

Postollec, F., Falentin, H., Pavan, S., Combrisson, J., & Sohier, D, 2011, Recent advances in quantitative PCR (qPCR) applications in food microbiology. *Food Microbiology,* Vol. *28* no. 5, pp. 848-861.

Quainoo, S., Coolen, J. P. M., van Hijum, S. A. F. T., Huynen, M. A., Melchers, W. J. G., van Schaik, W., & Wertheim H. F. L, 2017, Whole-Genome Sequencing of Bacterial Pathogens: the Future of Nosocomial Outbreak Analysis. *Clinical Microbiology Reviews.* Vol. 30, no. 4, pp. 1015- 1063.

Radmehr, B., Zaferanloo, B., Tran, T., Beale, D. J., & Palombo, E. A, 2020, Prevalence and characteristics of *Bacillus cereus* Group isolated from raw and pasteurised milk. *Current Microbiology,* Vol. 77, no. 10, pp. 3065-3075.

Ramarao, N., Tran, S.-L., Marin, M., & Vidic, J, 2020, Advanced methods for detection of *Bacillus cereus* and its pathogenic factors. *Sensors,* Vol. *20*, no. 9, e2667.

Ranjbar, R., Tabatabaee, A., Behzadi, P., & Kheiri, R, 2017, Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR) Genotyping of *Escherichia coli* Strains Isolated from Different Animal Stool Specimens, *Iranian Journal of Pathollogy.* Vol. 12, no. 1, pp. 25-34.

Reller, L. B., Weinstein, M., Jorgensen, J. H., & Ferraro, M. J, 2009, Antimicrobial Susceptibility Testing: A Review of General Principles and Contemporary Practices. *Clinical Infectious Diseases,* Vol. *49*, no. 11, pp. 1749-1755.

Rey, J., Sanchez, S., Blanco, J. E., Hermoso de Mendoza, J., Hermoso de Mendoza, M., García, A., & Alonso, J. M, 2006, Prevalence, serotypes and virulence genes of Shiga toxin-producing *Escherichia coli* isolated from ovine and caprine milk and other dairy products in Spain. *International Journal of Food Microbiology,* Vol. *107,* no. 2, pp. 212-217.

Ribeiro, M. C. E., da Silva Fernandes, M., Kuaye, A. Y., & Gigante, M. L, 2019), Influence of different cleaning and sanitisation procedures on the removal of adhered *Bacillus cereus* spores. *International Dairy Journal,* Vol. *94*, pp. 22-28.

Robinson, R. K, 2005, *Dairy microbiology handbook: the microbiology of milk and milk products*: John Wiley & Sons.

Rohde, A., Hammerl, J. A., Boone, I., Jansen, W., Fohler, S., Klein, G., & Dahouk, S, 2017, Overview of validated alternative methods for the detection of foodborne bacterial pathogens. *Trends in Food Science & Technology,* Vol. *62*, pp. 113-118.

Rossoni, E. M., & Gaylarde, C. C, 2000), Comparison of sodium hypochlorite and peracetic acid as sanitising agents for stainless steel food processing surfaces using epifluorescence microscopy. *International Journal of Food Microbiology,* Vol. *61,* no. 1, pp. 81-85.

Rozenberg, S., Body, J.-J., Bruyere, O., Bergmann, P., Brandi, M. L., Cooper, C., & Reginster, J.Y, 2016, Effects of Dairy Products Consumption on Health: Benefits and Beliefs, A Commentary from the Belgian Bone Club and the European Society for Clinical and Economic Aspects of Osteoporosis, Osteoarthritis and Musculoskeletal Diseases. *Calcified tissue international,* Vol. *98,* no. 1, pp.1-17.

Rugeles, L. C., Bai, J., Martinez, A. J., Vanegas, M. C., & Gomez-Duarte, O. G, 2010, Molecular characterization of diarrheagenic *Escherichia coli* strains from stools samples and food products in Colombia. *International Journal of Food Microbiology,* Vol. *138*, no. 3, pp. 282-286.

Sabate, M., Prats, G., Moreno, E., Balleste, E., Blanch, A. R., & Andreu, A, 2008, Virulence and antimicrobial resistance profiles among *Escherichia coli* strains isolated from human and animal wastewater. *Research in Microbiology,* Vol. 159, no. 4, pp. 288-293.

Samet-Bali, O., Roua, L., Felfoul, I., Attia, H., & Ayadi, M, 2013, Detection of *Escherichia coli* in unpasteurized raw Milk. *International Journal of Agricultural and Food Science,* Vol. 3, no. 2, pp 53-55.

Sandrin, T. R., Goldstein, J. E., & Schumaker, S, 2013, MALDI TOF MS profiling of bacteria at the strain level: A review: Strail level MALDI TOF MS profiling. *Mass spectrometry reviews,* Vol. *32*, no. 3, pp. 188-217.

Santos, T., Capelo, J. L., Santos, H. M., Oliveira, I., Marinho, C., Goncalves, A., & Igrejas, G, 2015, Use of MALDI-TOF mass spectrometry fingerprinting to characterize *Enterococcus* spp. and *Escherichia coli* isolates. *Journal of Proteomics,* Vol. *127*, pp. 321-331.

Sauget, M., Valot, B., Bertrand, X., & Hocquet, D, 2016, Can MALDI-TOF Mass Spectrometry Reasonably Type Bacteria? *Trends Microbiology,* Vol. *25*, no. 6, pp. 447-455.

Scott, T. M., Parveen, S., Portier, K. M., Rose, J. B., Tamplin, M. L., Farrah, S. R., & Lukasik, J, 2003, Geographical variation in ribotype profiles of *Escherichia coli* isolates from humans, swine, poultry, beef, and dairy cattle in Florida. *Applied and Environmental Microbiology,* Vol. *69*, no. 2, pp. 1089-1092.

Scott, T. M., Rose, J. B., Jenkins, T. M., Farrah, S. R., & Lukasik, J, 2002, Microbial source tracking: current methodology and future directions. *Applied and Environmental Microbiology,* Vol. *68*, no. 12, pp. 5796-5803. 5803.2002

Seng, P., Drancourt, M., Gouriet, F., La Scola, B., Fournier, P.-E., Rolain, J. M., & Raoult, D, 2009, Ongoing Revolution in Bacteriology: Routine Identification of Bacteria by Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry. *Clinical Infectious Diseases,* Vol. *49*, no. 4, pp.

Seo, D. J., Choi, S., Jeon, S. B., Jeong, S., Park, H., Lee, B.-H., & Choi, C, 2017, Comparative sequence analysis of enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 identified in Korean and Japanese *Escherichia coli* strains. *International Journal of Food Microbiology,* Vol. *243*, pp.1-8.

Shemesh, M., & Ostrov, I, 2020, Role of *Bacillus* species in biofilm persistence and emerging antibiofilm strategies in the dairy industry. *Journal of the Science of Food and Agriculture,* Vol. 100, no. 6, pp. 2327-2336

Sheng, Y., Chancellor, W., & Jackson, T, 2020, Deregulation reforms, resource reallocation and aggregate productivity growth in the Australian dairy industry. *Australian Journal of Agricultural and Resource Economics,* Vol. *64*, no. 2, pp. 477-504.

Silva, H. O., Lima, J. A. S., Aguilar, C. E. G., Rossi, G. A. M., Mathias, L. A., & Vidal, A. M. C, 2018, Efficiency of Different Disinfectants on *Bacillus cereus* Sensu Stricto Biofilms on Stainless-Steel Surfaces in Contact With Milk. *Frontiers in Microbiology,* Vol. *9*, e2934).

Singh, P., Hung, Y.-C., & Qi, H, 2018, Efficacy of Peracetic Acid in Inactivating Foodborne Pathogens on Fresh Produce Surface. *Journal of Food Science,* Vol. *83*, no. 2, pp. 432-439.

Sloan, A., Wang, G., & Cheng, K, 2017, Traditional approaches versus mass spectrometry in bacterial identification and typing. *Clin Chim Acta,* Vol. *473*, pp.180-185.

Smith, J. L., Drum, D. J. V., Dai, Y., Kim, J. M., Sanchez, S., Maurer, J. J., & Lee, M. D, 2007, Impact of Antimicrobial Usage on Antimicrobial Resistance in Commensal *Escherichia coli* Strains Colonizing Broiler Chickens. *Applied and Environmental Microbiology,* Vol. *73*, no. 5, pp.1404.

Smith, J. L., & Fratamico, P. M, 2016, *Escherichia coli* and Other *Enterobacteriaceae*: Food Poisoning and Health Effects. In *Encyclopedia of Food and Health*, Oxford: Academic Press.

Smith, J. L., & Fratamico, P. M, 2017, Chapter 7 - *Escherichia coli* as a Pathogen. In *Foodborne Diseases,* 3rd edn, Academic Press.

Smith, J. L., Fratamico, P. M., & Gunther, N. W, 2007, Extraintestinal pathogenic *Escherichia coli. Foodborne Pathogen and Disease,* Vol. *4*, no. 2, pp.134-163.

Speranza, B., & Corbo, M. R, 2017, Chapter 11 - The Impact of Biofilms on Food Spoilage. In *The Microbiological Quality of Food*, Woodhead Publishing.

Srey, S., Jahid, I. K., & Ha, S.-D, 2013, Biofilm formation in food industries: A food safety concern. *Food Control,* Vol. *31*, no. 2, pp. 572-585.

Stenfors Arnesen, L. P., O'Sullivan, K., & Granum, P. E, 2007, Food poisoning potential of *Bacillus cereus* strains from Norwegian dairies. *International Journal of Food Microbiology,* Vol. *16*, no. 2, pp. 292-296.

Stepanovic, S., Cirkovic, I., Ranin, L., & Svabic-Vlahovic, M, 2004, Biofilm formation by *Salmonella* spp. and Listeria monocytogenes on plastic surface. *Letters in Applied Microbiology,* Vol. *38*, no 5, pp. 428-432.

Stepanovic, S., Vukovic, D., Dakic, I., Savić, B., & Svabic-Vlahovic, M, 2000, A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *Journal of Microbiological Methods,* Vol. *40*, no. 2, pp. 175-179.

Sudhaus, N., Nagengast, H., Pina-Perez, M. C., Martinez, A., & Klein, G, 2014, Effectiveness of a peracetic acid-based disinfectant against spores of *Bacillus cereus* under different environmental conditions. *Food Control,* Vol. *39*, pp. 1-7.

Sudhaus, N., Pina-Perez, M. C., Martinez, A., & Klein, G, 2012, Inactivation Kinetics of Spores of *Bacillus cereus* Strains Treated by a Peracetic Acid–Based Disinfectant at Different Concentrations and Temperatures. *Foodborne Pathogen Disease,* Vol. *9*, no. 5, pp. 442-452.

Sukumar, D, 1980, *Outlines of dairy technology.* Oxford University Press.

Sumner, J., Petrenas, E., Dean, P., Dowsett, P., West, G., Wiering, R., & Raven, G, 2003, Microbial contamination on beef and sheep carcases in South Australia. *International Journal of Food Microbiology,* Vol. *81*, no. 3, pp. 255-260.

Sumner, J., Ross, T., Jenson, I., & Pointon, A, 2005, A risk microbiological profile of the Australian red meat industry: risk ratings of hazard-product pairings. *International Journal of Food Microbiology,* Vol. *105*, no. 2, pp. 221-232.

Suojala, L., Pohjanvirta, T., Simojoki, H., Myllyniemi, A.-L., Pitkala, A., Pelkonen, S., & Pyorala, S, 2011, Phylogeny, virulence factors and antimicrobial susceptibility of *Escherichia coli* isolated in clinical bovine mastitis. *Veterinary Microbiology,* Vol. 147, pp. 383-388.

Tang, X., Flint, S. H., Bennett, R. J., & Brooks, J. D, 2010, The efficacy of different cleaners and sanitisers in cleaning biofilms on UF membranes used in the dairy industry. *Journal of Membrane Science,* Vol. *352*, no. 2, pp. 71-75.

Te Giffel, M. C., Beumer, R. R., Granum, P. E., & Rombouts, F. M, 1997, Isolation and characterisation of *Bacillus cereus* from pasteurised milk in household refrigerators in the Netherlands. *International Journal of Food Microbiology,* Vol. *34*, no. 3, pp. 307-318.

Te Giffel, M. C., Beumer, R. R., Van Dam, W. F., Slaghuis, B. A., & Rombouts, F. M, 1995, Sporicidal effect of disinfectants on *Bacillus cereus* isolated from the milk processing

environment. *International Biodeterioration & Biodegradation,* Vol. *3*6, no. 3, pp. 421-430.

The Australian Institute of Food Safety, 2016, https://www.foodsafety.com.au/resources/articles/top-7-causes-of-food-poisoning

Teh, K. H., Flint, S., Palmer, J., Andrewes, P., Bremer, P., & Lindsay, D, 2014, Biofilm − An unrecognised source of spoilage enzymes in dairy products? *International Dairy Journal,* Vol. *34*, no. 1, pp. 32-40.

Uddin, S. M. K., Hossain, M. A. M., Chowdhury, Z. Z., & Johan, M. R, 2021, Detection and discrimination of seven highly consumed meat species simultaneously in food products using heptaplex PCR-RFLP assay. *Journal of Food Composition and Analysis.* Vol. 100, 103938e.

Uyttendaele, M., Rajkovic, A., Ceuppens, S., Baert, L., Coillie, E. V., Herman, L., & Imberechts, H, 2014, PCR Applications in Food Microbiology, *Encyclopedia of Food Microbiology, 2nd edn,* Oxford Academic Press.

Veen, S. Q. v., Claas, E. C. J., & Kuijper, E. J, 2010, High-Throughput Identification of Bacteria and Yeast by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry in Conventional Medical Microbiology Laboratories. *Journal of Clinical Microbiology,* Vol. *48*, no. 3, pp. 900-907.

Veloo, A. C. M., Knoester, M., Degener, J. E., & Kuijper, E. J, 2011, Comparison of two matrix-assisted laser desorption ionisation-time of flight mass spectrometry methods for the identification of clinically relevant anaerobic bacteria. *Clinical Microbiology and Infection,* Vol. 17, no. 10, pp. 1501-1506.

Ventola, C. L, 2015, The antibiotic resistance crisis: part 1: causes and threats. *P & T : a peer-reviewed journal for formulary management,* Vol. 40, no. 4, pp. 277-283.

Versalovic, J., Koeuth, T., & Lupski, R, 1991, Distribution of repetitive DNA sequences in eubacteria and application to finerpriting of bacterial enomes. *Nucleic Acids Research,* Vol. *19*, no. 24, pp. 6823-6831.

Vertes, A., Telekes, A., & Vekey, K, 2008, *Medical Applications of Mass Spectrometry*. Amsterdam, 1st edn, Elsevier Science.

Visvalingam, J., Zhang, P., Ells, T. C., & Yang, X, 2019, Dynamics of Biofilm Formation by *Salmonella Typhimurium* and Beef Processing Plant Bacteria in Mono- and Dual-Species Cultures. *Microbial Ecology,* Vol. *78*, no. 2, pp. 375-387.

Vithanage, N. R., Yeager, T. R., Jadhav, S. R., Palombo, E. A., & Datta, N, 2014, Comparison of identification systems for psychrotrophic bacteria isolated from raw bovine milk. *International Journal of Food Microbiology,* Vol. *189*, pp. 26-38.

Vivek Prabhu, S. I., Balu, M., Suryanarayana, V.V.S., Rathnamma, D, 2010, Genotyping by ERIC-PCR of *Escherichia coli* isolated from bovine mastitis cases. *Indian Journal of Biotechnology,* Vol. 9, pp. 298-301.

Wang, L., Nakamura, H., Kage-Nakadai, E., Hara-Kudo, Y., & Nishikawa, Y, 2017, Prevalence, antimicrobial resistance and multiple-locus variable-number tandem-repeat analysis profiles of diarrheagenic *Escherichia coli* isolated from different retail foods. *International Journal of Food Microbiology,* Vol. 249, pp. 44-52.

Wang-Wang J. H, Bordoy AE, Martro E, Quesada MD, Perez-Vazquez M, Guerrero-Murillo M, Tiburcio A, Navarro M, Castella L, Sopena N, Casas I, Saludes V, Gimenez M, Cardona P. J, 2022, Evaluation of Fourier Transform Infrared Spectroscopy as a First-Line Typing Tool for the Identification of Extended-Spectrum β-Lactamase-Producing *Klebsiella pneumoniae* Outbreaks in the Hospital Setting. *Frontiers in Microbiololgy,* Vol. 9, no. 13, e897161.

Weiss, A., Heinold, S., Brunisholz, R., Schmidt, H., & Drissner, D, 2019, Application of MALDI-TOF mass spectrometry and specific PCR for tracking of *E. coli* O157:H− strain 431/97 in Batavia lettuce. *Chemical and Biological Technologies in Agriculture,* Vol. *6*, no. 2.

Welker, M., & Moore, E. R, 2011, Applications of whole-cell matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry in systematic microbiology. *Systematic and Applied Microbiology,* Vol. *34*, no. 1, pp. 2-11.

Wenning, M., Breitenwieser, F., Konrad, R., Huber, I., Busch, U., & Scherer, S, 2014, Identification and differentiation of food-related bacteria: A comparison of FTIR spectroscopy and MALDI-TOF mass spectrometry. *Journal of Microbiol Methods,* Vol. *103*, pp. 44-52.

Wieser, A., & Schubert, S, 2016, MALDI-TOF MS entering the microbiological diagnostic laboratory – from fast identification to resistance testing. *TrAC Trends in Analytical Chemistry,* Vol. *84*, pp. 80-87.

Wilson, K, 2001, Preparation of Genomic DNA from Bacteria. *Current Protocols in Molecular Biology,* Vol. *56*, no. 1, pp. 241-245.

Wilson, L. A., & Sharp, P. M, 2006, Enterobacterial repetitive intergenic consensus (ERIC) sequences in *Escherichia coli:* Evolution and implications for ERIC-PCR. *Molecular Biology and Evolution ,* Vol. *23*, no. 6. pp. 1156-1168.

Wong, Y. P., Othman, S., Lau, Y. L., Radu, S., & Chee, H. Y, 2018, Loop-mediated isothermal amplification (LAMP): a versatile technique for detection of micro-organisms. *Journal of Applied Microbiology,* Vol. *124*, no. 3, pp. 626-643.

Yu, S., Yu, P., Wang, J., Li, C., Guo, H., Liu, C., & Ding, Y, 2020, A Study on Prevalence and Characterization of *Bacillus cereus* in Ready-to-Eat Foods in China. *Frontiers in Microbiology,* Vol. 10, e3043.

Zaman, S. B., Hussain, M. A., Nye, R., Mehta, V., Mamun, K. T., & Hossain, N, 2017, A Review on Antibiotic Resistance: Alarm Bells are Ringing. *Cureus,* Vol. 9, no. 6, e1403-e1403.

Zhang, S., Wu, Q., Zhang, J., Lai, Z., & Zhu, X, 2016, Prevalence, genetic diversity, and antibiotic resistance of enterotoxigenic *Escherichia coli* in retail ready-to-eat foods in China. *Food Control,* Vol.*68*, pp. 236-243.

Zhang, Y., Yan, C., Yang, H., Yu, J., & Wei, H, 2017, Rapid and selective detection of *E .coli* O157:H7 combining phagomagnetic separation with enzymatic colorimetry. *Food Chemistry,* Vol. 234, pp. 332-338.

Zhou, G., Liu, H., He, J., Yuan, Y., & Yuan, Z, 2008, The occurrence of *Bacillus cereus, B. thuringiensis* and *B. mycoides* in Chinese pasteurized full fat milk. *International Journal of Food Microbiology,* Vol. 121, no. 2, pp.195-200.

Appendices

A1 Publications arising from this thesis

I. Radmehr, Behrad; Palombo, Enzo A. Rapid identification of *Escherichia coli* isolated from raw milk using MALDI-TOF MS. Oral presentation, Food, Nutrition & Analytical Chemistry (FNAC) Symposium, Royal Australian Chemical Institute (RACI), Melbourne, August 2017

II. Radmehr, Behrad; Zaferanloo, Bita; Palombo, Enzo A. Prevalence, Characteristics and behaviour of bacterial dairy contaminants. Poster presentation, Australian Society for Microbiology Annual Meeting, Brisbane, July 2018

III. Radmehr, Behrad; Zaferanloo, Bita; Tran, Thien; Beale, David J.; Palombo, Enzo A. (2020). Prevalence and Characteristics of *Bacillus cereus* Group Isolated from Raw and Pasteurised Milk, *Current Microbiology*, Vol. 77, No. 10, pp. 3065-3075

IV. Radmehr, Behrad. Characteristics and diversity of *Bacillus cereus* group Isolates from raw and pasteurised milk in Victoria, Australia; Oral presentation (Public speech), Australian Society for Microbiology, Agriculture and Food Microbiology Night, October 2020

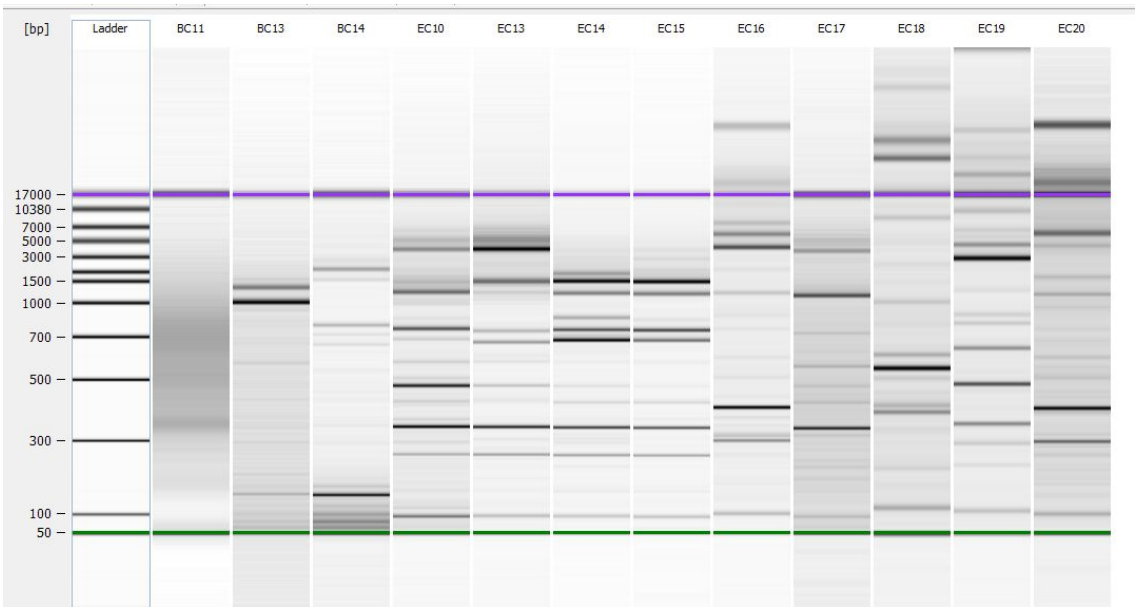

Figure A.1 ERIC PCR fingerprint for *E. coli* (EC) isolates using Agilent 2100 Bioanalyzer system.
Lanes 1: Agilent DNA 12000bp ladder; 2-14: *E. coli* isolates number EC11 to EC20

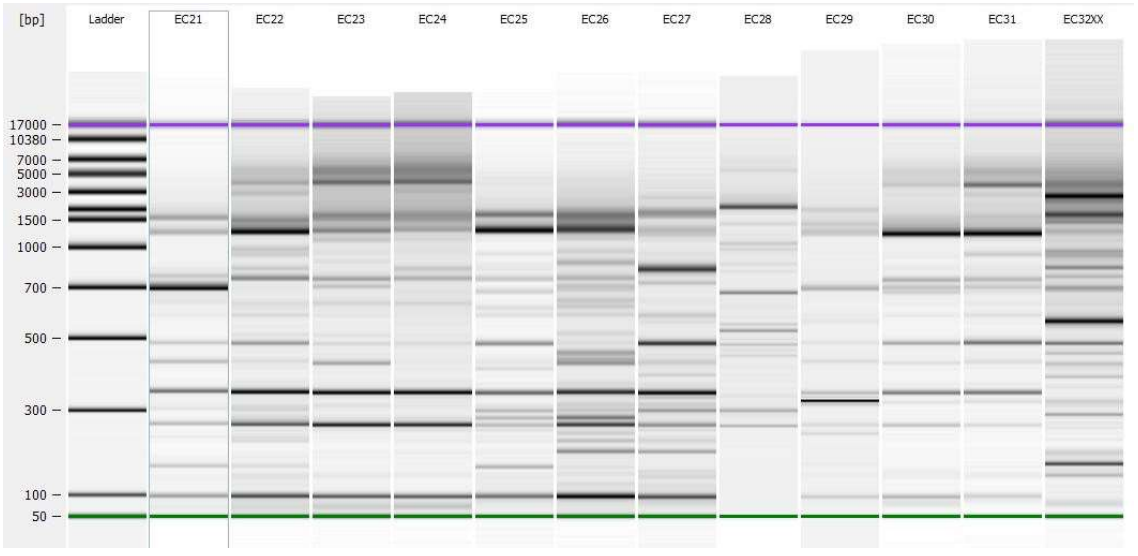

Figure A.2 ERIC PCR fingerprint for *E. coli* (EC) isolates using Agilent 2100 Bioanalyzer system.
Lanes 1: Agilent DNA 12000bp ladder; 2-14: *E. coli* isolates number EC21 to EC32

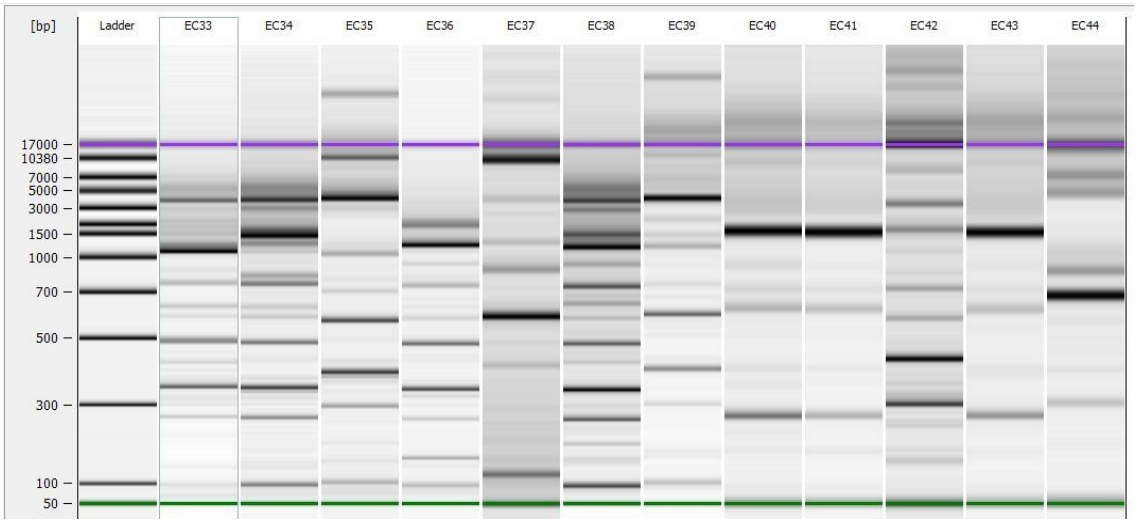

Figure A.3 ERIC PCR fingerprint for *E. coli* (EC) isolates using Agilent 2100 Bioanalyzer system.

Lanes 1: Agilent DNA 12000bp ladder; 2-14: *E. coli* isolates number EC33 to EC44

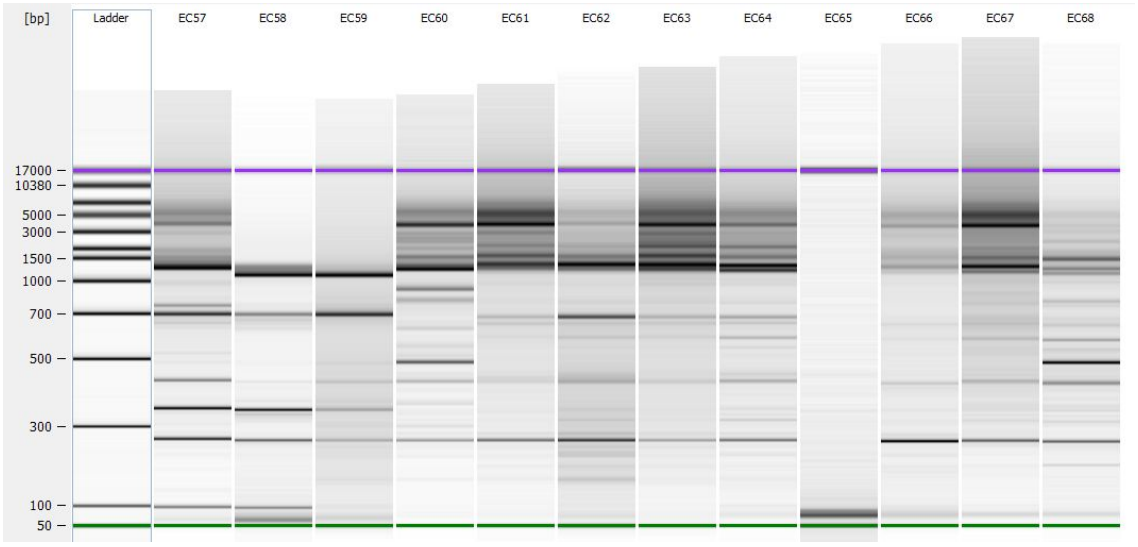

Figure A.4 ERIC PCR fingerprint for *E. coli* (EC) isolates using Agilent 2100 Bioanalyzer system.

Lanes 1: Agilent DNA 12000bp ladder; 2-14: *E. coli* isolates number EC57 to EC68

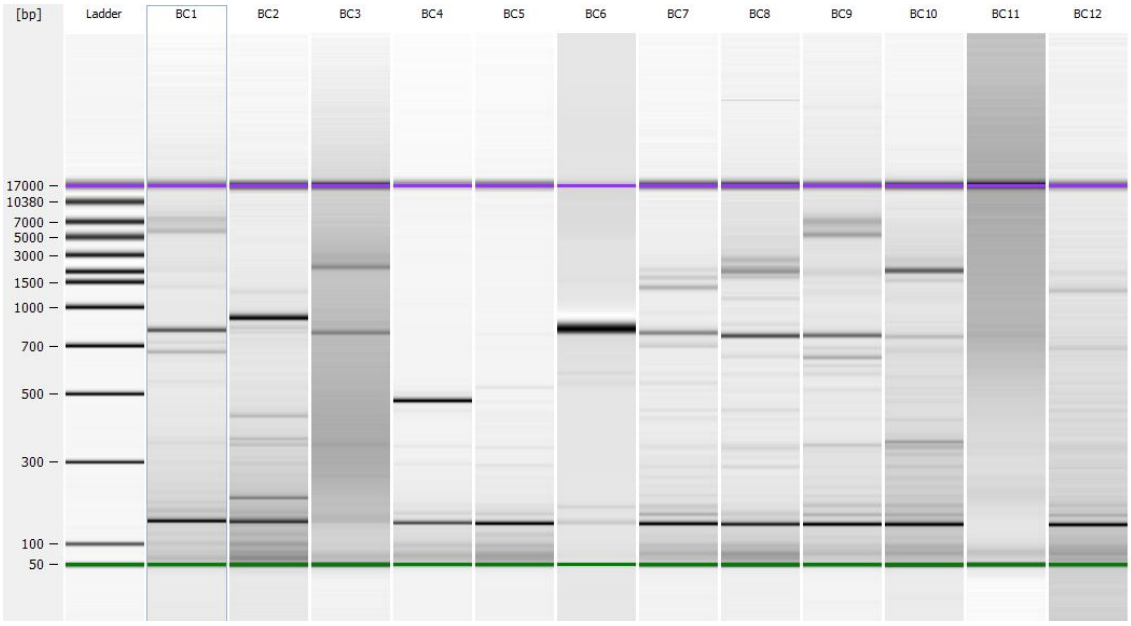

Figure A.5 ERIC PCR fingerprint for *B. cereus* group (BC) isolates using Agilent 2100 Bioanalyzer system.
Lanes 1: Agilent DNA 12000bp ladder; 2-14: *B. cereus* group isolates number BC1 to BC12

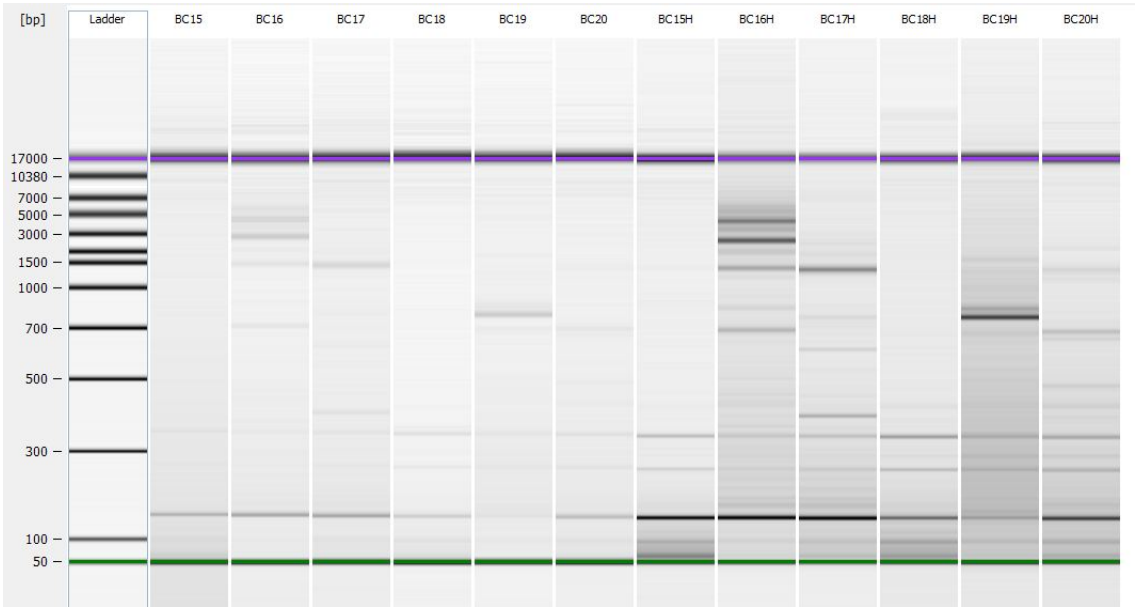

Figure A.6 ERIC PCR fingerprint for *B. cereus* group (BC) isolates using Agilent 2100 Bioanalyzer system.
Lanes 1: Agilent DNA 12000bp ladder; 2-7: *B. cereus* group isolates number BC15 to BC20; 8-14: *B. cereus* group isolates number BC15 to BC20 using higher DNA concentration

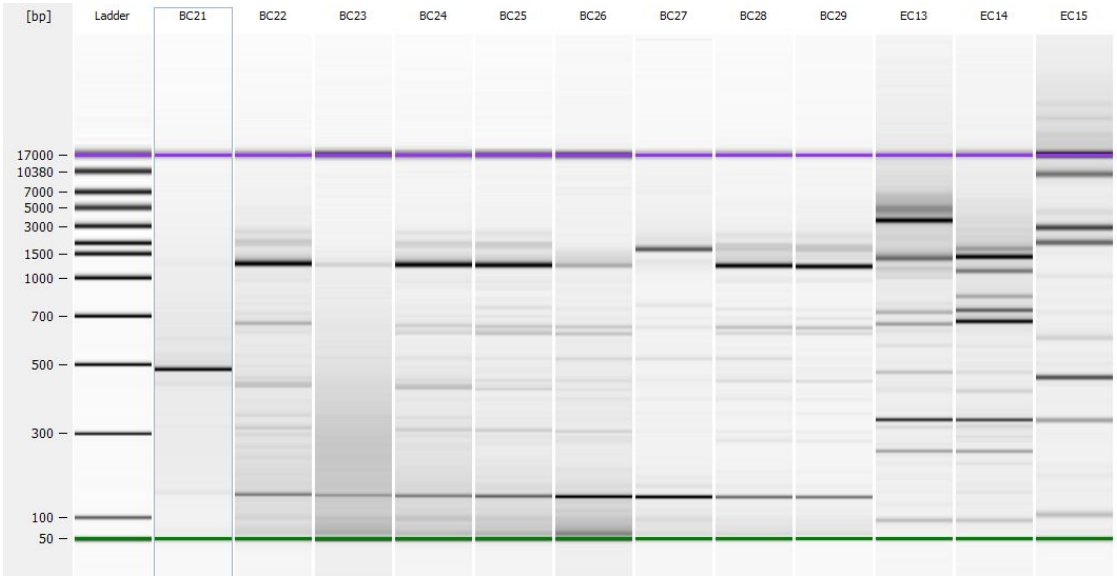

Figure A.7 ERIC PCR fingerprint for *B. cereus* group (BC) isolates using Agilent 2100 Bioanalyzer system.
Lanes 1: Agilent DNA 12000bp ladder; 2-11: *B. cereus* group isolates number BC21 to BC29; 12-14: *B. cereus* group isolates number BC13 to BC15