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**Biofilm formation by *B. licheniformis* isolated from
whey protein concentrate 80 powder as a potential
source of product contamination**

A thesis presented in partial fulfilment of the requirements for the degree of

**Doctor of Philosophy
in
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ABSTRACT

This study aimed to examine biofilm formation of *Bacillus licheniformis* isolated from whey protein concentrate 80 (WPC80) as a potential source of contamination in the manufacture of WPC.

Six WPC80 powder samples from one whey processing plant in New Zealand were used in this study. Six *Bacillus* species including (percentage of isolates in brackets) *B. licheniformis* (66%), *Bacillus cereus*/*Bacillus thuringiensis* (18%), *Bacillus subtilis* (4%), *Bacillus pumilus* (4%), *Paenibacillus glucanolyticus* (2%) and *Lactobacillus plantarum* (6%) were identified using colony morphologies, biochemical tests, species specific PCR and 16S ribosomal DNA gene sequencing and subsequent analysis using the BLAST and Seqmatch databases.

Preliminary screening for biofilm formation by the predominant contaminant, *B. licheniformis* using a microtitre plate assay with the bacteria grown in laboratory medium tryptic soy broth (TSB) at three different temperatures (30°C, 37°C and 55°C) showed most biofilm formation at 37°C with 9/33 isolates forming strong biofilm. In total 13/33 isolates formed strong biofilm at three different temperatures on the polystyrene microtitre plate surface.

Subsequent tests for biofilm formation on stainless steel (SS) showed an increased frequency of biofilm formation with 32/33 strains forming strong biofilm in TSB at 37°C. This demonstrates the limitation of the microtitre plate assay for screening for biofilm formation and suggests that biofilm growth of *B. licheniformis* favours a SS surface.

The attachment and biofilm formation was further investigated using SS coupons and reconstituted whey medium at different concentrations (1%, 5%, and 20%). The best medium for *B. licheniformis* isolates to form biofilm on SS at its best growth temperature (37°C) was 1% reconstituted WPC80. Interestingly, when 1% reconstituted WPC80 was supplemented with lactose and minerals (mainly calcium and magnesium)

to replicate the composition of Mozzarella cheese whey before ultrafiltration (UF), the *B. licheniformis* biofilm counts increased at least by one log.

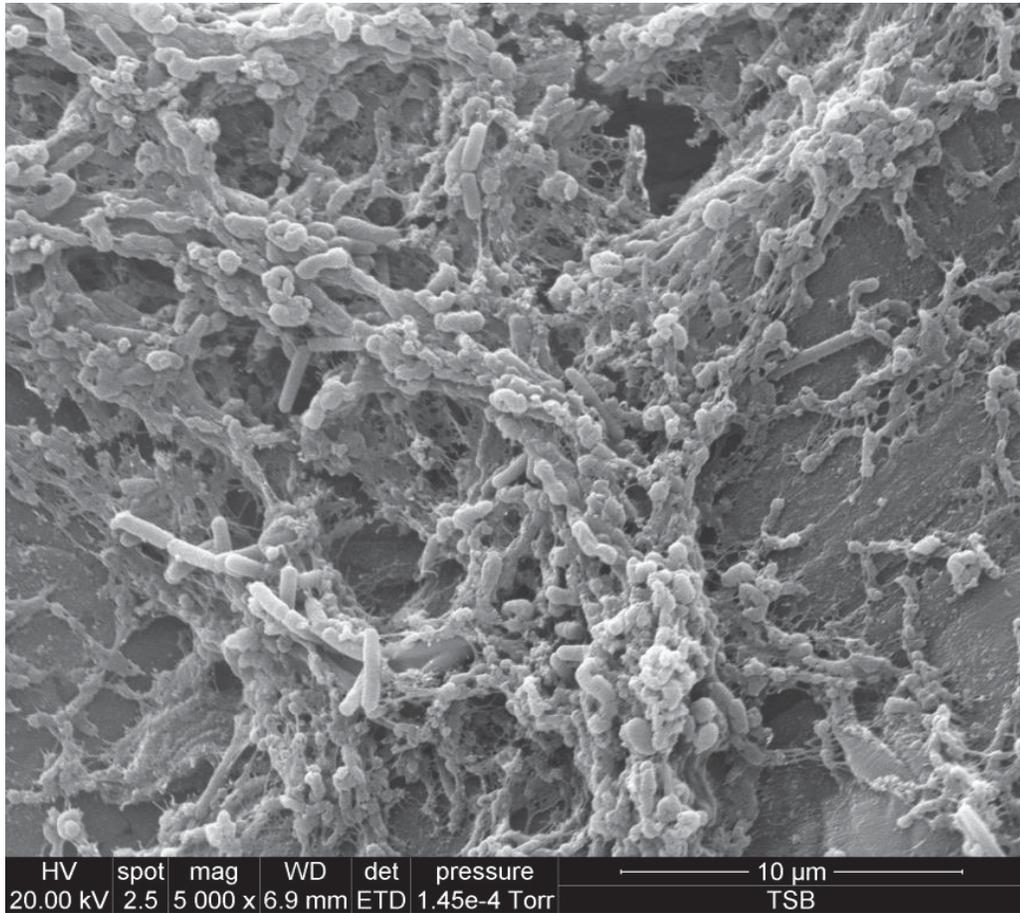
The production of protease enzyme, extracellular polymeric substances (EPS) and nitrate reduction by *B. licheniformis* showed the potential of *B. licheniformis* to influence the quality of dairy products. Biosurfactant production by *B. licheniformis* identified as lichenysin consisting of lipopeptide was detected and this may influence biofilm formation on SS. The inability of the *B. licheniformis* isolates to ferment lactose as their major carbon source was confirmed by lactose fermentation tests and shows that *B. licheniformis* is not ideally suited to a dairy environment. The *B. licheniformis* vegetative cells were found to be heat resistant with a $< \log_{10}$ reduction at the three temperatures tested; 72°C, 75°C and 80°C during 15 s, 30 s and 60 s heating intervals.

In order to thrive in a dairy system, synergistic interactions with other microflora were investigated as a possible mechanism to use lactose that has been broken down by other microflora. *Lactobacillus plantarum* (*L. plantarum*), another isolate from the WPC80 samples, has the ability to produce glucose and galactose from lactose. This was grown with each of two *B. licheniformis* isolates (E30C11 and F30C02) with different abilities to form biofilm. Interestingly this did not enhance the growth of *B. licheniformis* suggesting that another carbon source, most likely whey protein, must provide the energy source for this bacterium in a whey environment.

A review of the WPC80 processing plant showed the UF membranes had the largest surface area (3500 – 7500 m²), providing most potential for biofilm growth. However, UF was run at 10°C, too low for the growth of *B. licheniformis* which has a minimum growth temperature of 20°C. The hypothesis that sections of the processing plant before the UF step are the sites for *B. licheniformis* biofilm growth was supported by analysing several samples from the raw whey balance tank, clarifier, thermaliser and separator where 7 *B. licheniformis* strains were isolated. This shows that *B. licheniformis* is present at several early stages of WPC processing, with the most likely areas for growth being the certain sections of the clarifier, thermaliser and the separator where temperatures are close to the best growth temperature for this bacterium (37°C).

Preventing *B. licheniformis* contamination of WPC needs to focus on adjusting the conditions in these sections of the processing plant to limit biofilm growth.

Keywords: dairy, *Bacillus* species, *L. plantarum*, lichenysin, stainless steel, membrane processing plant.



FRONTISPIECE Biofilm of *Bacillus licheniformis* embedded in extracellular polymeric substances on 304 grade stainless steel after 24 h incubation.

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1. Xuemei Tang, Steve Flint, Rod Bennett, John Brooks and **Siti Norbaizura Md Zain** (2015). Biofilm in the dairy industry. Chapter 8: Biofilm contamination of ultrafiltration and reverse osmosis plants. John Wiley & Sons. Oxford.
2. **Siti Norbaizura Md Zain**, Steve Flint, Rod Bennett and Tay Hong Soon (2016). Characterisation and biofilm screening of the predominant bacteria isolated from whey protein concentrate. *Dairy Science & Technology*, 96(3), 285-295. doi:10.1007/s13594-015-0264-z
3. **Siti Norbaizura Md Zain**, Rod Bennett and Steve Flint (2017). The Potential Source of *B. licheniformis* Contamination During Whey Protein Concentrate 80 Manufacture. *Journal of Food Science*, 82(3), 751-756. doi: 10.1111/1750-3841.13633

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5. **Siti Norbaizura Md Zain,** Steve Flint, Rod Bennett and Tay Hong Soon. *Biofilm formation by *B. licheniformis* isolated from whey protein concentrate 80 powder on two different media and surfaces.* New Zealand Microbiological Society Conference, Rotorua, 2 - 5 November 2015.

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Contamination of whey products with bacteria is an economic problem for the dairy industry. Langeveld et al, (1995) noted the high concentration bacterial cells in milk was from their detachment from biofilms into the final product resulting in bacterial counts of $>10^6$ per mL, increasing the potential for product spoilage and economic loss. The role of biofilms as the source of the contaminating bacteria in whey products is unknown. Biofilms can form on any surface, but as a large amount of surface area (3500 -7500 m²) is included in the UF membranes used in whey processing, this is one key potential site for biofilm development leading to product contamination.

Whey provides nutrients to enable bacterial growth and the temperatures in different sections of dairy manufacture are suitable for the growth of many different types of bacteria. Growth of bacteria in the dairy liquids (milk and whey) during processing may occur anywhere but the length of time these liquids are at temperatures suitable for microbial growth is limited. Bacterial growth on surfaces (biofilm) is believed to be the main source of bacterial contamination of dairy products (Teh et al, 2015). There is ample opportunity for biofilm development in dairy manufacturing plant and it is likely that the contaminants seen in whey powder originate from biofilms shedding bacteria into the product. In addition, whey protein, along with other organic or inorganic molecules can form a conditioning layer on surfaces that may make them more likely to attract bacteria and start biofilm colonisation.

1.2 RESEARCH QUESTIONS

- 1) What is the microbial profile of WPC80?
 - a. *What are the predominant contaminants?*
 - b. *What are the characteristics of these contaminants that influence their growth and persistence in whey processing plants?*

- 2) What are the relationships between the isolated microorganisms and biofilm formation?
- a. *What conditions favour biofilm formation of the main contaminant (s) in WPC80?*
 - b. *What is the best test method to examine biofilm formation of these isolates that reflects growth on surfaces in whey processing plants?*
 - c. *Where is the potential source/location of the contaminant during WPC80 manufacture?*

1.3 HYPOTHESES

Whey powder quality is affected by the manufacturing process which is related to:

1. Biofilm/ biofouling formation by microorganisms in the whey processing plant.
2. The potential source/location of the contamination is at UF membranes.

1.4 OBJECTIVES OF THE STUDY

Microbial biofilms are a major problem in in the dairy industry due to the release of bacteria and enzymes from biofilms into the product. Biofilm growth is supported by the nutritional composition of dairy liquids. Prolonged manufacturing runs and insufficient Clean-In-Place (CIP) increases biofilm accumulation. A better understanding of the biofilms formation of bacteria contaminating WPC and the potential source/location of contamination in a whey processing plant will help the dairy manufacturer in modifying dairy processes to control these bacteria and ensure product quality. Key objectives were:

1. To identify the contaminants of WPC80 powder (**Chapter 3**).
2. To investigate the attachment and biofilm formation of the isolated bacteria (**Chapter 4**).
3. To characterise the contaminants (**Chapter 5**).

4. To investigate the co-culture and spore production of the contaminants **(Chapter 6)**.
5. To identify the potential sources of contamination in the whey processing plant **(Chapter 7)**.

1.5 SIGNIFICANCE OF RESEARCH

This study was designed to explore the fundamental properties of the bacteria isolated from whey and their ability to form biofilm, produce spoilage enzymes in whey processing plants from either single or mixed cultures and the potential sources of microbial contamination. This knowledge was aimed at helping design strategies to prevent microbial contamination in WPC manufacture.

CHAPTER 2

LITERATURE REVIEW

2.1 MICROORGANISMS IN THE DAIRY INDUSTRY

There are many different types of bacteria in the dairy industry. Raw milk contains a variety of bacteria from the cow and the milk harvesting process. Throughout the handling and processing of milk into manufactured products, conditions that the milk is exposed to result in changes in the microbial population of the milk. These microbial populations can result in contamination of the product with associated spoilage and undesirable high numbers of bacteria in the final product. Pathogenic bacteria and many of the spoilage bacteria are killed early in the manufacturing process by heat treatment.

2.1.1 Thermophilic, mesophilic and psychrophilic bacteria

Many thermophilic and thermotolerant bacteria survive heat treatment such as pasteurisation and can survive manufacturing processes to contaminate the final product. The common bacteria that survive heat treatment and contaminate dairy products are thermophilic streptococci such as *S. thermophilus* and various bacillus species such as *Anoxybacillus flavithermus* (*A. flavithermus*) and *Geobacillus* species. Thermophilic bacilli are used as hygiene indicators in processed product as they are believed to grow in the manufacturing plant as biofilms, so their presence is an indicator of inadequate cleaning. Furthermore, they are able to produce enzymes and acids that may lead to off-flavours in the final product (Flint et al., 2011b; Burgess et al, 2009; Burgess et al, 2010; Rucket et al, 2004).

Streptococcus species are facultative anaerobic, non-spore forming, catalase-negative, homo fermentative bacteria and have complex nutritional requirements (Flint et al, 1999a). They are lactic acid producing bacteria which can survive high temperatures (Delorme, 2008). They can be found in raw milk (Flint et al., 2011a) and have the ability to survive pasteurisation, hence they are termed thermotolerant. Their optimum growth temperature is around 40°C therefore they cannot be termed “thermophilic” as the in the dairy industry, thermophilic bacteria are those that grow at 55°C. *S. thermophilus* is also used as a starter to manufacture dairy products especially yogurt and some types of cheese. According to Delorme (2008), this bacterium is used in fermentation due to its ability to rapidly convert lactose into lactic acid resulting in a rapid change in pH, an important part of cheese manufacture. Furthermore, it is able to

produce extracellular polymeric substances (EPS) that contribute to the texture of fermented milk products (Delorme, 2008). Even though *S. thermophilus* can be desirable in the manufacture of some dairy products, it is still a concern when it is able to adhere to surfaces as it can contaminate products that are not supposed to contain high levels of this bacterium, resulting in products exceeding customers' specifications for microbial levels and associated sensory problems.

One thermophilic bacterium commonly isolated from dried dairy product is *A. flavithermus*. It is a non-pathogenic bacillus and spore former. The spores are heat resistant (surviving pasteurisation) and the vegetative cells are able to grow up to 65°C for 6 h (Burgess et al, 2009). *A. flavithermus* is a potential contaminant in milk powder and may deteriorate the quality of the product (Palmer et al, 2010).

Geobacillus species are another group of thermophilic, spore forming non-pathogenic bacteria also commonly found in dairy products. The spores of these microorganisms tend to be somewhat more heat resistant at 100°C with less log reduction (Witthuhn et al, 2011), D-values at 121°C (Dogan et al, 2009), than those of *A. flavithermus* and the maximum temperature for growth tends to be higher than that for *A. flavithermus* (Burgess et al, 2010; Seale et al, 2008).

Bacillus species as well as the closely related *A. flavithermus* and *Geobacillus* species are common contaminants in dried dairy products. This is because they produce spores that enable their survival. Pasvolsky et al, (2014) reported that *Bacillus* species contribute to hygiene and sanitation problems since they can form biofilm on the surface of dairy equipment as well as milking pipelines.

Thermophilic bacilli isolated from the dairy industry at 55°C, the temperature routinely used in the dairy industry to isolate thermophilic bacteria, can be divided into two groups: obligate thermophiles and facultative thermophiles. The obligate thermophiles grow only at elevated temperatures of approximately 40 - 68°C (Burgess et al, 2010). Facultative thermophiles belong to the *Bacillus* genus and are able to grow at both mesophilic and thermophilic temperatures depending on strains (Burgess et al, 2010). Thermophilic bacilli are regarded as indicators of poor hygiene in the dairy industry. Furthermore, thermophiles are potential spoilage microorganisms as they are

capable of producing enzymes and acids that may lead to off-flavours in the final product (Teh et al, 2012; Teh et al, 2013; Sadiq et al, 2016b). Thermophilic bacilli are likely to form biofilms in specific unit operations in dairy manufacture where temperatures are 40 – 65°C. These include separators, plate heat exchangers used during pasteurisation, cream heaters in anhydrous milk fat plants and UF plant that operating at hot temperatures (50 -55°C) (Flint et al, 1997).

A study by Yuan et al, (2012) on commercial powder milk in China examined the different species of thermophilic bacilli found in their product. They identified 801 isolates from 22 milk powder samples with 80.5% identified as *Bacillus* species (*B. licheniformis*, *A. flavithermus* and *G. stearothermophilus*). *B. licheniformis* was the dominant strain representing 27.8% of the isolates. They also reported that four heat treatments were used to study the heat resistant of 5 *B. licheniformis* isolates. For the vegetative cells of 4 out of 5 *B. licheniformis* isolates survived pasteurisation 72°C for 15 s; 3 out of 5 *B. licheniformis* isolates survived heat treatment at 85°C for 1 min and 2 out of 5 *B. licheniformis* vegetative cells survived 93°C for 3 min. Spores of *B. licheniformis* survived all of the three mentioned temperatures. However, both vegetative cells and spores were reduced by 8 log CFU/mL at 121°C for 15 s.

Parkar et al, (2001) showed a variety of thermophilic/thermotolerant bacilli strains including *Geobacillus*, *Anoxybacillus*, *B. licheniformis*, *B. coagulans* and *B. pumilus* were able to attach to both SS and milk foulant in similar numbers. Gilmore & Rowe, (1990) noted that some strains of *B. licheniformis* can produce extracellular substances that appear slimy and sticky and that these may affect the quality of pasteurised cream and milk. Agents targeting the EPS components are frequently reported to induce biofilm dispersion. *B. licheniformis* excretes an extracellular Dnase (NucB) that rapidly disperses the biofilms formed by both Gram-positive and Gram-negative bacteria (Nijland et al, 2010). *B. licheniformis* is also associated with a variety of clinical syndromes, such as enteric disease, septicaemia, peritonitis, ophthalmitis and food poisoning. It is also responsible for food spoilage such as ropy bread (Fernández-No et al, 2011).

Studies by Cook & Sanderman, (2000), Dhakal, (2013) and Sadiq et al, (2016a), reported *B. licheniformis* was the dominant thermophilic spore former in dairy products where the latter concluded that *B. licheniformis* contamination occurs in milk and dairy products as many different genotypes that vary in terms of heat resistance and biofilm capabilities. Thermotolerant *B. licheniformis* is a common species isolated from raw milk and contaminating the dairy production chains with spore production up to 5 log CFU/mL (Crielly et al, 1994). It was identified as the predominant bacteria colonising manufacturing UF plant used for standardising milk used in cheese manufacture (Lehmann, 1995). Growth most likely starts in the pre-heating plate heat exchangers of the UF plant.

Raats et al, (2011) found that raw milk communities did not contain *B. licheniformis*. They did, however detect *Bacillus* and *Corynebacterium* species in 2/3 of the samples. The dominant species they detected were *Streptococcus* and *Lactococcus*. *Lactococcus* species can also dominate in whey UF membranes. Chamberland et al, (2017b) suggesting that these species can survive through many dairy manufacturing processes.

Another raw milk contaminant is *Bacillus cereus* (*B. cereus*). This bacterium is ubiquitous in nature, and can be isolated from a variety of processed and raw foods, including milk. However, its presence in foods is not a significant health threat unless it is present in high numbers ($>10^6$ CFU/g) or able to grow (Logan, 2011). It has been recognised as a causative agent of food poisoning for more than 40 years producing emetic and diarrhoea symptoms (Fernandéz-No et al, 2011). *B. cereus* is a spore forming bacterium and a good biofilm former.

Bacillus thermoamylovorans is an emerging spore forming bacilli that is believed to be a threat to dairy product quality, with the ability to survive through heat sterilisation (UHT). *B. thermoamylovorans* has the ability produce lipolytic and β -galactosidase enzymes are linked to food spoilage (Flint et al, 2017).

Another spore forming genera associated with the dairy industry is the psychrophilic *Paenibacillus*. Since 1993, the genus *Paenibacillus* has grown from 11 species to over 26 species (Daane et al, 2002). *Paenibacillus* are Gram positive, rod

shaped, spore formers, motile with peritrichous flagella, some of which are facultative anaerobes while others are strict aerobes (Vithanage et al, 2014; Ivy et al, 2012). *Paenibacillus* are ubiquitous in nature and have been isolated from soil, water, food, faeces, plant materials and diseased insect larvae (Yoon et al, 1998). They produce spores that are heat resistant and able to survive heat treatment such as pasteurisation (De Jonghe et al, 2010). Scheldeman & Goossens, (2004) reported *Paenibacillus* as the pre-dominant microorganisms (95%) in refrigerated milk at 6°C after 10 days of storage. *Paenibacillus* can be distinguished from *Bacillus* species based on their ability to proliferate under refrigeration temperatures to high numbers after an extended storage (Ranieri et al, 2009).

Paenibacillus also were reported able to survive pasteurisation such as high temperature short time (HTST) and low temperature long time (LTLT) (Ranieri et al, 2009). In addition, some *Paenibacillus* strains are capable of producing spores that can survive ultra-high temperature (UHT) of 140°C to 145°C for 2 s to 5 s as well as retort processes of 110°C to 120°C for 12 to 20 minutes (Burgess et al, 2010).

Many of these bacteria in the dairy industry have been found to produce biofilms on dairy manufacturing plant surfaces (Flint, 1998; Burgess et al, 2010; Teh et al, 2012), and therefore are a potential source of dairy product contamination and quality issues.

2.2 BIOFILM

A biofilm is a population of microbial cells growing on a surface and enclosed in an amorphous extracellular matrix (Donlan, 2002). In most environments, microorganisms will grow and develop biofilms as their dominant form and exist on a solid liquid interface. The depletion of dissolved oxygen triggers the formation of floating biofilms. This floating cellular community is called a pellicle (a type of biofilm). Biofilm may consist of either single or multi species microorganisms and represents an important role in many ecosystems (Costerton, 1995; Stewart & Costerton, 2001; O'toole et al, 2000). The formation of biofilms of microorganisms is a protective mechanism for the survival of bacteria in harsh and hostile environments, protecting them from environmental stresses and nutrients depletion (Palmer et al, 2007). Under suitable conditions taking account intrinsic and extrinsic factors, all microorganisms can form biofilms. However, it was noted that some bacteria have a greater tendency than others to form a biofilm. Poulsen, (1999) reported that *Pseudomonas*, *Enterobacter*, *Flavobacterium*, *Alcaligenes*, *Staphylococcus* and *Bacillus* are the most common bacteria associated with biofilms.

Biofilm development starts with the adhesion/attachment of the bacteria to a substrate. This process consists of two phases: the reversible and the irreversible. The reversible phase includes various long distance interactions such as electrostatic, hydrophobic interactions and van der Waals forces. Next, the irreversible phase includes various short range forces such as dipole-dipole, hydrophobic, ion-dipole, ion-ion, covalent bonds and hydrogen bonds (Hood & Zottola, 1995; Kumar & Anand, 1998).

2.2.1 Factors affecting biofilm formation

Attachment of bacteria to a substrate is the first stage in biofilm formation. Both the substrate and the bacteria are important in this initial attachment phase. The physicochemical properties of the substrate also influence bacterial attachment. Microorganisms attach rapidly on hydrophobic (non-polar surfaces) such as Teflon and polystyrene. Meanwhile, hydrophilic surfaces such as glass and metal are suitable for their attachment (Husmark, 1997). The hydrophobicity of the microbial cell surface is another crucial factor in the adhesion process because of hydrophobic interactions between microbial cell surface and substratum likely to increase with the increasing non-polar properties of one or both surfaces (Wienczek et al, 1991).

Attachment on hydrophobic surfaces, such as microtitre plates, is a good screening tool for biofilm formation however not all microtitre plates are suitable as different types polystyrene amphipathic plasticizing agents are added which affect the results (Donlan, 2002). Polystyrene may also carry net negative charge which may influence adhesion as electrostatic forces are also involved in the adhesion process (Husmark, 1993). SS is another example of a hydrophobic surface. From studies by Flint et al, (1999a; 1999b) on the removal of thermo-resistant streptococci, *S. thermophilus* was able to adhere to SS and withstand various cleaners and sanitisers especially in warm zones of dairy manufacturing plants, where *Streptococci* attach directly to SS. The dairy industry has used SS for more than 60 years in almost all milk handling systems (Marchand et al, 2012). SS is the most common material used in food processing plant surfaces as it is corrosion resistant and easy to clean as described by Holah & Gibson (2000). The properties of SS such as grade (304 or 316) and surface finish do impact the SS hydrophobicity with 316 SS more hydrophilic than 304 SS with 2B finish (Teh et al, 2015). Further modification of SS surfaces might be useful to reduce or mitigate bacterial attachment and biofilm formation. The surface properties of SS show some crevasses that bacteria can be trapped in (Figure 2.1). This microscopic appearance is playing a vital role for bacterial attachment or cleaning or both.

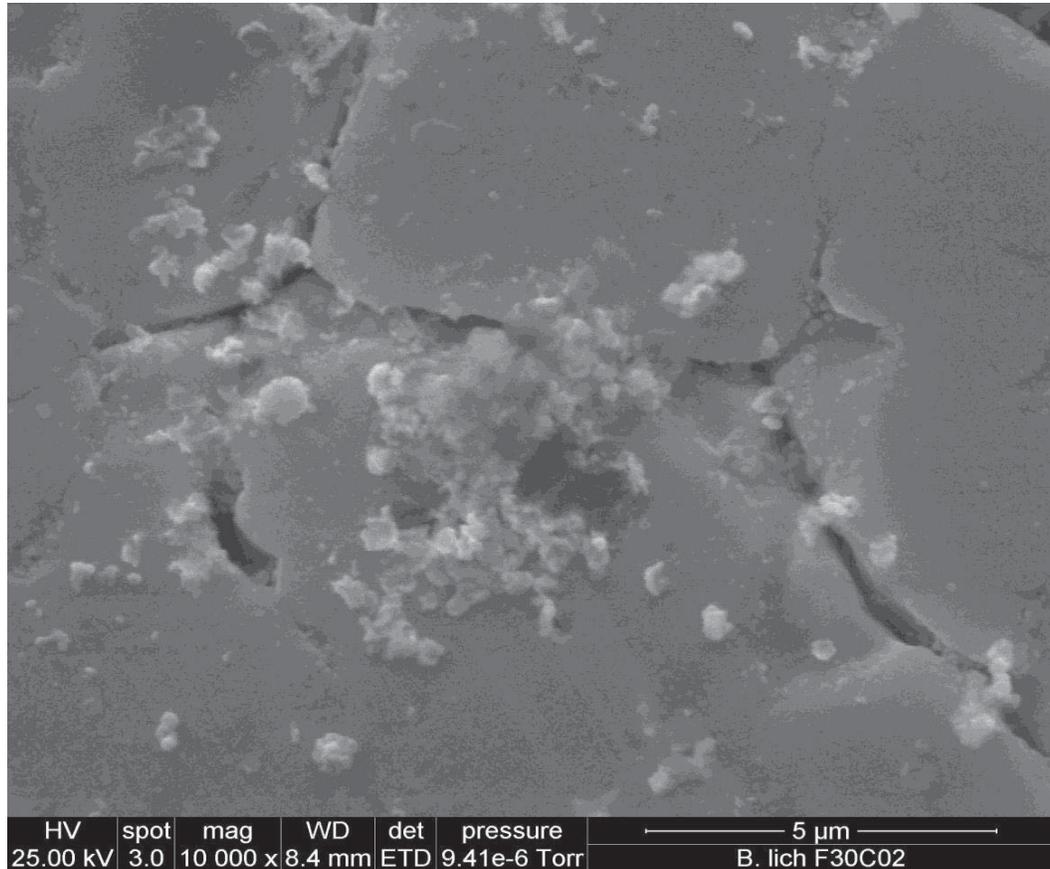


Figure 2.1 Image of SS on contact with whey for 24 h which. There are deposits on the SS surface (presumably protein) that may attract bacterial attachment. Note the crevices and pits on the surface which can trap microbial cells.

Bacterial attachment occurs best on surfaces that are hydrophobic, rough and coated with conditioning films. A conditioning film is a layer of organic material that can alter the conditions on the substrate, making it more or less likely to support bacterial attachment (Tang et al, 2015; Teh et al, 2015). Zaky et al, (2012) studied the role of conditioning films in the early stage of membrane fouling. In addition, nutrient concentration, temperature and flow velocity do enhance the attachment process. (Anand et al, 2014; Yang et al, 2012). Hydrophobic interaction depends on the hydrophobic properties of the cell surfaces and hydrophobic properties of the substrate. Heat treatment (80°C for 10 min) induces *Bacillus* spores to be more hydrophobic (Husmark, 1993). Hydrophobic interaction increased with temperature. The more

hydrophobic the substrate and the cell surface the higher the percentage of adhering spores. The contaminating microorganisms' adherent spores or bacteria develop into biofilms on every surface including SS, floors, belts or rubber seals in the food industry (Costerton, 1995; Kumar & Anand, 1998).

Both the vegetative cells and the spores of *Bacillus* species are involved in the formation of a biofilm. The spore surfaces are hydrophobic due to proteins on their outer surface (Wiencek et al, 1990). The high relative hydrophobicity of *Bacillus* spores is reflected in a high degree of attachment and the hydrophobic interactions increase proportionally with temperature. Spores from different *Bacillus* species vary in their attachment to solid surfaces. Husmark, (1993) discovered that attachment was higher in the spore state than the vegetative state in five different *Bacillus* species and *B. cereus* is the most hydrophobic species. The attachment process is quite rapid and within 1 hour, a maximum level of adhesion is reached for specific spore concentration. In general, the mechanism of microbial attachment and growth as biofilm on any surface is a very complex process with internal and external factors affecting the process. Although spores, if present, dominate in the attachment process, Faille et al, (2014) found no correlation between biofilm and sporulation within biofilms of *Bacillus* species.

Seale et al, (2008) noted, “the outer layer of a *Bacillus subtilis* spore consists of protein and carbohydrates while in species such as *Bacillus cereus* and *Bacillus anthracis*, the spores exhibit an additional layer, known as an exoporium comprising proteins, carbohydrates and lipids”. The factors influencing spore attachment to surfaces are primarily hydrophobicity, electrostatic interactions and surface polymer conformation (Seale et al, 2010). Surface polymer conformation controls the attachment of thermophilic spores such as *Geobacillus* and *A. flavithermus*. Surface hydrophobicity enhances bacterial attachment with spores being more hydrophobic and hence more likely to attach compared with vegetative cells (Wiencek et al, 1991; Husmark, 1993).

Another factor that contributes to attachment of bacteria to surfaces is cell motility. Korber et al, (1989) reported motile strains of *Pseudomonas fluorescens* (*P. fluorescens*) attach rapidly and in higher numbers compared to non-motile strains in

continuous flowing conditions. Non-motile *P. fluorescens* strains appeared to be slow biofilm formers as they are unable to re-colonise and seed the empty areas of the substratum compared to motile strains. They concluded that bacterial appendages such as flagella play an important role in attachment and biofilm formation by overcoming the repulsive electrostatic forces associated with the substratum. Lemon et al, (2007) studied the *Listeria monocytogenes* (*L. monocytogenes*) with flagella biofilm formation in which flagella helped the *L. monocytogenes* motility by propelling them towards the surface to aid attachment in static conditions. Meanwhile in continuous laminar flow conditions, *L. monocytogenes* without flagella resulted lower attachment in the early stages of biofilm development however the biofilm formation increased with time. Abee et al, (2011) reported the same observation on biofilm formation by *B. cereus* and the role of flagella to aid the motility of *B. cereus* in the attachment process.

Biofilm will grow with time and there will be an increase in microorganisms encapsulated in a polymer matrix. Diffusion through this matrix becomes a major factor when determining the structure of the biofilm. The production of EPS by bacteria during the biofilm formation plays many functions. The functions including facilitation of the initial attachment of bacteria to a surface, maintaining the formation of a micro colony and biofilm structure and enhanced resistance of the bacteria in the biofilm to environmental stress and anti-microbial agents (Poulsen, 1999; Yang et al, 2012). EPS are responsible for filtration membrane fouling by irreversibly binding to the membranes. Tang, (2011) quoted that “EPS enhance the survival and robustness of the biofilm microorganisms by forming a chemically reactive diffusion transport barrier for the bacterial cells, impeding convective flow and slowing the penetration of biocide into the biofilm”.

EPS acts as protective layers in holding cells from hostile environments. Although there is evidence of biofilm formation from single species, most biofilms in nature are consist of multi species of microorganisms (Flemming & Wingender, 2010; Mielich-Süss & Lopez, 2015). Van Dyk et al, (2012) reported that different carbon sources (glucose, arabinose, sucrose, and xylose) and nitrogen sources did affect the production of *B. licheniformis* EPS production.

Quorum sensing (QS) is also believed to be involved in biofilm development. QS is cell-to-cell signalling which provides communication between bacterial cells to form mature biofilm (Anand et al, 2014). The QS-coordinated process is achieved by producing, releasing and detecting small signal molecules known as autoinducers (AI). The AI increased with the increasing bacterial cell density. The regulator proteins are triggered once the concentration of AI is at its peak, leading to transcription of QS-regulated genes resulting in changes in bacterial properties. QS has a role in biofilm formation and dispersion. Inhibition of QS systems of microorganisms appears to be a promising method for controlling microbial attachment and membrane fouling (Xiong & Liu, 2010)

A summary of the factors affecting biofilm formation on any surface is presented in the Table 2.1 which includes properties of the substratum, bulk fluid and cells.

Table 2.1 The effects of the substratum, conditioning films forming on the substrate, hydrodynamics of the aqueous medium, characteristics of the medium and various properties of the cell surface on biofilm formation. (Adapted from Donlan, (2002) and Simões et al, (2010)).

Properties of the substratum	Properties of the bulk fluid	Properties of the cell
Texture or roughness	Flow velocity	Cell surface hydrophobicity
Hydrophobicity	pH	Flagella / Fimbriae
Conditioning film	Temperature	Quorum sensing
	Cations	Extracellular polymeric substances
	Presence of antimicrobial agents	Spores

There are 5 key factors that affecting biofilm formation in the food industry (Teh et al, 2015);

- 1) Types of microorganisms.
- 2) Types of product being processed.
- 3) The operation conditions of the manufacturing plant (length of operation and temperature).
- 4) Types of surfaces.
- 5) Cleaning and sanitising regime.

2.2.2 Controlling biofilm formation

Increasing numbers of bacteria in food from a manufacturing plant can be due to biofilm growth and release of bacteria from the biofilm during processing and is exacerbated by an accumulation of residues from incomplete cleaning between production runs (Flint, 1997; Burgess et al, 2010; Murphy et al, 1999; Parkar et al, 2003). Control of microbial colonisation within processing lines in the dairy industry requires CIP procedures. Sodium hydroxide (NaOH) at 1-2% w/v at temperatures $> 70^{\circ}\text{C}$ is widely used in the food and beverage industries (Faille et al, 2013). This procedure has proven effective in dissolving both proteinaceous and fatty soils as well as having emulsifying properties.

Anand et al, (2014) proposed the use of surfactants together with enzymes and chelating agents to prevent bacterial attachment and improving cleaning process. Tang et al, (2010) recommended QuatroZyme which is composed of mixed enzymes to be used as a more effective cleaning system than the standard caustic and acid cleaning systems generally used for membrane processing plants.

Commercially produced microbiological products such as bacteriocin (nisin) and biosurfactant (subtilin) can be used to control biofilm. The biosurfactant has anti-adhesive properties that can prevent the attachment of cells and spores to surfaces. Busscher et al, (1996) used biosurfactants produced by thermophilic dairy streptococci (*S. thermophilus*) for fouling control on heat exchanger plates in pasteurisers. Earlier, Busscher et al, (1990) reported a biosurfactant layer on the surface to which freshly cultured cells did not adhere. This layer was due to biosurfactants produced by the adhering cells themselves. Such a biological anti-adhesive coating might be a potential technique for the control of microbial fouling in the dairy industry. Another role of biosurfactants in a biofilm is their contribution to surface activity (surface tension) in the, early stages of micro-colony formation, aiding surface associated bacterial migration preventing colonisation of channels within biofilms and taking a role in biofilm dispersion (Flemming & Wingender, 2010; Pamp & Tolker-Nielsien, 2007).

A biosurfactant that is produced by *B. licheniformis*, is lichenysin. Some information on lichenysin is presented in Table 2.2. Lichenysin synthesis is coded by the lch A operon (Ron & Rosenberg, 2001). The production of lichenysin may be regulated by cell density, which sparks several quorum sensing control elements either stimulate or inhibit its production. Lichenysin A is produced by *B. licheniformis* (Yakimov et al, 1995; 1997) and Lichenysin B is produced by *B. licheniformis* JF-2 (Lin et al, 1994).

Table 2.2 Description on biosurfactant produced by *B. licheniformis* identified as lichenysin.

Properties	Description	Source
Heat stable	<ul style="list-style-type: none"> Lichenysin A is a lipopeptide lactone that can be produced aerobically and anaerobically (100°C, 20 mins). 	Mikkola et al, 2000
Surface tension	<ul style="list-style-type: none"> Lipopeptide high surface activity and can reduce surface (biofilm) colonisation by <i>E. coli</i>. Lower surface tension of water for 72 to 27 mN/m. 	Rivardo et al, 2011 Anuradha, 2010
Emulsion/surfactant	<ul style="list-style-type: none"> Secondary metabolites that solubilise hydrophobic substrates, and regulation of attachment-detachment of microorganisms from surfaces. Act as antimicrobial and pseudo-surfactant (synergistic effect). 	Anuradha, 2010 Coronel-León et al, 2017

Bacillus species producing biosurfactant in biofilms that reduce the surface tension between the cell surface and the substrate reduce biofilm formation. Faille et al, (2014) noted that *Bacillus* biofilms adherent poorly to SS in their lab and were even susceptible to detachment during a single rinsing procedure, believed to be due to

surfactants produced by these bacteria. A similar observation was made by Wijman et al, (2007).

The formation of biofilm in different parts of dairy manufacturing plant is likely to be influenced by the types of contaminants present that may enhance or reduce the potential for biofilm formation. Some parts of dairy manufacturing plant are likely to be influenced by biofilm formation more than others. As whey originates from the result of several manufacturing processes, this is likely to be influenced by microbial contamination more than other dairy products.

2.3 WHEY

Whey was traditionally a waste product from the manufacture of cheese or casein that needed to be discarded. Whey is difficult to dispose of or use due to its unfavourable lactose-to-protein ratio and high biological oxygen demand of 30 000 – 50 000 ppm (Madaeni & Mansourpanah, 2004). The need to reduce the impact on the environment plus the realisation that whey contains components of value has resulted in the manufacture of numerous whey products. Whey is the liquid remaining after the production of cheese or the removal of fat and casein (80% of the proteins) from milk (Macgibbon, 2014). The worldwide trade of whey ingredients (all types combined) were estimated at 1.5 million metric tonnes in 2013 with European Union the leading exporters (Lagrange et al, 2015). Most of the whey (92%) is cheese whey, the liquid remaining from the production of cheese. Whey contains about 50% of the nutrients present in milk, comprising milk sugar (lactose), serum proteins (whey proteins), minerals, a small amount of fat, and most of the water soluble minor nutrients from milk such as vitamins (Zadow, 1992; Smithers, 2008) (Figure 2.2).

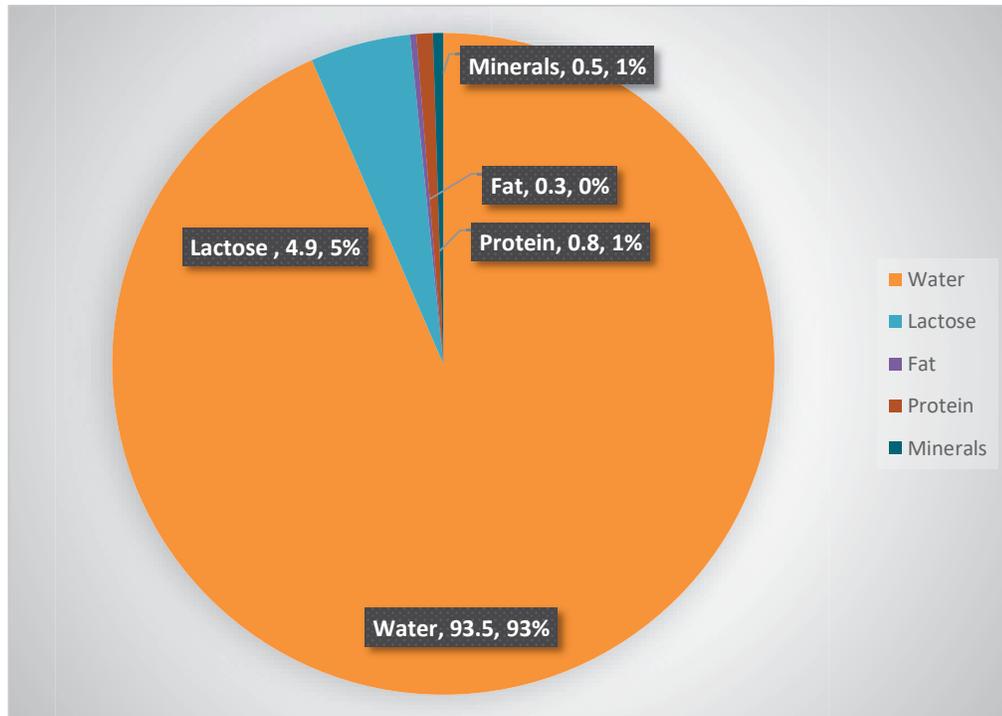


Figure 2.2 The standard whey content before the UF process. Information from Wisconsin Centre for Dairy Research, (<http://www.cdr.wisc.edu>)

The whey constitutes of 90% of the original milk volume with 50% of the original milk components remaining when the casein is removed (Onwulata & Huth, 2008). Whey protein is concentrated and spray dried to provide a product that can be used as an ingredient in many foods (Figure 2.3). The most abundant component of whey after water is the milk sugar lactose can be considered as having nutraceutical roles besides being the main source of energy for the newborn (Morr & Ha, 1991; Morr & Ha, 1993). Lactose is now in very strong demand for protein standardisation in milk powder manufacture. Its disposal is no longer an issue in New Zealand.

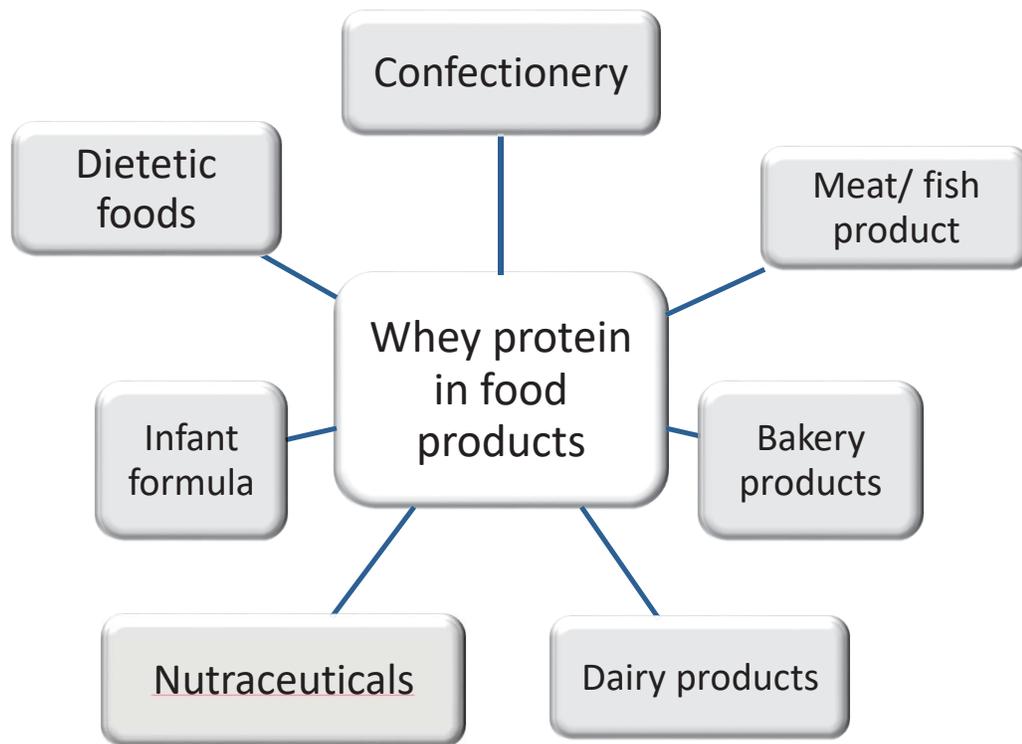


Figure 2.3 The use of whey protein in the food industry

WPC prepared by the UF process of and drying of whey contains 68.4% β -lactoglobulin, 21.3% α -lactalbumin and 10.3% serum proteins (Delaney, 1976). The lactose, salts and much of the water are removed during UF and dialysis. The remaining water is removed by evaporation and spray drying. The ratio of individual proteins in any ultrafiltered WPC will be influenced by factors such as:

- 1) type and source of whey.
- 2) rejection characteristics of the UF membrane used.
- 3) degree of concentration achieved.

Whey became of interest to food processing industries as its physical and chemical properties, especially its protein, have unique nutritional qualities. In addition,

the undenatured whey proteins have unique functional properties. The major whey product is WPC (Zadow, 1992). WPC can be defined as the product derived from milk whey containing 50, 65 or 80% native proteins (Luck et al, 2013). WPC contains higher tryptophan (non-polar) amino acid and cysteine compared to skim milk but falls below Food and Agriculture Organization (FAO) whole egg reference protein values for valine, tyrosine, phenylalanine and methionine content (Delaney, 1976). The high percentage of lysine, tryptophan, methionine and cysteine in WPC should be noted. However, phenylalanine and tyrosine are limited in gel filtered and ultra-filtered WPC. Low mineral content is desired in WPC because of the demand of certain food formulations such as infant formula and healthy food products. The vitamin content of WPC is rich in B₁₂ and folic acid. The other water soluble vitamins of whey in the free form is released through the UF membrane during WPC manufacture.

2.3.1 Whey manufacturing process

A schematic diagram of the whey protein concentrate manufacturing process at one dairy plant in New Zealand is presented in Figure 2.4. The first step is clarification to remove particles of cheese or any precipitated proteins and possibly some bacterial contamination. Thermalisation reduces the microbial load and chilling slows any further microbial growth during UF and dialysis. The thermaliser heat plate exchanger used in this manufacturing plant has temperatures from 36°C to 77°C (Appendix 1). The separator used to remove fat from whey. Once concentrated to approximately 50% of its original volume, and the lactose and salts have been removed by dialysis, the remaining protein solution is concentrated by evaporation by another 50% before spray drying.

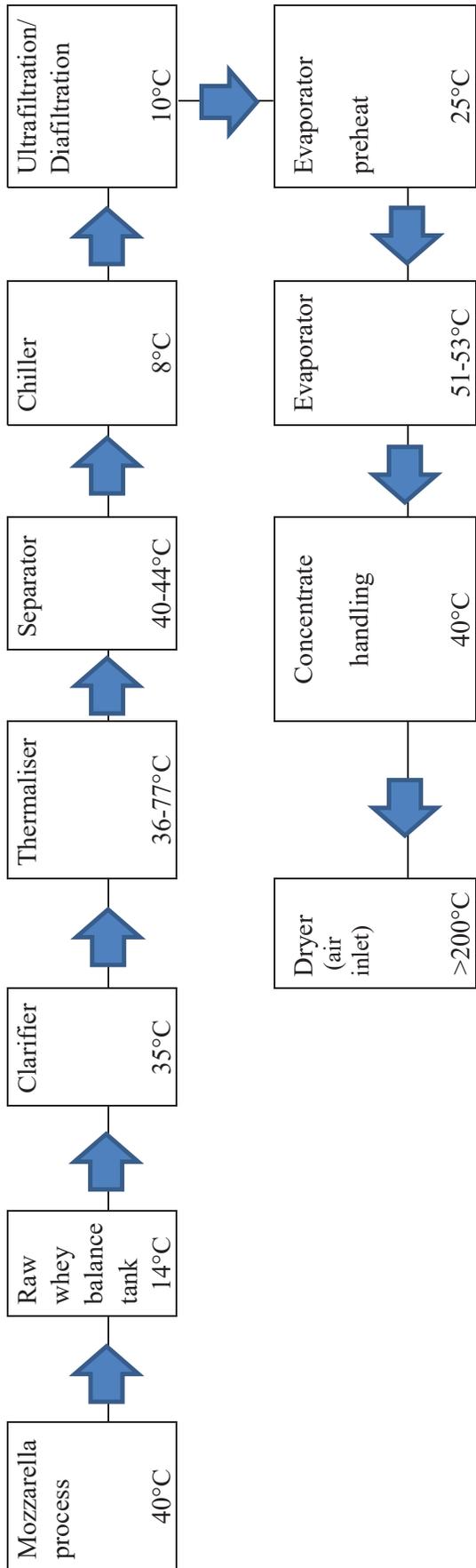


Figure 2.4 A flowchart of the whey manufacture processes.

A key part of the manufacturing process is UF. This separation takes place through semipermeable membranes, using a hydrostatic pressure gradient as the driving force. UF is molecular sieving on a commercial scale in the manufacture of whey products. UF is a pressure-driven flow-dependent filtration process in which porous membranes are used to separate the components of a solid-liquid mixture on the basis of shape and size and in some instances, charge. When the pressure gradient is applied across the membrane, the liquid is forced to flow through the pores to the low-pressure side, transporting any components that are smaller than the size of the membrane pores. The pressure gradient required for UF is generally low and typically around 500 Kpa. The pore dimensions are typically in the range of 1 to 100 nm diameter. The molecular weight cut-off in the range from 10 000 – 50 000 Daltons used for the fractionation of whey protein in UF membranes. Low molecular weight solids such as lactose, minerals and water will pass through the membrane as permeate, while proteins and residual fat are rejected and stay inside as retentate (Mohammadi et al, 2002; Zadow, 1992) (Table 2.3).

Table 2.3: Different types of filtration process and their function (Zadow, 1992).

Type	Pore size (nm)	Components retained	Molecular weight of component (kDa)
MF	200-4000	Bacteria, casein micelles, fat globules	100-500
UF	20-200	Whey proteins	1-100
NF	< 2	Lactose	0.1-1
RO	< 2	Ions	< 0.1

Whey separation by UF is normally performed at temperatures below 55°C with an inlet pressure around 300 kPa and a membrane pore size of 250 nm. Whey retentate consists of protein, fat and insoluble salts, lactose, soluble mineral and much water (Onwulata & Huth, 2012). Initially UF plants operated at 50°C for reasons of improved flux and microbiological control. Since the advent of spiral wound membranes with a high surface area to volume ratio, it has become economic to operate plants at 10 °C for improved microbiological control and increased membrane life.

Processing operations (pasteurisation, pre-heating, evaporation and spray drying) may result in Maillard type browning and concomitant reduction in available lysine content. The UF step has little adverse effect on the available lysine content as UF is a gentle, low temperature process involving no phase change.

Higher degrees of whey fractionation result in the viscosity of the retentate becoming too high to pump. A process called “Diafiltration” involves adding water during UF to remove salts and lactose thus solved this problem. The composition of a WPC depends on the properties of the membrane, the duration of the filtration process and the use of water (Diafiltration). The UF process is able to produce all types of WPC, with protein contents ranging from 25% to 80% total solids (De Wit, 2001).

Milk fractionation using different pore sized membranes can separate the casein micelles and bovine serum albumin leaving whey serum as the retentate. Further separation of the whey occurs using UF which separates the whey protein consisting of α -lactalbumin and β -lactoglobulin as the retentate and leaves the lactose and peptides as permeate. Nanofiltration is used to fractionate and concentrate the whey protein into its individual components (of α -lactalbumin and β -lactoglobulin). Meanwhile reverse osmosis is used to concentrate the lactose for drying (Morr & Ha, 1993).

Membrane fouling limits the operation of UF membrane plants. Fouling is defined as the attachment and growth of microorganisms and irreversible collection of materials trapped on the membrane surface which results in a flux decline. Fouling of UF membranes in the dairy industry is mostly due to the colonisation of microorganisms, and residual protein, fats and minerals on the membrane surfaces either at its pore openings or within its pores (Mohammadi et al, 2002; Mohammad et al, 2012; Chamberland et al, 2017a). Zaky et al, (2012) defined abiotic and biotic fouling on membranes as:

- 1) Abiotic fouling: conditioning layer: fouling deposition of organic, salts, dead and cell debris from the feed water on the membrane surface.
- 2) Biotic biofouling: fouling due to microbial growth from active cells on the membrane surface.

Whey is a foulant for membranes in dairy plants with the foulant being mainly made up of protein. In whey, β -lactoglobulin is the most well-known foulant (Fouladitajar et al, 2014). Inorganic salts, including calcium phosphate also contribute to fouling. This salt acts as a binding bridge between the membrane and the protein, leading to hydraulic resistance of the protein layer (Madaeni & Mansourpanah, 2004; Pattaranawik & Leiknes, 2011; Herzberg & Elimelech, 2007; Laborie et al, 1998; Liu et al, 2012). However, biotic fouling such as microbial growth on the membrane surface may result in poor quality product. Studies on biofilm formation on UF membranes by Tang, (2011) showed Gram negative *Klebsiella oxytoca* were dominant and believed to have originated from water used in the cleaning of the plant and dialysis. Chamberland et al, (2017b) studied the biofilm formation on UF membrane operated at 10°C and found that *Lactococcus* spp were the dominant contaminant.

2.4 CONCLUSIONS

Microbial contamination of dairy products is controlled by heat treatment such as pasteurisation. However, some thermophilic and mesophilic organisms as well spore forming bacteria can survive and contribute to the spoilage of the milk and dairy products. These bacteria include *S. thermophilus* and spore-forming bacteria *Anoxybacillus*, *Geobacillus* and *Bacillus* species (Flint, 1998).

Dairy product contamination is enhanced by the growth of biofilms in the manufacturing plant. Biofilms develop from bacteria that survive heat treatment and contaminate any surface where the conditions are suitable for bacterial growth. The contribution of biofilms of these thermo-resistant bacteria to the contamination of whey products is unknown.

Biofilms reduce heat transfer in heat exchangers and cooling towers. The EPS matrix provides protection and resistance to antimicrobial treatments and contributes to a higher transformation frequency (transfer of DNA) among the bacteria comprising the biofilm than planktonic cells (Vlamakis et al, 2013; Flemming & Wingender, 2010). The contribution of different stages of WPC manufacture to contamination of WPC through biofilm development is unknown.

Fouling, including biofilm, results in an increase in operational costs, due to an increased energy demand, additional labour for maintenance, cleaning solution costs, shorter membrane life and limitations in the amount of product that can be processed. Heat exchangers or/and thermaliser are cleaned every day by CIP as fouling with whey protein occurs rapidly and reduces heat transfer (Bansal & Chen, 2006). Even this, however, is not always effective in controlling microbial contamination of WPC.

CHAPTER 3

ISOLATION AND IDENTIFICATION OF MICROORGANISMS FROM WPC80 POWDER

3.1 INTRODUCTION

Whey is the liquid remaining after the production of cheese or the removal of fat and casein (80% of the proteins) from milk, and contains predominantly Gram-positive organisms from the starter population used in cheese and casein manufacture (lactic acid bacteria) or thermo-resistant species such as spore-forming *Bacillus* species (Schreiber, 2001). There are some starter bacteria, such as *S. thermophilus* and thermo-resistant *Bacillus* species can tolerate heat treatment such as thermalisation, pasteurisation and evaporation used in the manufacture of WPC. Whey contains about 50% of the nutrients present in milk, comprising milk sugar (lactose), serum proteins (whey proteins), minerals, a small amount of fat and most of the water soluble minor nutrients from milk such as vitamins (Smithers, 2008; Zadow, 1992). This whey is selectively concentrated by UF and evaporation then spray dried to provide a high protein product that can be used as an ingredient in many foods.

Whey, formerly a waste product, became of interest to the food processing industries due to its physical and chemical properties, especially its protein and nutritional qualities. The major whey product is WPC (Zadow, 1992). It can be defined as the product derived from milk whey containing 50%, 65% or 80% native proteins (Luck et al, 2013) and called WPC50, WPC65 and WPC80, respectively. The individual protein composition in ultra-filtered WPC is about 68% β -lactoglobulin, 21% α -lactalbumin and 10% serum proteins. This is approximately the same as present in whole milk (Delaney, 1976). The amino acid composition of WPC and skim milk were compared and the results showed WPC contains higher tryptophan and cysteine. This is due to the activity of starter bacteria in the manufacture of cheese whey (Macgibbon, 2014).

The microflora in WPC varies depending on the type of whey with sweet whey showing a higher prevalence of thermophilic and mesophilic spore formers compared with acid whey (Watterson et al, 2014). Aerobic spore formers of particular concern in dairy products include the psychrotolerant *Paenibacillus* species, mesophilic *Bacillus* species; for example, *B. licheniformis*, *B. subtilis* and *B. pumilus* and thermophilic *A. flavithermus* and *Geobacillus* species in a group that is not well defined (Burgess et al, 2009; Burgess et al, 2010; Watterson et al, 2014). Spore

formers found in the dairy industry are the resistant forms of *Bacillus* and *Clostridium* species that withstand heat and chemical treatment that is used to control most bacteria in dairy manufacture.

Generally, the microbial contaminants in products such as WPC are recorded as either thermophilic or mesophilic bacteria in routine testing. Dairy manufacturers have specifications for these groups of bacteria but there is no requirement for further testing to identify the bacteria comprising these groups. The isolation and identification of these bacteria could help in determining the effect on product quality and the risk of the manufacture of unacceptable product and the risk of product spoilage. The study in this chapter is aimed to isolate and identify the contaminants in the six Mozzarella WPC80 powder samples.

3.2 MATERIALS AND METHODS

3.2.1 Source of samples

Six Mozzarella WPC80 samples from different manufacturing runs were obtained from one dairy manufacturing site in New Zealand. The samples in powdered form were received in 100 g foil lined paper pouches used to store samples from the manufacturing process for testing. The Mozzarella WPC80 samples are listed in Table 3.1. Six different Mozzarella WPC80 samples provided due to reported high bacterial counts were analysed to obtain the total microbial load using the aerobic plate count technique as used in the dairy industry to monitor the quality of the product.

Table 3.1 Six Mozzarella WPC80 samples from one dairy company in New Zealand.

Samples	Details
A	Cypher: CX15
B	Cypher: CX06
C	Cypher: CX07
D	Cypher: CX09
E	Cypher: CX17
F	Cypher: CX22

3.2.2 Isolation of bacteria

Ten grams of each WPC80 samples were diluted in 90 g of sterile 0.1% peptone water (pH 7.0) and homogenised using a peristaltic blender for 1 min prior to serial 10-fold dilution to 1×10^{-6} in 0.1% peptone. The pour plate technique was performed using milk plate count agar (MPCA) (Merck, BDH, Palmerston North, New Zealand) in duplicate to obtain numbers in the range of 30 - 300 colonies per plate. The agar plates were incubated aerobically at 30°C for 24 h for mesophilic bacteria and at 55°C for 18 h. Two to three single isolated colonies of different colony morphology were taken from the plates of each Mozzarella WPC80 samples and restreaked onto MPCA to obtain pure cultures. Pure strains were streaked on beads in glycerol and stored at -80°C (Microbank, Pro-Lab Canada). Mesophilic spores from the samples were determined by adding 1g of each WPC80 samples into 99 mL sterile distilled water and heated at 85°C for 10 mins. Serial dilutions were performed until 10^{-5} and plated on MPCA in duplicate. The plates were incubated for 48 h at 30°C.

3.2.3 Phenotypic characterisation of isolates

The pure cultures grown on MPCA were screened using microscopic observation of Gram stained cells. The motility tests were done by the hanging drop technique, motility agar and observed under light microscopy (Olympus, USA). Some of the isolates samples were sent to the Manawatu Microscopy Imaging Centre for scanning electron microscope (SEM) and transmission electron microscope (TEM) images. These were used as additional confirmatory tests for some of the isolates,

selected on the basis of colony morphology as presumptive *B. licheniformis*. Supportive information on the identification of the bacterial isolates was obtained by biochemical testing using the conventional culture methods in selective growth media or miniaturised API 50CHB system. The *Bacillus* species were isolated on MYP agar (mannitol-egg yolk polymyxin; Oxoid) for presumptive *B. cereus* identification. After incubation of plates for 24 to 48 h at 30°C, characteristic pink colonies, non-fermenting mannitol and surrounded by a zone of white precipitation of lecithin were recorded (Appendix 2). Further identification was done by biochemical tests using the following media in the Table 3.2 below, incubating at 30°C for 24 h. A 24 h culture was prepared and 1 mL of the culture was pipetted into the API 50CHB inoculation medium and subsequently pipetted into each tube but not the cupule. The strip was incubated at 30°C for 48 hours and observed after 24 and 48 h. After 48 h of incubation, the results were coded and interpreted using the API website (bioMérieux).

Table 3.2 Biochemical tests done to help identify unknown *Bacillus* species.

No	Media	Manufacture	Purpose
1	Mannitol egg-yolk polymyxin agar	Becton Dickson, USA	<i>B. cereus</i> identification
2	Simmons citrate	OXOID, England	Citrate utilisation
3	Gelatine agar	OXOID, England	Gelatine production
4	Nutrient broth with 7% NaCl	Becton Dickson, USA	NaCl tolerance

3.2.4 Identification by PCR

Gram positive *Bacilli* with colonies indicative of *B. licheniformis*, were further tested using species specific PCR targeting the gyrase B gene (Huang et al, 2012) using one set of species specific primers Blich-F1 5'-AKACGGAAGTGACGGGAAC-3' and Blich-R1 5'-AGAAACTTTTCRAGCGCTT-3'. The DNA was extracted by boiling the bacterial culture. PCR amplification involved 20 µL Master mix (5 Prime MasterMix-100 Rxns GmbH, Germany) consisting of dNTPs, magnesium chloride and Taq DNA polymerase, 24 µL UltraPure™ DNase/RNase-Free Distilled water (Invitrogen), 1 µL forward primer, 1 µL reverse primer and 4 µL DNA template (unknown culture) to achieve a final volume of 50 µL. Amplification conditions were as follows; denaturing step at 94°C for 7 min followed by 35 cycles with denaturation (49°C for 60 s), annealing (55°C for 60 s) and extension (72°C for 60 s) followed by a final extension at 72°C for 15 min. All PCR assays were carried out on the Techno thermal cycler (TC-400, Total Lab Systems, Auckland, New Zealand). The PCR products were visualised (E-Gel iBASE™, Invitrogen) using pre-made 2% agarose electrophoresis gel (E-Gel® EX with SYBR Gold II) then visualised under UV Transilluminator, UVP, Inc. (Chromato-Vue, San Gabriel, California, USA) and UVITEC (Cambridge, UK).

3.2.5 Partial 16S rDNA gene sequencing

For the colonies that were not indicative of *B. licheniformis* and for those isolates that did not produce a positive result with the species-specific primers for the *gyrB* gene of *B. licheniformis*, universal primers Bac27F 5'-AGAGTTTGATCCTGGCTCAG-3' and U1492R 5'-TACGGCTACCTTGTTACGACTT-3' were used to amplify a 1000bp part of the 16S rDNA genome for sequencing, using the conditions in Flint et al (1999b). Prior to sequencing, the PCR products were purified using a Zymo DNA Clean & Concentrator TM-5, USA kit and sent to UV/VIS spectrophotometry to check the DNA concentration (1 µL – 2 µL) using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA). Sequencing was done at the Massey University Sequencing

Unit using the BigDye terminator v3.1 cycle sequencing kit and the results analysed using Seqmatch and BLAST GeneBank.

3.3 RESULTS

3.3.1 Isolation of bacteria

Six different Mozzarella WPC80 samples were analysed to obtain the total microbial load using the total plate count technique. Two temperature profiles (30°C and 55°C) were selected for incubation to isolate both mesophilic and thermophilic microorganisms. The predominant bacteria in the WPC80 samples were mesophilic bacteria (Table 3.3) with total mesophilic bacterial counts close to or exceeding the specification limits. The Australia New Zealand Food Safety Authority guideline for the mesophilic plate count for dried milk including whey powder is 5×10^4 CFU/g (acceptable level) and 2×10^5 CFU/g (maximum). The thermophilic bacterial counts were all $< 10^3$ CFU/g (Table 3.4). There is no guideline for thermophilic plate count. Mesophilic spore counts mostly below 100 CFU/g except in Sample A which did not yield any positive result. The results are given in Table 3.5

Table 3.3 Microbial load WPC80 samples incubated at 30°C for 24 h determined by plate counting on MPCA.

Sample	Total plate count (CFU/g)
A	5.5×10^4
B	5.9×10^5
C	1.0×10^4
D	7.3×10^4
E	1.2×10^5
F	3.3×10^4

Table 3.4 Microbial load from WPC80 samples incubated at 55°C for 18 h determined by plate counting on MPCA.

Sample	Total plate count (CFU/g)^a
A	< 10 ²
B	< 10 ³
C	< 10 ³
D	< 10 ³
E	< 10 ²
F	< 10 ³

^a Spreading colonies for the thermophilic aerobic plate count meant no accurate counts could be obtained for this test.

Table 3.5 Mesophilic spore counts determined by plate counting on MPCA from WPC80 samples incubated at 30°C for 48 h.

Sample	Spore counts (CFU/g)^b
A	0
B	< 100
C	< 100
D	< 100
E	< 100
F	< 100

^b Spreading colonies after 48 h of incubation.

The list of isolated bacteria from six WPC80 powder is listed in Table 3.6. Mozzarella WPC80 samples were labelled A, B, C, D, E, and F and bacteria isolated from each sample was labelled according to their cypher sources.

Table 3.6 List of the bacteria isolated from Mozzarella WPC80 samples and their basic physical properties.

WPC80 Sample	Number of Isolates	Gram staining		Incubation temperature	
		G +ve bacilli	G -ve bacilli	30°C	55°C
A	30C01	+		+	
	30C11	+		+	
	30C21	+		+	
B	30C11	+		+	
	30C12	+		+	
	30C13	+		+	
	30C14	+		+	
	30C21	+		+	
	30C22	+		+	
	30C24	+		+	
	55C01B	+			+
	55C02	+			+
55C11	+			+	
C	30C01	+		+	
	30C11A	+		+	
	30C11B	+		+	
	30C21	+		+	
	55C01	+			+
	55C02	+			+
	55C11	+			+
	55C12	+			+
D	30C01A	+		+	
	30C01B	+		+	
	30C02	+		+	
	30C03	+		+	
	30C04	+		+	
	30C12	+		+	
	30C14	+		+	
	55C01	+			+
	55C02	+			+
	55C03	+			+
	55C04	+			+
	55C11	+			+
E	30C01	+		+	

	30C03	+	+	
	30C04	+	+	
	30C11	+	+	
	30C12	+	+	
	30C21	+	+	
	30C22	+	+	
	55C01	+		+
	55C02	+		+
F	30C01	+	+	
	30C02	+	+	
	30C11	+	+	
	30C14	+	+	
	55C01	+		+
	55C02	+		+
	55C11	+		+
	55C12	+		+

3.3.2 Phenotypic characterisation of isolates

The isolates obtained from the six WPC80 samples were predominantly *Bacillus* species with rod shaped purple-violet colour on Gram staining, as expected from colony morphology. All of the isolated bacteria were Gram positive, mostly spore formers and motile based on light microscopic observation. (Olympus, USA) (Figure 3.1).

The colonies formations of *B. licheniformis* on MPCA were mucoid, large opaque, adherent, wrinkled and irregular edges (Figure 3.2). The isolates were retrieved from MPCA and incubated at the appropriate temperature (30°C or 55°C). The growth indicated that isolates were facultative anaerobes (the colonies grew best within the solid agar) and others were strict aerobes (the colonies grew best on the agar surface). Although some of the isolates were observed to be facultative anaerobes, their growth in strict anaerobic conditions was not tested.

The summary of biochemical tests is listed in Table 3.6. Biochemical testing was performed to identify the isolates prior to bio-molecular testing. Supportive information on the identification of the bacterial isolates was obtained by conventional culture methods in selective growth media for *Bacillus* (Table 3.7) followed by biochemical testing using the miniaturised API 50CHB (bioMérieux) (Table 3.8).

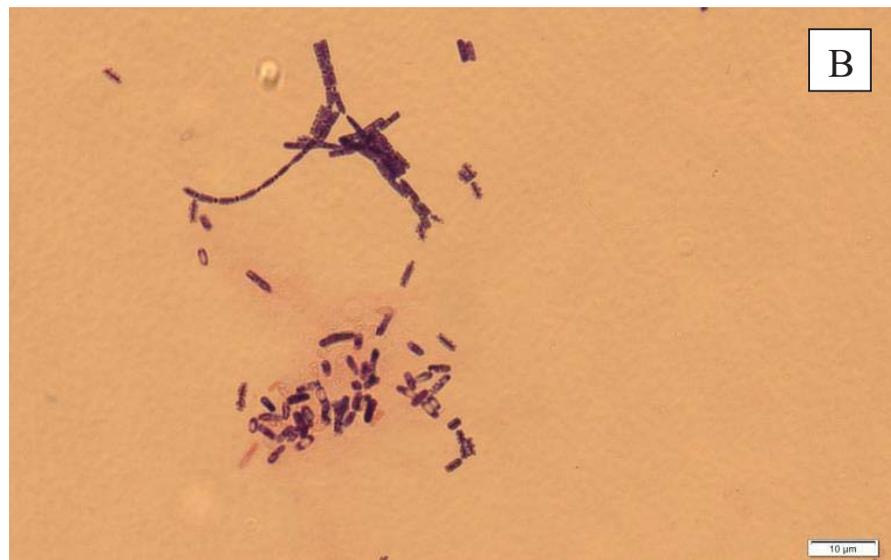
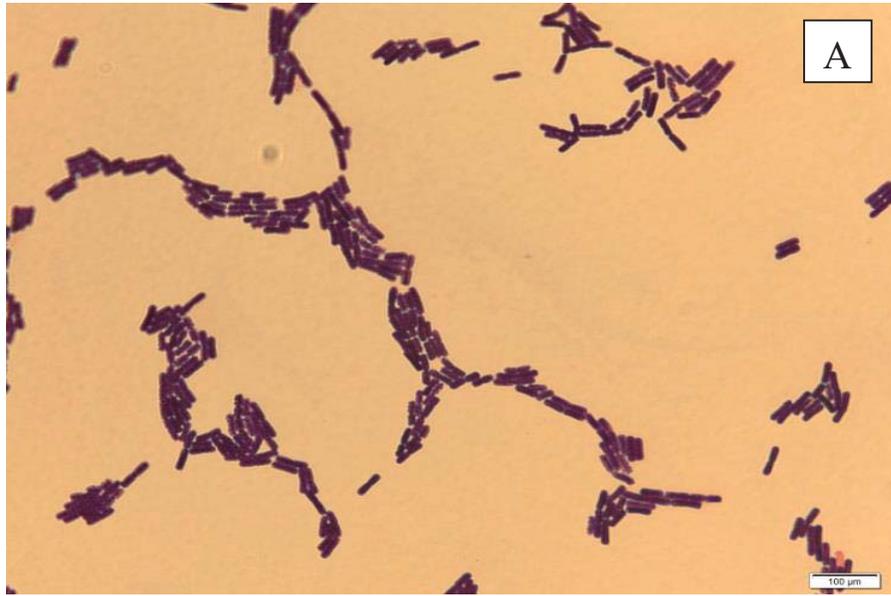


Figure 3.1 Image of E30C11 strain (A) and D30C02 strain (B) shows the cell the morphology typical of *Bacillus* species isolated from WPC80. Some endospores were spotted within the cell (B).



Figure 3.2 Typical single colony formations (wrinkled, mucoid) of *B. licheniformis* E30C11 strain on MPC agar.

Table 3.7 Summary of the biochemical tests and expected identity of the *Bacillus* species (Appendix 2) excluding the *B. licheniformis* isolates already identified by PCR specific gene (Appendix 3).

Isolates	Simmons citrate agar	Mannitol yolk polymyxin agar	NaCl tolerance	Gelatine test	Expected identity
A30C11	+	-	-	+	<i>B. subtilis</i>
A30C21	+	+	-	+	<i>B. cereus</i>
B30C12	+	-	-	+	<i>B. subtilis</i>
B30C21	-	+	-	+	<i>B. cereus</i>
B30C22	-	+	-	+	<i>B. cereus</i>
B30C24	-	+	-	+	<i>B. cereus</i>
B55C01B	-	-	+	+	<i>B. subtilis</i>
C30C11A	-	+	-	+	<i>B. cereus</i>
C30C21	-	+	-	+	<i>B. cereus</i>
C55C02	-	-	-	+	<i>B. subtilis</i>
D30C01A	-	+	-	+	<i>B. cereus</i>
D30C01B	-	+	-	+	<i>B. cereus</i>
D30C02	-	+	-	+	<i>B. cereus</i>
D30C03	+	-	-	+	<i>B. subtilis</i>
D30C04	-	+	-	+	<i>B. cereus</i>
D30C12	-	-	-	+	<i>B. subtilis</i>
E30C03	+	+	-	+	<i>B. cereus</i>
E30C12	-	-	-	+	<i>B. subtilis</i>
E30C22	-	+	-	+	<i>B. cereus</i>
E55C01	-	-	-	+	<i>B. subtilis</i>
F30C01	-	-	-	+	<i>B. subtilis</i>
F30C02	-	-	-	+	<i>B. subtilis</i>
F55C11	-	-	+	+	<i>B. subtilis</i>

Table 3.8 Results from carbohydrate profile API 50CHB kit after 24 to 48 h of incubation towards *Bacillus* species from Mozzarella WPC80 samples.

Test	Carbohydrate	<i>B. licheniformis</i>	<i>B. thuringiensis/B. cereus</i>	<i>B. subtilis</i>	<i>B. pumilus</i>
Control (Ng= No growth)					
GLY	Glycerol	+	-	+	+
ERY	Erythritol	-	-	-	-
DARA	D-arabinose	-	-	-	-
LARA	L-arabinose	+	-	+	+
RIB	D-ribose	+	-	+	+
DXYL	D-xylose	+	-	+	+
LXYL	L-xylose	-	-	-	-
ADO	D-adonitol	-	-	-	-
MDX	Methyl-BD-xylopyrannoside	-	-	-	-
GAL	D-galactose	+	-	-	-
GLU	D-glucose	+	+	+	+
FRU	D-fructose	+	+	+	+
MNE	D-mannose	+	-	+	+
SBE	L-sorbose	+	-	-	-
RHA	L-rhamnose	+	-	-	-
DUL	Dulcitol	-	-	-	-
INO	Inositol	+	-	+	-
MAN	D-manitol	+	-	+	+
SOR	D-sorbitol	+	-	+	-
MDM	Methyl α D- annopyranoside	-	-	-	-
MDG	Methyl α D- glucopyranoside	+	-	-	+
NAG	N-acetylglucosamine	+	+	-	+
AMY	Amygdaline	+	-	+	+
ARB	Arbutine	+	+	+	+
ESC	Esculin	+	+	+	+
SAL	Salicin	+	+	+	+
CEL	D-cellobiose	+	-	+	+
MAL	D-maltose	+	+	+	+
LAC	D-lactose	-	-	-	-
MEL	D-melobiose	-	-	+	-
SAC	D-saccharose	+	-	+	+
TRE	D-trehalose	+	+	+	+
INU	Inulin	+	-	-	-
MLZ	D-melezitose	-	-	-	-
RAF	D-rafinose	+	-	+	-
AMD	Amidon	+	+	+	-
GLYG	Glycogene	+	+	+	-
XLT	Xylitol	-	-	-	-
GEN	Gentiobiose	-	-	+	-
TUR	D-turanose	+	-	-	+
LYX	D-lyxose	-	-	-	-
TAG	D-tagatose	+	-	-	+

DFUC	D-fructose	-	-	-	-
LFUC	L-fructose	-	-	-	-
DARL	D-arabitol	-	-	-	-
LARL	L-arabitol	-	-	-	-
GNT	Potassium gluconate	+	-	-	-

3.3.3 Identification by PCR

The identification of suspect *B. licheniformis* isolates based on colony morphology was confirmed as expected using species specific PCR based on *gyr B* gene. A negative control (no template) DNA was included. *B. licheniformis* was the predominant isolate making up 33 of the 50 isolates (Table 3.9).

3.3.4 Partial 16S rDNA gene sequencing

B. cereus and *B. subtilis* identified from biochemical tests were subjected to partial 16S rDNA gene sequencing and the results are showed in Table 3.9.

The following isolates (A30C11, B30C12, C55C02, D30C03, F30C01 and F30C02) confirmed as *B. licheniformis* instead of *B. subtilis* and E30C21 confirmed as *P. glucanolyticus* using partial 16s rDNA gene sequencing. All the *B. licheniformis* isolates identified by PCR and biochemical tests were 16S rDNA sequenced for confirmation.

Table 3.9 The summary of isolates retrieved from WPC80 and their frequency based on specific PCR and 16s rDNA analysis.

No.	Bacterial Identity	16S rDNA (% similarity)	Frequency
1	<i>Bacillus licheniformis</i>	98-99	33
2	<i>Bacillus cereus/ Bacillus thuringiensis</i>	98	9
3	<i>Bacillus subtilis</i>	99	2
4	<i>Bacillus pumilus</i>	99	2
5	<i>Paenibacillus glucanolyticus</i>	98	1
6	<i>Lactobacillus plantarum</i>	98	3
Total			50

SEM images show *B. licheniformis* and *P. glucoanolyticus* on membrane surfaces (Figures 3.3 and 3.4 respectively). The spores of *P. glucoanolyticus* have quite a distinctive morphology (Figure 3.5).

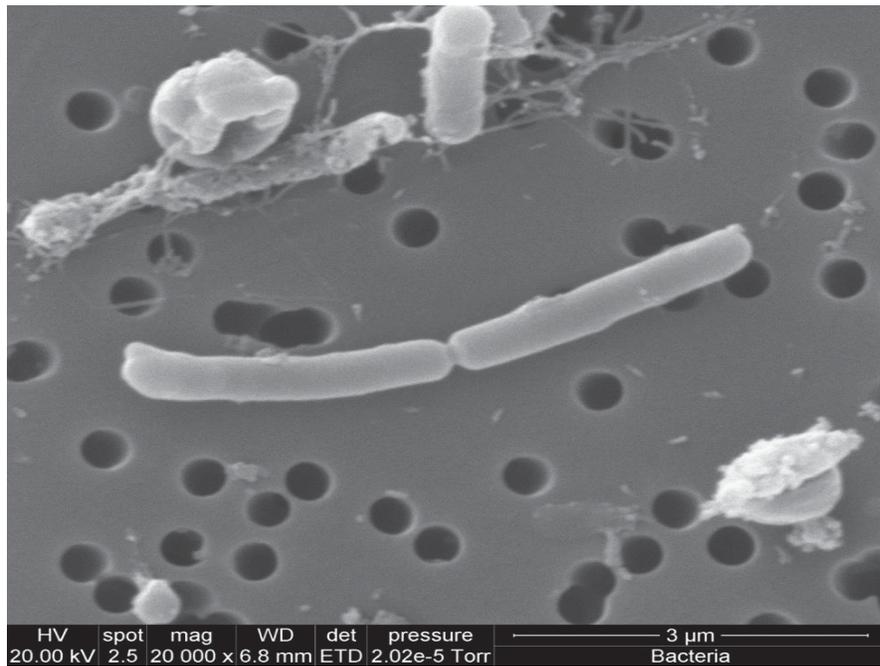


Figure 3.3 SEM image of *B. licheniformis* cell with 3 μm length.

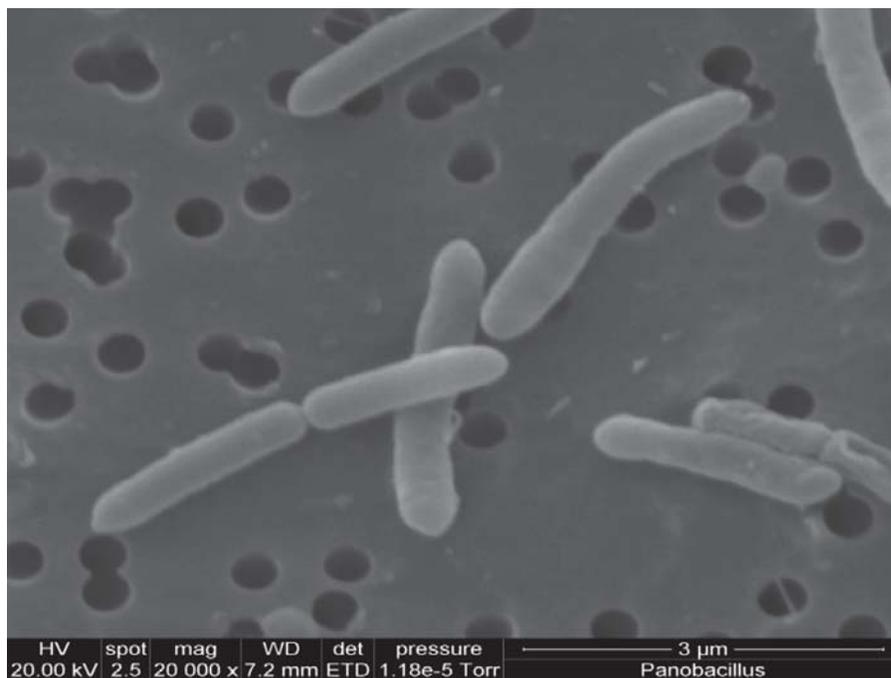


Figure 3.4 SEM image of *P. glucanolyticus* cell with 3 µm length.

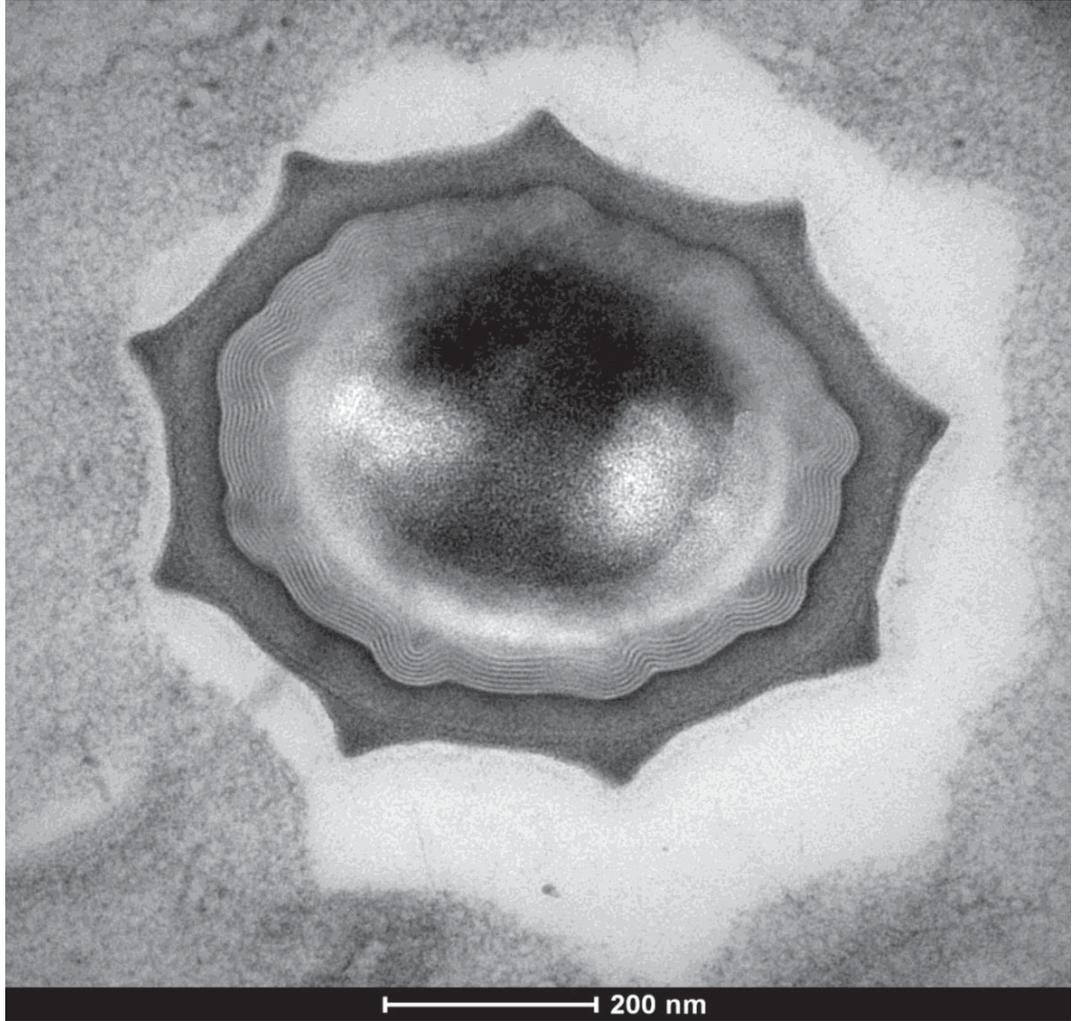


Figure 3.5 TEM image of *P. glucanolyticus* spore (Appendix 4).

3.4 DISCUSSION

The source of microbiological contamination of Mozzarella WPC80, a quality problem for the dairy industry, has not been thoroughly investigated. The objective of this chapter was to identify the bacteria isolated from Mozzarella WPC80 powder. Six *Bacillus* species including (percentage of isolates in brackets) *Bacillus licheniformis* (66%), *Bacillus cereus*/*Bacillus thuringiensis* (18%), *Bacillus subtilis* (4%), *Bacillus pumilus* (4%), *Lactobacillus plantarum* (6%) and *Paenibacillus glucanolyticus* (2%) were identified based on biochemical and further confirmation were done using molecular analysis in six different WPC80 powder batches (Zain et al, 2016). Mesophilic spore counts from 5 of the 6 WPC80 samples were less than 100 CFU/g and one sample (Sample A) did not yield any colonies on MPCA.

The dominant isolates were *B. licheniformis* with 33 isolates. These aerobic-spore forming bacteria are frequent contaminants isolated from dairy milk or dairy processing environments (Aouadhi et al, 2013; Buehner et al, 2014; Burgess et al, 2013; Alvarez-Ordóñez et al, 2014; Lücking et al, 2013; Masiello et al, 2014; Frank, 1997), predominant in milk powder (Yuan et al, 2012; Reginensi et al, 2011) and but not commonly reported in whey powder. Buehner et al, (2014) reported that *B. licheniformis* was the major contaminant in milk regardless of the seasons (winter or summer). *B. licheniformis* can contribute to product spoilage through the production of protease enzymes (Teh et al, 2012).

Infrequently, *Bacillus* species such as *B. licheniformis*, *B. amyloliquefaciens* and *B. pumilus* have been reported to produce toxic components which may play role in food poisoning (Lücking et al, 2013). The origin of *B. licheniformis* is believed to be from soil and the heat resistant spores survive pasteurisation of milk and further survive the cheese manufacturing process thus probability contaminating the cheese product too. Although the isolates were recovered at 30°C (mesophiles), some grew at 55°C (thermophilic)(Lücking et al, 2013). Watterson et al, (2014) reported that sweet whey and non-fat dry milk showed higher prevalence of thermophilic and mesophilic spore-formers compared with acid whey and WPC80. Three of the *B.*

licheniformis isolated in the present trial grew and formed biofilm at both 30°C and 55°C. Facultative thermophiles in dairy processing belong to the *Bacillus* genus and include species such as *B. coagulans*, *B. licheniformis* and *B. pumilus* that are able to grow at both mesophilic and thermophilic temperatures (Burgess et al, 2013; Flint et al, 2011a; Flint et al 2011b).

The second most isolated *Bacillus* species in the present study was *B. cereus*/*B. thuringiensis* with 9 isolates. *B. cereus* is capable of producing emetic and diarrhoeal toxin that can cause to food poisoning (Burgess et al, 2013; De Jonghe et al, 2010). The next most isolated species were *B. subtilis* and *B. pumilus*. These three *Bacillus* species are also common contaminants in dairy products (Masiello et al, 2014; Pasvolosky et al, 2014). Certain mesophilic *Bacillus* species. (e.g., *B. subtilis*, *B. pumilus*, and *B. licheniformis*) can also grow at temperatures that are used to detect and enumerate thermophilic organisms (i.e., 55°C) (Yuan et al, 2012). In one recent trial, WPC80 contained thermophilic spore counts detected by direct plating (Watterson et al, 2014). Three *Lactobacillus plantarum* isolates were also isolated from the Mozzarella WPC80 samples. This is not the first time that *Lactobacillus* species have been associated with whey as Tang et al, (2011) isolated *Lactobacillus* species from the permeate side of UF membrane used in a whey processing plant.

Based on the 16s rDNA gene sequence analysis (Table 3.6), one isolate was identified as *P. glucanolyticus*. This is the first time that this bacterium has been isolated from Mozzarella WPC80 and there are no records of this bacterium being isolated in New Zealand. It was identified using 16S rDNA sequence with confident level 99% (BLAST) and 94% (Seqmatch) and on the basis of a biochemical profile obtained by the use of API 50CHB (bioMérieux). The result was two possible identities for the *P. glucanolyticus*. There was a 78.7% similarity to *P. amylolyticus* and 19.0% similarity to *P. glucanolyticus* based on the API 50CHB test, demonstrating the difference between biochemical and molecular typing methods.

The *P. glucanolyticus* was observed using SEM (Figure 3.4) and the intercellular properties of the cells together with the spores were observed using TEM by Manawatu Microscopy Imaging Centre. The cells were long (> 3.0 µm) and thin (<

0.9 mm) and produced oval terminal spores that markedly distended the sporangium. Interestingly, their spores portrayed a very unique shape, different from spores of *Bacillus* species (Figure 3.5).

This bacterium formerly included in the genus *Bacillus* is a facultative anaerobic, long, thin rod-shaped bacterium with terminal spore formation and may be isolated from various soils. *Paenibacillus* species are psychrotrophic spore-forming bacteria that have been isolated from farm environments and raw and pasteurised milk and have the potential to cause spoilage (Lorentz et al, 2006; Ferrand et al, 2013). Lorentz et al, (2006) studied the antimicrobial activity by *Paenibacillus* species against bacteria, filamentous fungi and yeast isolates with the purpose of finding new bacteria for microbiological control. His findings noted that *P. glucanolyticus* produced antibiotic peptides that inhibited *Xanthomonas axonopodis* growth with inhibition zones from 12 to 18 mm. Ferrand et al, (2013) published a case report on *P. glucanolyticus* infection in a 65-year old patient with type 2 diabetes who developed a cardiac device-related endocarditis. This is believed to be the first ever report associating *P. glucanolyticus* with human infection.

3.5 CONCLUSIONS

Mesophilic bacteria, in particular, *B. licheniformis*, may predominate in Mozzarella WPC80 contributing to aerobic plate counts close to or exceeding specification limits. Preliminary attempts to identify the contaminants using biochemical tests gave an indication to bacterial identity and later this was confirmed by bio-molecular tests. Both biochemical and bio-molecular were in agreement in this study. One bacterium, *P. glucanolyticus* which recorded as the first *Paenibacillus* species isolated from Mozzarella WPC80 in New Zealand should be highlighted as it is a psychrophilic, spore forming bacteria that can potentially thrive during cold UF process. Dairy practice should include a lower incubation temperature than 30°C to enable isolates of this bacterium to be detected from routine inspection of their finished product. However, *B. licheniformis* is the predominant isolate and this cannot thrive in the temperatures used for UF process. The growth of *B. licheniformis* in other parts of a whey manufacturing plant will be the focus of this study.

CHAPTER 4

ATTACHMENT AND BIOFILM FORMATION BY *B. licheniformis*

4.1 INTRODUCTION

Contamination of dairy plants by *Bacillus* species can cause product quality problems as their spores cannot be destroyed by heat treatment such as pasteurisation or thermalisation (De Jonghe et al, 2010). Bacteria contaminating dairy manufacturing plant are often growing as a biofilm (Flint, 1998; Flint et al, 2000; Tang et al, 2009). Initially, UF process was thought to be the most likely source of biofilm and product contamination in a WPC manufacturing plant however the results identifying the predominant contaminant from Chapter 3, suggest this is unlikely. Other surfaces in the WPC manufacturing plant will now be considered as the site for biofilm growth. These will include heat exchangers (SS surface) used to treat the whey before WPC manufacture (thermalisation), the preheaters for the concentrated whey before evaporation and the evaporators used to concentrate the whey before drying.

The conditions at these points in the manufacturing process such as temperature, water activity, whey protein, salt composition and lactose content influence biofilm formation and will determine the most likely zone for biofilm growth. All these factors will vary during the process and are likely to have an influence on biofilm development. For example, Somerton et al, (2013) found that changes in salt concentration influenced the attachment of *Geobacillus* and *Anoxybacillus* species to SS surfaces. Understanding the source of contamination and development of *B. licheniformis* biofilms will be important in developing methods to control or prevent biofilm formation and the contamination of WPC. This chapter aimed to investigate the attachment and biofilm formation abilities by *B. licheniformis* isolated from Mozzarella WPC80 samples.

4.2. MATERIALS AND METHODS

4.2.1 Source of strains

Thirty three *B. licheniformis* strains isolated from WPC80 samples as retrieved from WPC80 samples in Chapter 3, were used in this study. The isolates were maintained on nutrient agar slants at 4°C and in glycerol beads stock at -80°C.

4.2.2 Attachment and biofilm screening

A microtitre plate assay (Oh et al, 2007) was used to determine the ability of isolates to attach (adhere to a surface) and grow (reproduce on a surface) and form biofilm based on crystal violet (CV) absorbance. A sterile 96-well flat-bottomed polystyrene microtitre plate (Falcon® 96, 35 3072, Becton, Dickinson & Company, USA) was filled with 230 µL of TSB. Three wells were filled with TSB only as negative controls. Overnight culture (20 µL) was added into the test wells, three wells per culture, and the plates were incubated aerobically for 1 h for attachment studies at 30°C and 55°C to reflect the temperatures used to isolate them from WPC80 powder. A time of 1 h was selected to ensure irreversible attachment of the cells to the surface with minimal opportunity for growth.

For the biofilm study, overnight culture (20 µL) was added into the test wells, three wells per culture, and the plates were incubated 24 h at 30°C, 37°C and 55°C. This reflected the temperatures used to isolate these bacteria but also the best growth temperature for *B. licheniformis* and to reflect temperatures in different parts of the manufacturing plant. A time of 24 h was considered sufficient to allow biofilm to develop.

After incubation, the contents of the microtitre plates were removed by inverting the plates, and then the wells were washed three times with 300 µL of sterile distilled water. The remaining attached cells were fixed with 250 µL of pure methanol per well for 15 minutes. The liquid was discarded and the microtitre plates air-dried. The microtitre plate wells were stained with 250 µL of 0.5% (w/v) CV for five minutes. The excess stain was rinsed off by placing the microtitre plate under running

distilled water. After the microtitre plates were air-dried, the dye bound to the adherent cells was re-solubilised with 250 μ L of 33% (v/v) glacial acetic acid per well. The optical density (OD) of each well was measured at 570 nm using an automatic 96-well microplate reader (BMG Labtech Spectrostar microplate reader, Bio-Tek Instruments, INC, Winooski, VT, USA). The biofilm formation of each *B. licheniformis* isolate (the predominant isolate) was tested using the microtitre plate assay with the strength of biofilm formation based on the following range of OD values based on (Oh et al, 2007). The cut-off OD (OD_c) was defined as three SDs above the mean OD of the negative control. Strains were classified as follows: OD < OD_c = no biofilm former, OD_c < OD < (2 x OD_c) = moderate biofilm former and OD > (2 x OD_c) = strong biofilm former.

4.2.3 Biofilm formation on SS using three different concentrations of reconstituted whey

Reconstituted whey (RWPC80) medium and RWPC80 with lactose and minerals (artificial whey permeates) were prepared in concentrations of 1%, 5% and 20% (pH 5.7 - 6.5) as described in Tang, (2011) to copy the composition of whey protein concentration in three different stages of UF process. The minerals of artificial whey permeate listed in Table 4. 1, were prepared by mixing the listed minerals in deionised water to make up 1 L (pH 6.0 - 6.5) and sterilised. WPC80 powder was sterilised by gamma irradiation (25 kGy, MSD, Upper Hutt, Wellington, New Zealand) prior to medium preparation to avoid any competition from the natural microflora of whey. An initial inoculum of 6 log₁₀ CFU/mL of one *B. licheniformis* isolate (E30C11) was cultured in each RWPC80 concentration. SS is the most common material used in food processing plant surfaces as it is corrosion resistant and easy to clean as described by Holah & Gibson, (2000). A 304 grade SS coupon (1 cm²) was used as the surface for this *B. licheniformis* biofilm study. Each RWPC80 solution contained one SS coupon (1 cm x 1 cm) submerged into the medium and incubated at 37°C for 24 h. The SS coupon was aseptically removed from the medium and rinsed twice using sterilised deionised water and the cells that had colonised the surface were

removed by bead beating using 3g of sterile glass beads (d= 2 mm) by vortex mixing at high speed for 2 minutes. The detached cells were serially diluted to 10^{-3} and MPCA was used for pour plating in duplicate and incubated at 37°C for 24 to 48 h. Colonies forming on MPCA were counted. Mean and standard errors were taken from duplicates.

Table 4.1 The mineral content of artificial whey permeate.

Mineral	Amount	Source
KOH	52.7 mL 2 mol per litre	BDH, Poole, England
$C_6H_5O_7Na_3 \cdot 2H_2O$ (Trisodium citrate dehydrate)	24.29 g	Merck KGaA, Darmstadt, Germany
$C_6H_5O_7K_3 \cdot 2H_2O$ (Tripotassium citrate dehydrate)	4.99 g	UNIVAR, Auckland, NZ
$CaCl_2 \cdot 2H_2O$ (Calcium chloride)	3.67 g	Biolab, Clayton, Australia
$MgCl_2 \cdot 6H_2O$ (Magnesium chloride)	5.85 g	J.T. Baker, Phillipsburg, Mexico
KH_2PO_4	23.3 g	Merck KGaA, Darmstadt, Germany
H_2SO_4	17.1 mL 3 mol per litre	Biolab, Clayton, Australia

4.2.4 Biofilm formation on SS using three different media

An initial inoculum of $6 \log_{10}$ CFU/mL of *B. licheniformis* (diluted and confirmed with plate counts) was grown in three different media; tryptic soy broth (artificial media), 1% RWPC80 and 1% RWPC80 with additional lactose and minerals (artificial whey permeates) to mimic the composition of whey protein concentration

in the first stage of UF. Each of the three media preparations contained one SS coupon and was treated as in section 4.2.3.

4.2.5 Biofilm formation on SS with effects of individual cations

One *B. licheniformis* isolate derived from each WPC80 powder samples (A30C11, B55C11, C55C02, D55C03, E30C11 and F30C02) were chosen to study on the effects of individual cations; calcium (Ca^{2+}) and magnesium (Mg^{2+}). Each of these stock cations was prepared by dissolving 3.67g Calcium chloride ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) in 1 L of sterile deionised water; 5.85 g Magnesium chloride ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) in 1 L of sterile deionised water. These individual cations stocks were used to replace the artificial whey permeate (used previously) in 1% RWPC80 with lactose media for biofilm study on effects of these individual cations. The concentrations of individual cations were 0.025 mol per L for Ca^{2+} and 0.029 mol per L for Mg^{2+} and substituted the whey permeates as 6% from the 1% RWPC80 medium to replicate the concentration of whey medium in the formulation. The amount of each cation used were 200 mg/L. In real scenario, the value of minerals such calcium and magnesium are 423 mg/100g and 50 mg/100g respectively (Appendix 5).

4.2.6 Statistical analysis

Statistical calculation was performed on the log density values with the mean and standard deviations produced from the log density of 3 replicates. Anova one-way (Excel, 2016) was used to analyse the variance of individual cations affecting biofilm formation of *B. licheniformis* isolate on SS.

4.3 RESULTS

4.3.1 Attachment and biofilm study of *B. licheniformis* isolates

The attachment of a subset of mesophilic thermotolerant *B. licheniformis* isolates was determined using a microtiter plate with TSB as the growth medium. Successful attachment was based on an OD that exceed 3 x SD of negative control (TSB). Two temperatures were selected for this preliminary attachment study; 30°C and 55°C. Figure 4.1 showed 3 of 11 *B. licheniformis* did not attach on the microtitre plate surfaces at 30°C after 1 h of incubation as the reading was below the negative control. Ten selected *B. licheniformis* isolates were able to attach on the microtitre plate surface at 55°C after 1 h of incubation in a static condition (Figure 4.2).

The biofilm formation of each *B. licheniformis* isolate was tested using the microtitre plate assay with the strength of biofilm formation based on the following range of OD values (Table 4.2) (Oh et al, 2007). In this study, three temperatures were used; 30°C, 37°C and 55°C on the basis of the fact that *B. licheniformis* as it is considered as mesophilic/thermo-tolerant bacteria. The cut-off point (ODc) value at 570 nm was 0.5.

The biofilm formation by 33 *B. licheniformis* and the amount of biofilm at three different temperatures are given in Figure 4.3 and Table 4.3 respectively.

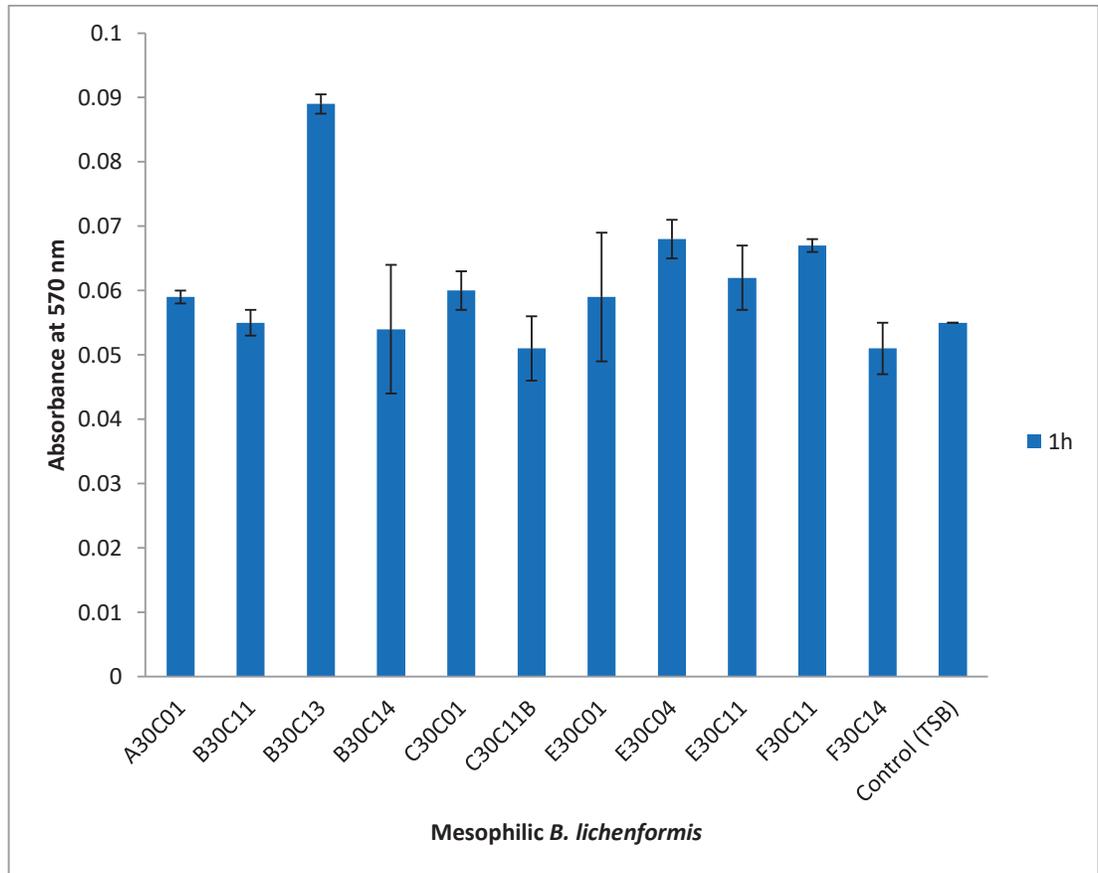


Figure 4.1 *B. licheniformis* attachment on a microtitre plate surface after 1 h of incubation at 30°C. Values and error bars represent the mean and standard deviation respectively for 8 independent replicates.

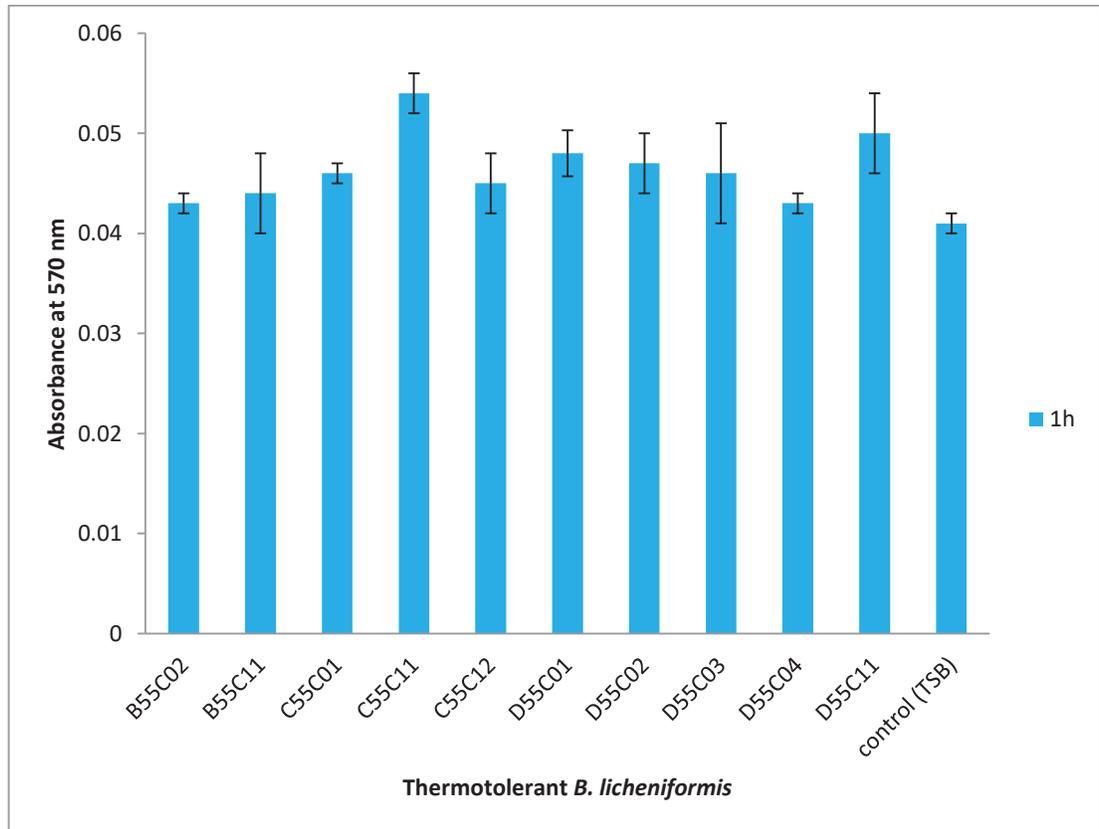


Figure 4.2 *B. licheniformis* attachment on microtitre plate surface after 1 h of incubation at 55°C. Values and error bars represent the mean and standard deviation respectively for 8 independent replicates.

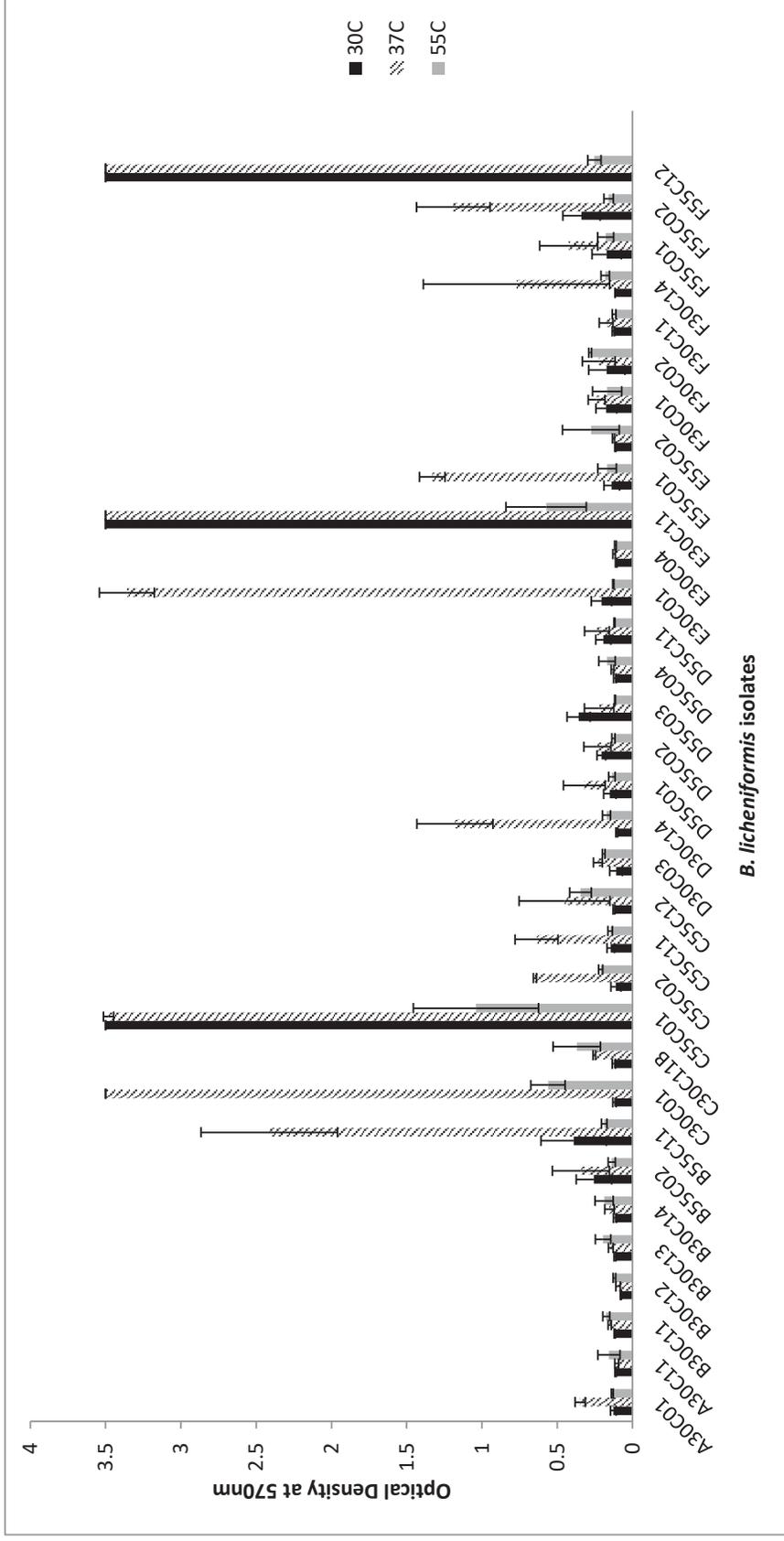


Figure 4.3 Biofilm formation by 33 *B. licheniformis* isolates in TSB at three different temperature profiles. Values and error bars represent the mean and standard deviation respectively for 20 independent replicates.

Table 4.2 Biofilm categories determined by OD absorbances at 570 nm.

Range	Indication
$X \leq 0.5$	No biofilm
$0.5 \leq X \leq 1.0$	Moderate biofilm
$X \geq 1.0$	Strong biofilm

Table 4.3 Summary of frequency of *B. licheniformis* biofilm categories at three different temperatures.

Tryptic Soy Broth & Temperature	Biofilm formation		
	None	Moderate	Strong
30°C	30	0	3
37°C	21	3	9
55°C	30	2	1

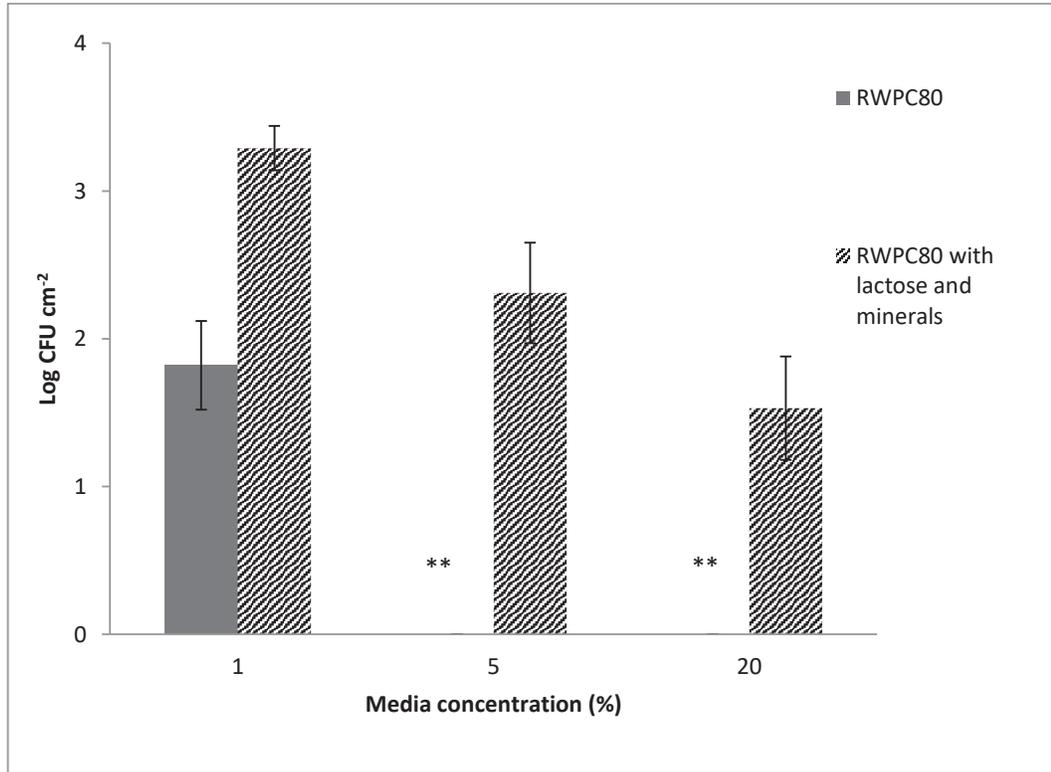


Figure 4.4 The 1% reconstituted WPC80 with and without lactose and minerals was the best medium for *B. licheniformis* E30C11 isolate to form biofilm on SS after 24 h incubation at 37°C compared to 5% and 20% RWPC80. The ** in the graph indicates no growth/biofilm formation. Values and error bars represent the mean and standard deviation respectively for 3 independent replicates.

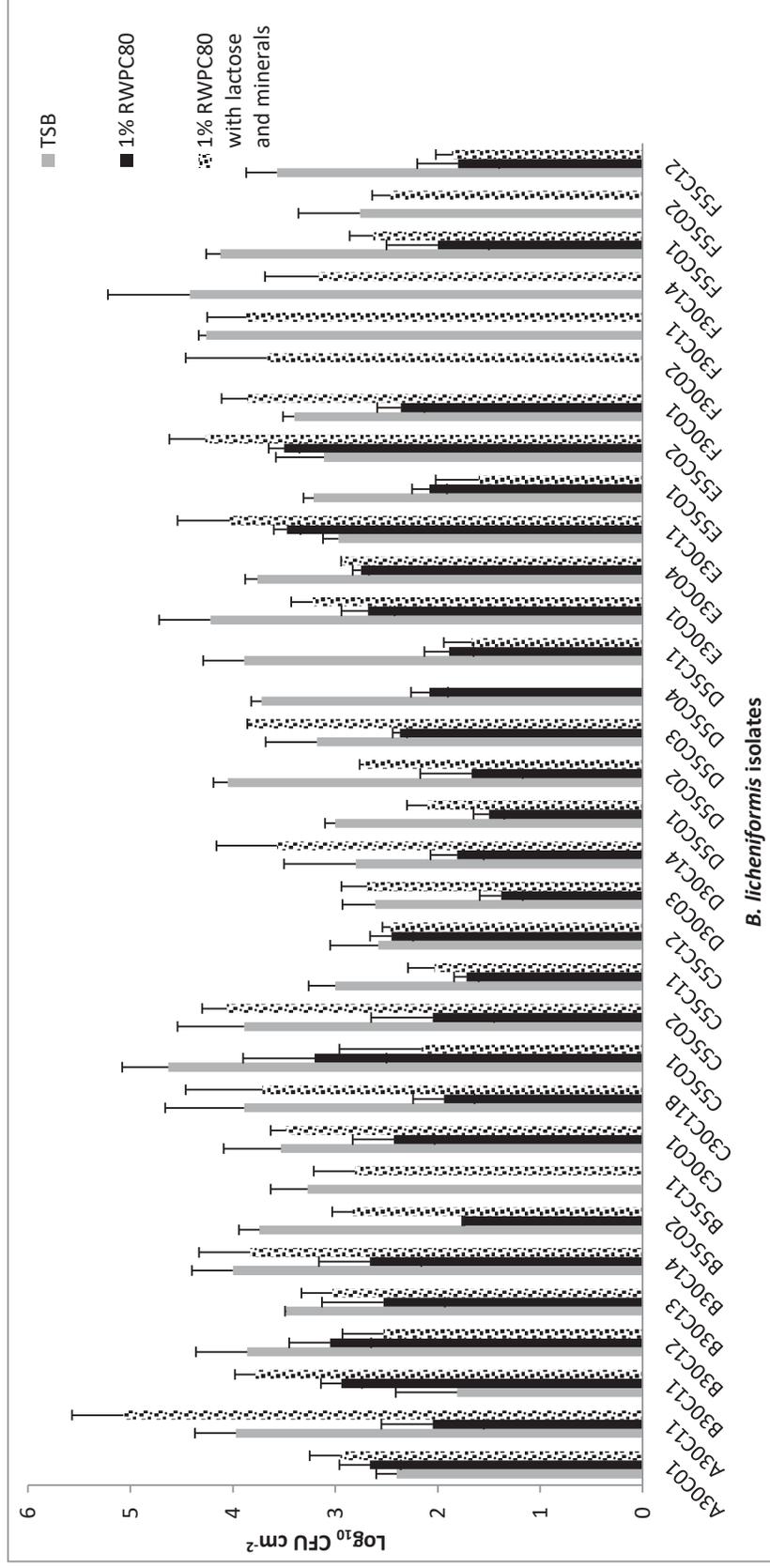


Figure 4.5 Biofilm formations by 33 *B. licheniformis* isolates on SS submerged in three different mediums at 37°C for 24 h. These 33 *B. licheniformis* isolates are from six Mozzarella WPC80 powder abbreviated A, B, C, D, E and F. Values and error bars represent the mean and standard deviation respectively for triplicate assays (N=3).

Prior to using SS as surface for biofilm study, biofilm formation by 33 *B. licheniformis* in WPC was assayed using the microtitre plate assay at 37°C but the CV absorbance readings were high (3.5 and above) at 570 nm (results not included). Protein precipitation was observed on the bottom and side of the microtitre plate wells after 24 h of incubation in 1% RWPC80 and 1% RWPC80 with lactose and minerals.

Three different RWPC80 concentrations (1%, 5% and 20%) were tested for *B. licheniformis* biofilm formation (Figure 4.4). The 1% RWPC80 was the best (with and without lactose and minerals) medium for *B. licheniformis* E30C11 to form biofilm on SS after 24 h incubation at 37°C.

Nine isolates that formed strong biofilm in the microtitre plate assay using TSB medium at 37°C showed strong biofilm on SS (\log_{10} CFU cm^{-2}) (Figure 4.5). Three isolates (C55C02, C55C11 and F30C14) that formed moderate biofilm in the microtitre plate assay in TSB, produced strong biofilm on SS in TSB but not in 1% WPC80 medium. Twenty one isolates that did not produce biofilm in the microtitre plate assay in TSB medium at 37°C, showed strong biofilm formation on SS in TSB medium. Seventeen isolates showed better biofilm with the enrichment of lactose and minerals. Four isolates from product batch F (Chapter 3) showed biofilm formation in 1% RWPC80 with the enrichment of lactose and minerals but none in 1% RWPC80 without minerals.

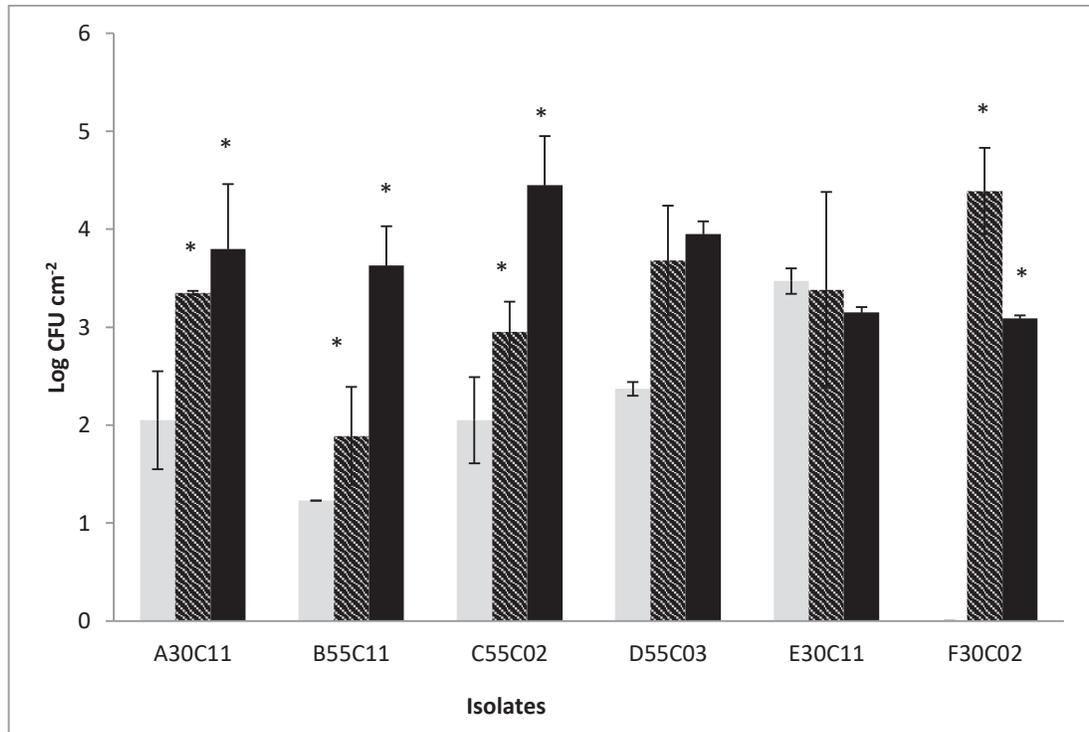


Figure 4.6 Biofilm formation by six *B. licheniformis* isolates from each isolate on SS in 1% RWPC80 with individual cations (Ca^{2+} and Mg^{2+}) for 24 h at 37°C. Values and error bars represent the mean and standard deviation respectively for triplicate samples in two separate runs. The asterisk (*) represent the significant differences at $p < 0.05$.

Grey bar: 1% reconstituted WPC80 without added minerals

Stripe bar: 1% reconstituted WPC80 with calcium

Black bar: 1% reconstituted WPC80 with magnesium

The individual effects of Ca^{2+} and Mg^{2+} were examined on 6 *B. licheniformis* isolates and there were significant different ($p < 0.05$) by one-way ANOVA between these two ions on tested strains and their effect on *B. licheniformis* biofilm formation on SS at 37°C. Magnesium resulted in the most biofilm formation of A30C11, B55C11, C55C02 and D55C03 while calcium resulted in the most biofilm of E30C11 and F30C02 isolates (Figure 4. 6).

4.4 DISCUSSION

There is a lack of information on the biofilm forming ability of *B. licheniformis* and the importance of biofilms of this bacterium in the contamination of dairy products. *B. licheniformis* are thermo-tolerant bacteria with the ability to attach and form biofilm. This biofilm is a potential source of microbial contamination of product, resulting in microbial specification limits being exceeded. Spoilage of product may result from the metabolic products, such as enzymes, produced by these biofilms. These bacteria are therefore likely to form biofilm on manufacturing plant surfaces where they can grow and then be released into the whey being processed.

Three *B. licheniformis* isolates (C55C01, E30C11 and F55C12) produced strong biofilm $OD_{570nm} \geq 0.5$ at 30°C with an O.D at 3.5 (the highest absorbance from microtitre plate reader) (Table 4.2). Nine *B. licheniformis* isolates produced strong biofilms at 37°C suggesting 37°C is the best temperature for *B. licheniformis* to form biofilm (Figure 4.3).

B. licheniformis is known as thermotolerant bacteria and able to grow at temperatures up to 55°C (Dhakal, 2013; Smithers, 2008). However, in this study, most of the *B. licheniformis* isolated from 6 different whey samples did not produce biofilm at 55°C. Biofilm formation at 55°C was generally poor with only three isolates showing a tendency to form biofilm at this temperature on microtitre plate surface. High temperature will cause high solubility although calcium showed less solubility compared to the minerals (Bansal & Chen, 2006). Calcium phosphate is the main mineral deposited at 70% - 80% on SS at temperature above 110°C. Moderate biofilm formation in TSB at 37°C was produced by three *B. licheniformis* isolates. The majority of *B. licheniformis* strains were unable to produce biofilm at any of the three temperatures (30°C, 37°C and 55°C) in the TSB medium. However, they may produce biofilm under the conditions of a dairy manufacturing plant on a protein fouled surface. Most of the facultative anaerobic and spore forming *B. licheniformis* were observed to produce pellicles after 24 h incubation at 30°C, 37°C and 55°C in TSB medium.

B. licheniformis (E30C11) was able to form biofilm in a microtitre plate assay using tryptic soy broth medium at 30°C, 37°C and 55°C. This strain was selected from 33 *B. licheniformis* isolates to determine the optimum concentration of whey to promote biofilm formation (Figure 4.4). A preliminary study using microtitre plates showed protein precipitation on the microtitre plate surfaces when using RWPC80 and RWPC80 with lactose and minerals as the substrate, hence giving high CV readings at 570 nm (results not included) that reflected the protein precipitation rather than the microbial colonisation. SS was chosen as the surface to study biofilm formation as it is a common surface material used in dairy manufacturing plant. E30C11 produced $1.82 \log_{10} \text{CFU cm}^{-2}$ in 1% RWPC80 while no biofilm formation was observed at 5% and 20% of RWPC80 at 37°C for 24 h in a static environment. On the other hand, E30C11 showed more biofilm in 1% RWPC80 with lactose and minerals (artificial whey permeates) with $3.29 \log_{10} \text{CFU cm}^{-2}$. There was biofilm formation observed in 5% and 20% RWPC80 with lactose and minerals with $2.31 \log_{10} \text{CFU cm}^{-2}$ and $1.53 \log_{10} \text{CFU cm}^{-2}$ respectively (Figure 4.4).

Thermo-tolerant *B. licheniformis* can survive pasteurisation. They can grow in subsequent manufacturing steps on equipment such as cream separators, heat exchangers, preheaters and evaporators during milk powder production (Dhakal, 2013). The plate heat exchanger temperature profile is presented in Appendix 1. The temperature ranges between 37°C to 77°C with cold and heated whey in the regenerative zones of the plate heat exchanger. Scott et al, (2007) and Murphy et al, (1999) concluded that spore forming bacteria contamination occurred in evaporators after a 20 h run time. Based on the results in this present study (Figure 4.4), *B. licheniformis* contamination most likely occurs before UF as biofilm counts were highest at 1% RWPC80 and the role of lactose and minerals, removed during dialysis in UF will have an impact on *B. licheniformis* biofilm on SS. The water activity at pre-UF sites is expected to have minimal effect on *B. licheniformis* biofilm growth however, after evaporation the lower water activity and higher total solids is expected to limit microbial growth. Figure 4.4 showed E30C11 strain able to form biofilm in 1%, 5% and 20% RWPC80 medium with lactose and minerals.

As 1% RWPC80 was favoured by E30C11 to form biofilm, further study was done using the 33 *B. licheniformis* isolates with three different media; 1% RWPC80, 1% RWPC80 with lactose and minerals and artificial medium (TSB). There were variations in biofilm formation by *B. licheniformis* on SS with each medium (Figure 4. 5). Twenty one *B. licheniformis* were non-biofilm formers using TSB as medium at 37°C using the microtitre plate assay. However, only one *B. licheniformis* isolate (F30C02) from 33 *B. licheniformis* did not form biofilm on SS whereby the rest produced between 1.81 – 4.63 log₁₀ CFU cm⁻² in TSB. Biofilm formation by 28 *B. licheniformis* on SS resulted in 1.38 to 3.47 log₁₀ CFU cm⁻² using 1% RWPC80 as media. Interestingly, five isolates from the same batch of WPC (B55C11, F30C02, F30C11, F30C14 and F55C02) did not produce any biofilm in 1% RWPC80 but produced between 2.46 to 3.87 log₁₀ CFU cm⁻² in 1% RWPC80 with lactose and minerals which indicates the importance of lactose and salts such as calcium and magnesium for these strains to form biofilm in a whey environment. The other *B. licheniformis* isolates showed increased biofilm count (1.6 to 5.07 log₁₀ CFU cm⁻²) with 1% RWPC80 with lactose and minerals substrates except for D55C04 which did not produce biofilm. Dhakal et al, (2013) reported that *B. licheniformis* occurs in milk and dairy products as many different genotypes that vary in the term of heat resistance and biofilm capabilities. This could explain what was seen in the present investigation.

Subsequent tests for biofilm formation on SS showed an increased frequency of biofilm formation with 32/33 strains forming biofilm in TSB at 37°C. This demonstrates the limitation of the microtitre plate assay for screening for biofilm formation. This suggests that biofilm growth of *B. licheniformis* favours a SS surface. The results of biofilm formation on SS coupons in this study are expected to differ from the actual plate heat exchanger in the industry as the experiments were conducted in batch not continuous condition. However, the role of *B. licheniformis* biofilm formation on SS as source of product contamination cannot be ruled out.

Ions have been reported as having a role in biofilm formation. Bellona & Drewes, (2005) reported that positively charged ions such as sodium, calcium, magnesium, and cationic surfactants can bind and neutralise negatively charged

surfaces and enhance bacterial attachment to a filter membrane. The role of precipitated minerals in biofilm development in WPC80 production is aiding the conditioning film formation (Zaky et al, 2012; Tang et al, 2015; Teh et al, 2015). Somerton et al, (2013) found that changes in salt concentration (calcium and magnesium) influenced the attachment of *Geobacillus* and *Anoxybacillus* species to SS surfaces. It has either positive (enhance) or negative (inhibit) biofilm development depending on the strains and concentration (Somerton et al, 2015). In this chapter, 1% RWPC80 with additional lactose and minerals (calcium and magnesium) did increase the magnitude of biofilm formation by *B. licheniformis* isolates by 1-2 fold except for one strain (D55C04) which was unable to form biofilm. Individual cations (Ca^{2+} and Mg^{2+}) at 200 mg/L in these experiments were significantly ($p < 0.05$) affecting *B. licheniformis* biofilm formation on SS with Mg^{2+} increasing the biofilm of 4 of the 6 *B. licheniformis* biofilm on SS. High minerals needed for the biofilm formation. Traces of minerals (calcium and magnesium) were recorded 423 mg per 100g and 50 mg per 100g (Appendix 5). In this study, both minerals were added at 200 mg/L using 1g of sterile WPC80 powder. On the other hand, Oknin et al, (2015) reported that magnesium ions mitigate biofilm formation by *Bacillus* species. They studied the effects of Mg^{2+} on biofilm formation of *B. subtilis* in chemically defined medium in flasks. The biofilm formation was quantified by the production of pellicles on the surface of the medium and images under confocal laser microscopy. This is a general result as different bacteria and methods were used, and different surfaces were compared in the present study.

4.5 CONCLUSIONS

B. licheniformis is a contaminant of WPC. Biofilm formation by *B. licheniformis* isolates from WPC varies between strains and is dependent on the substrate surface with SS supporting more growth than polystyrene. There were limitations on microtitre plate assay to study biofilm formation on polystyrene where protein precipitation was developed instead of bacterial biomass and hydrophobicity interaction cannot be ruled out.

Whey concentration of 1% RWPC80 with and without lactose and minerals supports biofilm formation of *B. licheniformis* with most biofilm forming in the presence of lactose and minerals. The ability of *B. licheniformis* to form biofilm has been demonstrated and this is important as this is the most likely source of contamination of high numbers of *B. licheniformis* and their enzymes in product.

CHAPTER 5

CHARACTERISATION OF ***B. licheniformis***

5.1 INTRODUCTION

B. licheniformis biofilm growth was tested at 30°C, 37°C and 55°C in Chapter 4. The origins of Mozzarella WPC80 powders were from cold UF (10°C) concentration and dialysis, followed by evaporation and spray drying. The hypothesis in Chapter 1.3 was that the contaminants formed biofilms on UF membrane as it represents the largest surface area in the WPC plant. Therefore, in this chapter, growth at 10°C was tested.

Lactose is a major carbohydrate in whey making up approximately 5% of the total of cheese whey. It is postulated that microorganisms that contaminate whey use lactose as their carbohydrate source using β -galactosidase to break down the lactose into fermentable sugars (Geiger et al, 2016; Juajun et al, 2011). *B. licheniformis* is one of the microorganisms commonly found in whey and is regarded as a potential cause of spoilage through the production of heat stable enzymes such as protease and lipase. Microbial spoilage enzymes produced by bacteria may result in product defects in terms of organoleptic and functionality changes. There is some concern that *B. licheniformis* may be involved in nitrate reduction resulting in high nitrite levels in whey products. The importance of enzyme production and nitrate reduction due to *B. licheniformis* in whey is unknown. The percentage of isolates of *B. licheniformis* from whey, producing protease or lipase and reducing nitrate that may affect the quality and safety of whey products respectively is unknown.

The bacterial contaminants, such as *B. licheniformis*, in many dairy products are believed to originate from biofilms on dairy manufacturing plant surfaces. These dairy biofilms contain extracellular polymeric substances (EPS) and milk residues such as protein and calcium phosphate (Flint, 1998). The EPS is an integral part of a biofilm. EPS consists of polysaccharides, eDNA, proteins and lipids played vital role in the adhesion and aggregation of bacterial cells. In addition, EPS form a protective barrier and provide a nutrient source for the biofilm communities as energy storage (Simões et al, 2010).

Dhakal, (2013) studied the heat resistance of *B. licheniformis* spore in milk and Husmark, (1993) investigated the high temperature (80°C) effects towards *B.*

licheniformis spore hydrophobicity. However, the heat resistance of *B. licheniformis* vegetative cells has not been reported. It is important to understand the heat resistance of *B. licheniformis* vegetative cells towards high temperature such as pasteurisation in dairy processing.

This chapter aimed to characterise 33 *B. licheniformis* isolates from whey product manufacture by studying their heat resistance, ability to ferment lactose, produce enzymes, convert nitrate to nitrite and produce EPS and biosurfactant that will contribute to their survival through dairy manufacture and determine their influence on product quality.

5.2 MATERIALS AND METHODS

5.2.1 Growth at 10°C

Twenty μL of 33 *B. licheniformis* isolates were inoculated in 230 μL TSB (pH 7.0 ± 0.2) in 96-well microtitre plate wells in 8 replicates and incubated at 10°C for 24 h. Three wells of microtitre plate were filled with sterile TSB as negative controls. Growth was measured at 570 nm using a microplate reader (Spectrostar Nano, BMG Labtech, Auckland, New Zealand). Similar growth trials were done in 1% RWPC80 with lactose and minerals, except cultures were prepared in 25 mL bottles (2 mL inoculum in 23 mL medium) instead of the microtitre plates and incubated at 10°C for 24 h. *B. licheniformis* growth was determined by serial dilution and colony formation on MPCA after incubation for 24 at 10°C . The purpose of these trials was simply to indicate the potential for growth at 10°C in either medium rather than do a direct comparison of the extent of growth.

5.2.2 Lactose fermentation

In order to determine the ability of *B. licheniformis* isolates to ferment lactose, phenol red broth was prepared following the instructions of the manufacturer (Merck, BDH, Palmerston North, New Zealand), distributed into test tubes and autoclaved. Lactose 10% m/v stock solution was prepared by adding 10.00 ± 0.001 g of lactose monohydrate into 100 mL beaker and made up the volume by adding sterile deionised water and mixed well. The lactose stock solution was sterilised by filtration using a 0.2 μm membrane filter and stored at 4°C before use. An amount of 1 mL of lactose stock solution was pipetted into a test tube containing 9 mL of sterile phenol red broth to make a 1% lactose solution. Lactose fermentation is indicated by the change in phenol red from red to yellow, indicating acid production.

5.2.3 Protease and lipase enzyme

To determine the ability of 33 *B. licheniformis* to produce protease, a single colony was streaked onto calcium caseinate agar (Composition: meat peptone, sodium chloride, beef extract, casein, calcium hydroxide, calcium chloride, bacteriological agar) (Conda, Spain). The plates were incubated at 37°C and checked daily for 2 days for the presence of clear zones along the streaked line.

To determine the ability of 33 *B. licheniformis* isolates strain to produce lipase, a single colony was streaked onto Tributyrin agar (Fort Richard Laboratories Ltd, NZ) and spirit blue agar (Composition: Pancreatic digest agar of casein, yeast extract, agar, spirit blue) (Difco™, Becton Dickinson, USA) with soy oil addition. The plates were checked between 24 to 48 h for the presence of clear zones along the streaked lines.

5.2.4 Nitrate conversion

B. licheniformis isolates were incubated in nitrate broth (9 g/L) at 37°C for 24 h. One drop of sulfanilic acid and one drop of a α -naphthylamine were added into each broth. A colour change from yellow (nitrate broth) to red as a result from the reaction between the added two reagents, indicates nitrate reduction. If the yellow colour of nitrate broth remains after the addition of two reagents, half teaspoon of zinc was added into the broth. This step was a confirmatory test which zinc is able to catalyse the reduction of nitrate to nitrite. No colour change means *B. licheniformis* reduced nitrate completely to ammonia and nitrogen gas. Therefore, no colour change at this point is a positive result. If the broth colour changed to red, it indicated negative results as the nitrate was reduced to nitrite by zinc and not by *B. licheniformis*.

5.2.5 Haemolysis on Columbia sheep blood agar

To indicate the production of lichenysin, haemolysis of sheep blood is used (Madslie et al, 2013). *B. licheniformis* isolates were streaked on to Columbia blood sheep agar plates with 5% sheep blood (Fort Richard Laboratories Ltd, NZ) and incubated at 37°C for 24 to 48 h. The presence or absence of clear zone surrounding the colonies (haemolysis) was recorded after 24 to 48 h.

5.2.6 Lichenysin synthetase gene (Lch AA)

Sheep blood haemolysis is an indicator of lichenysin production. However, haemolysis could also be due to other factors. To provide further evidence for the ability to produce lichenysin, a PCR test was used to determine whether the *B. licheniformis* isolates contained the Lch AA gene that is one of the genes that are responsible for the secretion of lichenysin. Lichenysin synthetase A (Lch AA) was indicated by PCR amplification using the following primers;

F: 5' –ACTGAAGCGATTCGCAAGTT- 3'

R- 5'- TCGCTTCATATTGTGCGTTC- 3'

The PCR conditions were; five minutes of denaturation at 95°C, 35 cycles denaturation at 95°C for 10s, annealing at 56°C for 10s, extension at 72°C for 30s and elongation step at 72°C for 7 minutes (Madslien et al, 2013). The PCR products were visualised (E-Gel iBASE™, Invitrogen) using pre-made 2% agarose electrophoresis gel (E-Gel® EX with SYBR Glod II) then visualised under UV Transilluminator, UVP, Inc (Chromator-Vue, San Gabriel, Californis, USA) and UVITEC (Cambridge, UK). A negative control (no template) was used.

5.2.7 Pellicle formation and Congo red binding assay

Pellicle formation and Congo red binding were used to indicate EPS production. The production of pellicles indicates the overproduction of polysaccharides by *B. licheniformis* and is indicative of biofilm formation. Pellicle formation was studied by inoculating 1 loop of *B. licheniformis* single colony in 10 mL sterile TSB in a 25 mL universal bottle (25 mm by 70 mm). The bottles were incubated in a static condition at 37°C for 2 days. Pellicle formation at the air-liquid interface was visually inspected and the images were captured using a digital camera (Nikon, Japan).

To quantify the amount of polysaccharide produced by *B. licheniformis*, a Congo red binding assay was used. This method was adapted and modified from Ghafoor et al, (2011) and Spiers et al, (2003). Each of the 33 *B. licheniformis* isolates was inoculated into 10 mL TSB broth for 48 h at 37°C in a static condition. The bacterial mass and polysaccharides of *B. licheniformis* isolate cultures were collected by centrifugation and the supernatant was discarded. The pellet was washed by using sterile deionised water and transferred into 2 mL microfuge tubes. The pellet was re-suspended in 1 mL of 40 µg/mL Congo red in sterile deionised water and incubated for 90 minutes with shaking (37°C at 100 rpm). Bacterial mass and bound Congo red

was sedimented by centrifugation at 13 500 x g for 6 minutes. The supernatant was collected and the amount of Congo red remaining in the supernatant was determined by measuring the OD_{490nm} of the solution and the amount of EPS was calculated based on the formula below to obtain the percentage of polysaccharides produce by *B. licheniformis* strains.

$$\frac{\text{OD}_{490 \text{ control}} - \text{OD}_{490 \text{ final}}}{\text{OD}_{490 \text{ control}}} \times 100\%$$

5.2.8 Heat resistance study at 72°C, 75°C and 80°C

A single colony of selected *B. licheniformis* isolates was isolated into 20 mL TSB and incubated for 24 h at 37°C. The *B. licheniformis* cells were observed using Gram staining under light microscopy to confirm the vegetative cells and no spore production prior the heat kinetic study. An immersed coil apparatus (Coil 100, Sherwood Instruments, MA, USA) was used to heat a suspension of *B. licheniformis* culture under selected controlled temperature (70°C, 72°C and 80°C). At the beginning, the water bath attached to the coil apparatus was set to the selected temperature before use. The heating time was set to the required time intervals; 15, 30 and 60 s.

The apparatus was cleaned by injecting 20 mL of NaOH, followed by 50 mL of sterile water, then 20 mL of H₂SO₄ and 50 mL of sterile distilled water.

Ten µL of the overnight *B. licheniformis* culture approximately 8.0 ± 0.5 log CFU/mL (confirmed by plate counting on MPCA) was injected into the Coil 100. At the pre-set temperature and time interval, a small sample of heated sample ≈ 1 µL was discharged from the discharge tube into a sterile sample tube. The sample tubes were covered immediately with lids and subjected to serial dilution up until 10⁻⁶. One mL each dilution was plated in triplicate on MPCA and incubated at 37°C for 24 to 48 h.

5.3 RESULTS

5.3.1 Lactose fermentation, protease, lipase, nitrate conversion, Columbia sheep blood agar haemolysis and lichenysin synthetase gene A

The results for *B. licheniformis* growth at 10°C, lactose fermentation, protease and lipase enzymes production, nitrate conversion, haemolysis on Columbia sheep blood agar and synthetase A (lch AA gene) analysis are shown in Table 5.1. These results were compared to Bergey's Manual (Logan & Von, 2015).

Table 5.1 The characterisation of *B. licheniformis* isolates from WPC80 samples

Isolates	Growth at 10°C ^a	Lactose fermentation ^b	Protease	Lipase ^c	Nitrite Production	EPS (%) ± SD	Blood agar haemolysis	Lichenysin synthetase gene A
A30C01	-	-	+	-	+	25 ± 0.01	+	+
A30C11	-	-	+	-	+	21 ± 0.02	+	+
B30C11	-	-	+	-	+	33 ± 0.03	+	+
B30C12	-	-	+	-	+	42 ± 0.03	+	+
B30C13	-	-	+	-	+	52 ± 0.02	+	+
B30C14	-	-	+	-	+	45 ± 0.04	+	+
B55C02	-	-	+	-	+	42 ± 0.03	+	+
B55C11	-	-	+	-	+	53 ± 0.02	+	+
C30C01	-	-	+	-	+	57 ± 0.02	+	+
C30C11B	-	-	+	-	+	48 ± 0.02	+	+
C55C01	-	-	+	-	+	57 ± 0.09	+	+
C55C02	-	-	+	-	+	66 ± 0.01	+	+
C55C11	-	-	+	-	+	39 ± 0.02	+	+
C55C12	-	-	+	-	+	74 ± 0.03	+	+
D30C03	-	-	+	-	+	75 ± 0.00	+	+
D30C14	-	-	+	-	+	37 ± 0.02	+	+
D55C01	-	-	+	-	-	71 ± 0.00	+	+
D55C02	-	-	+	-	+	56 ± 0.02	+	+
D55C03	-	-	+	-	+	62 ± 0.03	+	+
D55C04	-	-	+	-	+	53 ± 0.02	+	+
D55C11	-	-	+	-	+	57 ± 0.02	+	+
E30C01	-	-	+	-	+	72 ± 0.01	+	+
E30C04	-	-	+	-	+	63 ± 0.02	+	+
E30C11	-	-	+	-	+	51 ± 0.03	+	+
E55C01	-	-	+	-	+	71 ± 0.02	+	+
E55C02	-	-	+	-	+	42 ± 0.02	+	+
F30C01	-	-	+	-	-	69 ± 0.01	+	+
F30C02	-	-	+	-	+	63 ± 0.02	+	+
F30C11	-	-	+	-	-	68 ± 0.01	+	+
F30C14	-	-	+	-	+	53 ± 0.01	+	+
F55C01	-	-	+	-	+	34 ± 0.03	+	+
F55C02	-	-	+	-	+	54 ± 0.01	+	+
F55C12	-	-	+	-	+	62 ± 0.01	+	+

^a Results from the microtitre plate reading at 570 nm and MPCA plate counting.

^b Results indicates no colour changes and gas production.

^c Results from tributyrin and spirit blue agar.

5.3.2 Pellicle formation and Congo red binding assay

The pellicle formation (air-liquid interface) and EPS production of *B. licheniformis* assayed by Congo red binding are shown in Figures 5.1 and 5.2. All of *B. licheniformis* isolates produced pellicles but the degree of pellicle formation for each strain are varied based on the Congo red staining results at OD_{490 nm}.

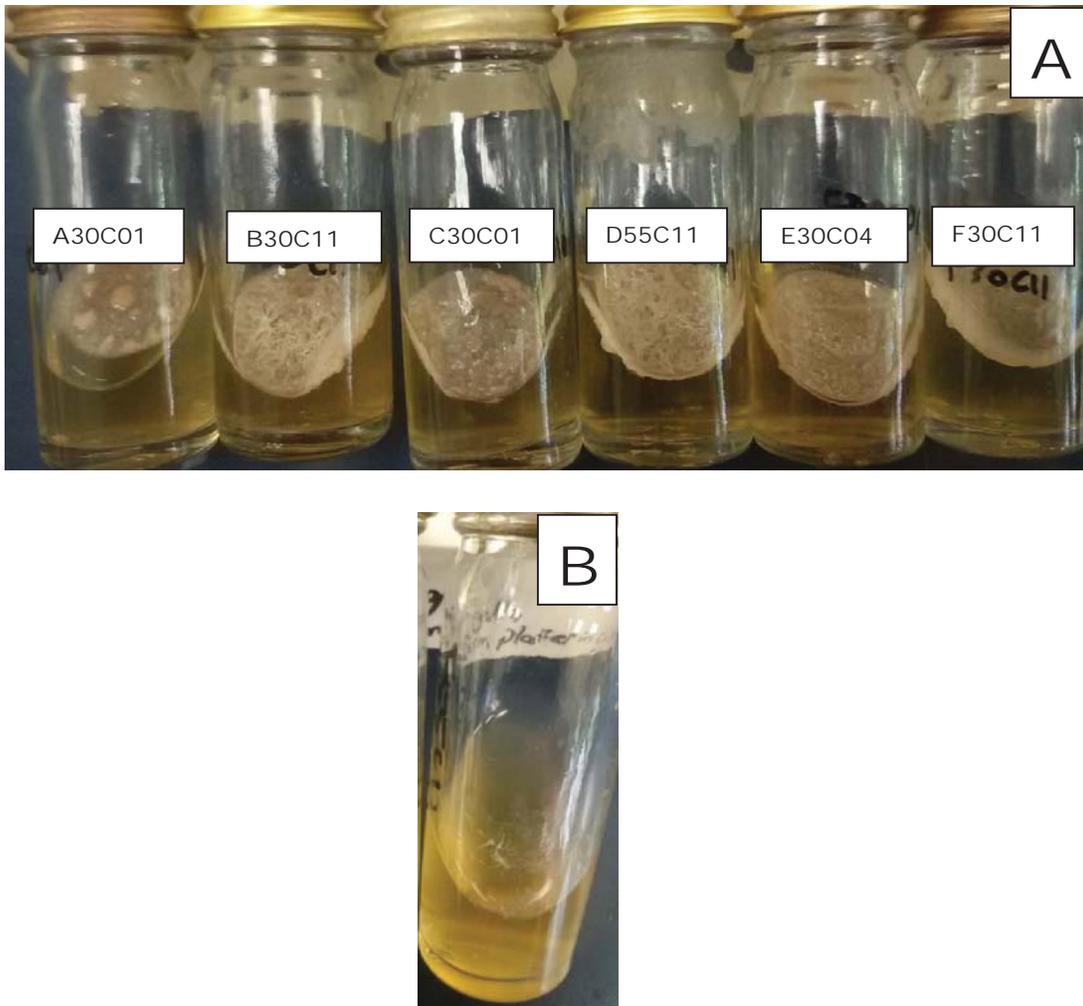


Figure 5.1 Image (A) showing pellicles (air-liquid interface) formation by six *B. licheniformis* (A30C01, B30C11, C30C01, D55C11, E30C04 and F30C11) in TSB after 48 h of incubation at 37°C (The remaining 27 *B. licheniformis* isolates pellicles formation was not shown here). Image (B) showed pellicle production by *B. subtilis* as control.

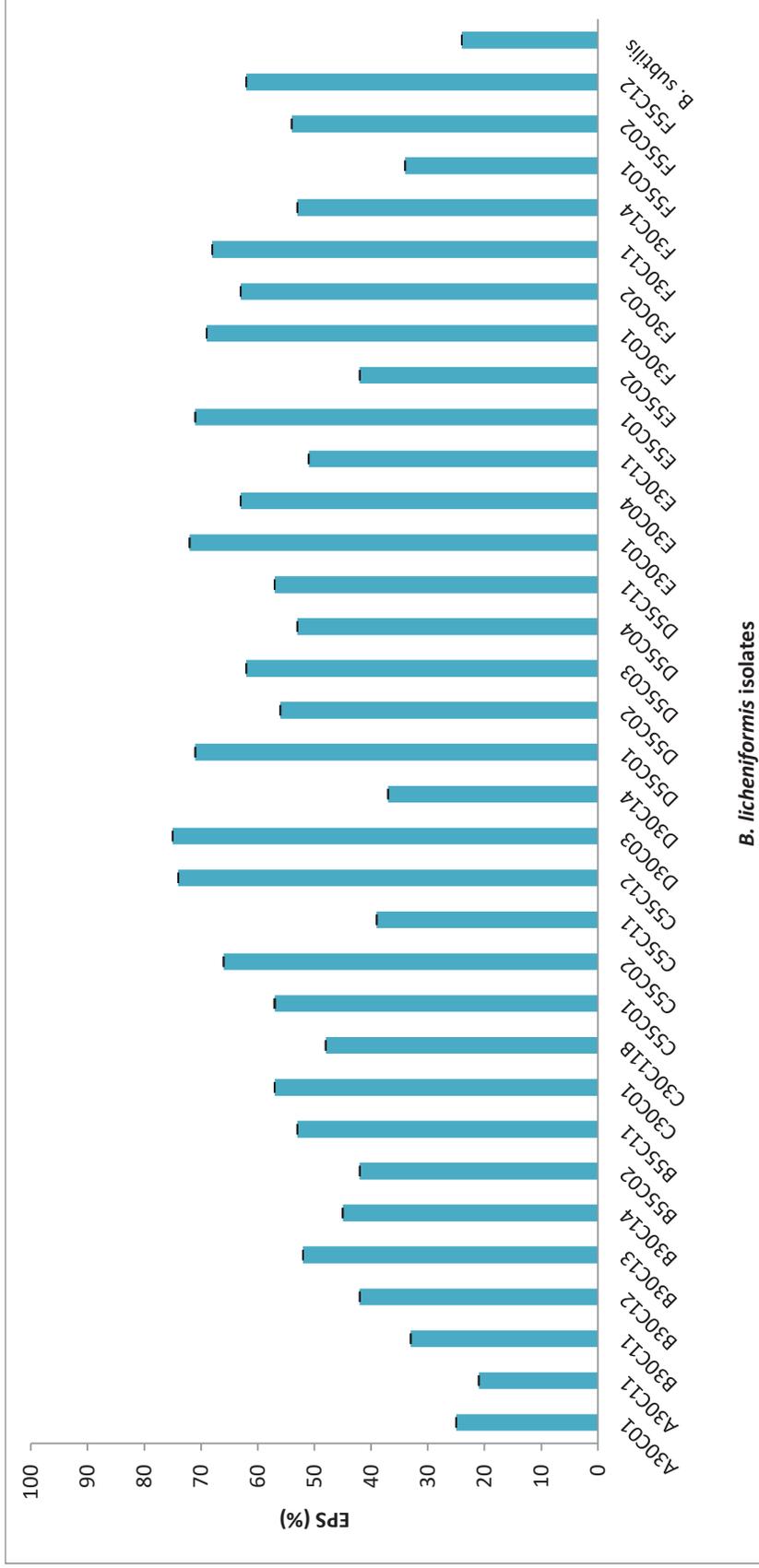


Figure 5.2 The percentage of Congo red bound indicates the amount of EPS produced by *B. licheniformis* isolates. The absorbance was measured at OD_{490 nm}. Mean and standard deviation were calculated from 4 replicates.

5.3.3 Heat resistance study at 72°C, 75°C and 80°C

The heat resistant study of selected *B. licheniformis* vegetative cells at 72°C, 75°C and 80°C are shown in Figure 5.3 to Figure 5.5.

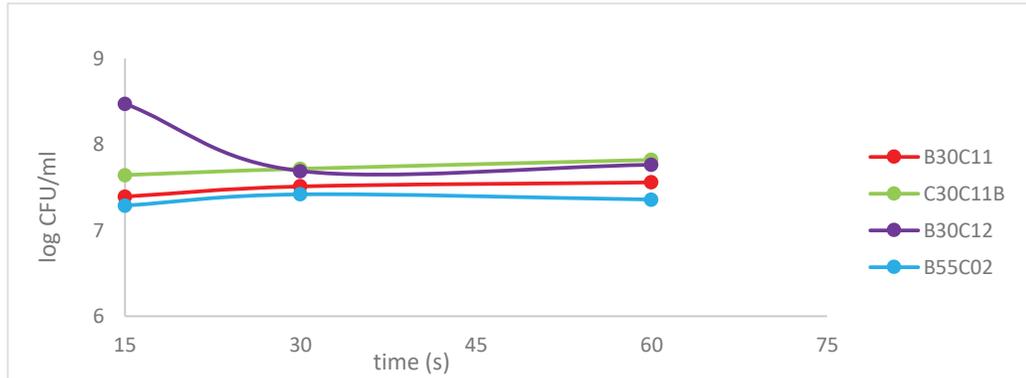


Figure 5.3 The heat resistance of *B. licheniformis* vegetative cells at 72°C.

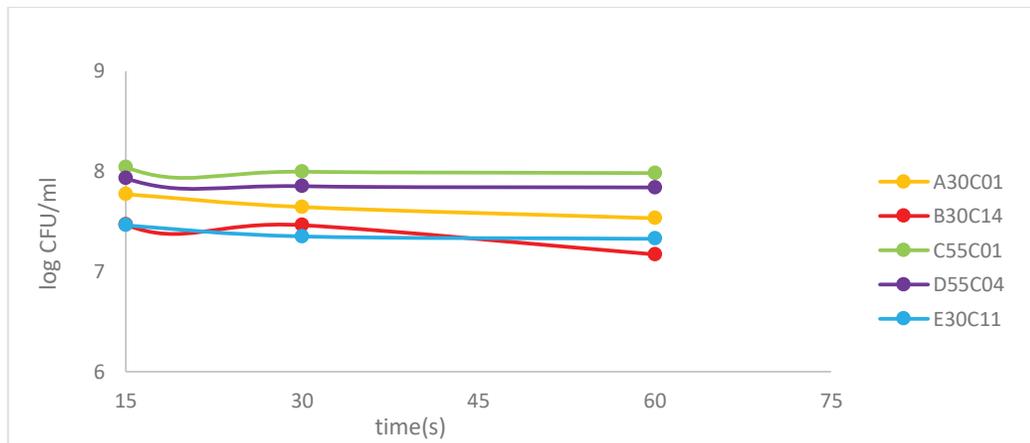


Figure 5.4 The heat resistance of *B. licheniformis* vegetative cells at 75°C.

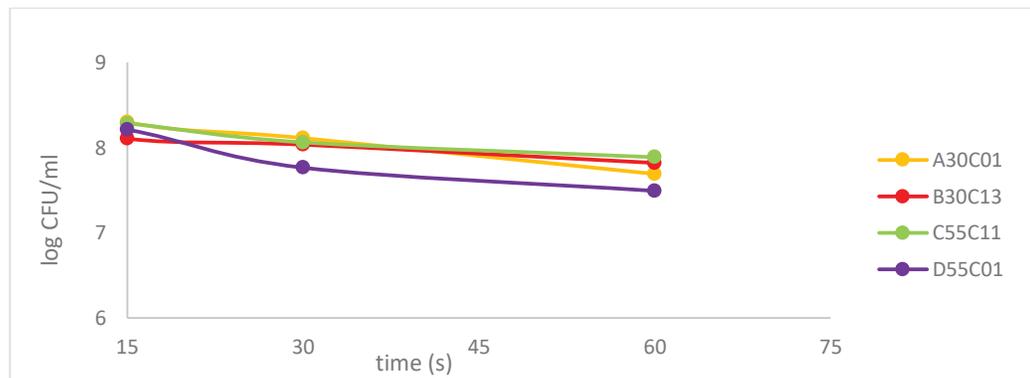


Figure 5.5 The heat resistance of *B. licheniformis* vegetative cells at 80°C.

5.4 DISCUSSION

B. licheniformis isolates from WPC80 powder are unable to grow at 10°C. Although they are thermotolerant and therefore able to survive pasteurisation, the inability to grow at 10°C means that they are unable to grow in the UF modules (inlet & outlet temperature is 10°C) of a whey manufacturing plant. This result agrees with Bergey's manual (Logan & Vos, 2015) which stated the minimum growth for *B. licheniformis* is 15°C - 20°C depending on strains. UF membranes represent the largest surface area in a WPC manufacturing plant providing opportunity for extensive biofilm growth. However, most WPC manufacturing plants operate their UF membranes at 10°C. The inability of these isolates to grow at this temperature is important as this eliminates this section of a WPC plant as a potential zone for *B. licheniformis* growth.

Lactose is a sugar mainly found in milk. β -galactosidase is used to break down lactose to galactose and glucose that can be used as an energy source by many microbial cells. However, 28/33 of the *B. licheniformis* isolates from WPC80 powders were able to form biofilm in 1% RWPC80 without lactose or minerals on SS (Figure 4.5). It was postulated that these bacteria are unable to ferment lactose in the whey medium. Lactose fermentation analysis was done on all 33 *B. licheniformis* isolates confirming that 33 cannot ferment lactose. This finding is supported by Sadiq et al, (2016b). This observation seems surprising with bacteria that have originated from a lactose rich environment and suggests that some other organic material, such as protein, may provide the carbon source for these bacteria in a dairy environment. This new information is crucial in order to understand the survivability of *B. licheniformis* in the dairy industry without using lactose.

All 33 *B. licheniformis* produced protease indicated by clear zones along the streaked line on calcium caseinate agar. This degradation of protein suggests that these bacteria may be able to use protein as an alternative carbon source in the absence of a fermentable carbohydrate. This would enable them to survive and grow in a whey protein solution and as biofilm in WPC manufacturing plants. The ability of *B. licheniformis* to adapt to a carbohydrate depleted environment was demonstrated by Wiegand et al, (2013) where they demonstrated the importance of protease in fermentation.

The production of protease enzymes that may influence the quality of milk and milk products, is reported by Teh et al, (2012). The effect of the enzymes produced by these *B. licheniformis* on the sensory quality of Mozzarella WPC80 is unknown. WPC80 is a major food ingredient in secondary processing such as in UHT beverages, yoghurt and infant formula products that also include casein. The WPC80 concentrate level varies in these different products but the ability to produce protease is likely to influence the quality of all these products.

Another factor that potentially influences product quality and acceptability is the ability to convert nitrate and nitrite. Nitrate and nitrite are permitted at low levels (50 mg/kg calculated as nitrate ion) in food products (Food Standards Australia and New Zealand). Elevated levels of nitrite in the WPC manufacturing might be experienced by dairy manufacture and are a cause for concern by some dairy product customers (personal communication). In this study, 30 *B. licheniformis* isolates were able to convert nitrate into nitrite. Three isolates (D55C01, F30C01 and F30C11) were unable to reduce nitrate. The ability for these bacteria to convert nitrate to nitrite depends on availability of a substrate.

One of the unique features of *B. licheniformis* is the ability to produce lichenysin. Lichenysin is a lipopeptide that has antimicrobial activity. Madslie et al, (2013) identified lichenysin was produced by most *B. licheniformis* strains and has the cytotoxicity and biosurfactant effects. The production of lichenysin is induced by haemolysis. The 33 *B. licheniformis* isolates from Mozzarella WPC80 samples produced haemolysis on Columbia sheep blood agar. The gene responsible for lichenysin production, Lichenysin synthetase A was detected in all 33 *B. licheniformis* isolates. The activity of what is now known to be lichenysin has been reported in a number of publications. Sayem et al, (2011) did an investigation on a broad spectrum anti biofilm exopolysaccharide from a marine strain of *B. licheniformis* that reduced the microbial colonisation of sponges. Batrakov et al, (2003) isolated *B. licheniformis* strains from drilling fluid and subsurface thermal water (56°C) that produce a lipopeptide that acted as an inhibitor for *Corynebacterium variabilis* adhesion on glass.

Two main biofilm components are microorganisms and EPS. Pellicle formation is a combination of bacteria and EPS forming a layer at a liquid-air

interface. Cells in pellicles are held together by an extracellular matrix consisting of exopolysaccharide, an amyloid-like fibres largely composed of protein (Kolodkin-Gal et al, 2012). Pellicle formation was observed for all 33 isolates of *B. licheniformis* when grown overnight in a microtitre plate with TSB as medium at 30°C, 37°C and 55°C (Chapter 3.3.1). This confirms their potential to form biofilm, which is believed to be the main source of contamination of WPC during manufacture.

Another indicator of EPS production and the potential to form biofilms is a Congo red binding assay. This involves treating a culture with Congo red which binds to the EPS produced by the bacteria. Ghafoor et al, (2011) used Congo red binding assay to investigate the glucose rich, *Pel* gene that associated with pellicles production of *Pseudomonas aeruginosa* (*P. aeruginosa*) between wild type and mutant. The mutant, *Pel*-negative did not produce pellicles and this was reflected in reduced Congo red binding compared with the wild type. The glucose rich gene, *Pel* is required for adherence and architecture at the later stages of biofilm maturity. *Pel* production indicates cell to cell interaction. This method was also used by Spiers et al, (2003) and Hay et al, (2009) for investigating the effects of, *Pel* production in artificial laboratory media (Luria bertani).

The results from the Congo red assay using the 33 *B. licheniformis* isolates showed that the amount of EPS production varied for each strain. There was no correlation between the amount of EPS produced during the Congo red assay and biofilm formation. It can be concluded that polysaccharide production is not the only factor responsible for biofilm formation of *B. licheniformis* isolated from WPC80. There is a high chance of *B. licheniformis* strains that produce more EPS might be more difficult to control by cleaning and sanitising in the dairy industry. *B. subtilis* was used as a control in this EPS assay as *B. subtilis* is the model in the *Bacillus* family for studying biofilm formation. Pellicle formation by *B. subtilis* when incubated in TSB showed a different wrinkled pattern from *B. licheniformis* as depicted in Figure 5.1. Roux et al, (2015) noted that poly-*N*-acetyl glucosamine is a major polysaccharide component of the *B. subtilis* biofilm matrix. This major carbohydrate component is essential for *B. subtilis* biofilms but may not be as critical for biofilms of *B. licheniformis*. This could be one explanation for the difference in pellical formation.

Several *B. licheniformis* isolates were subjected to heat treatment mimicking the thermalisation/pasteurisation temperature 72°C – 80°C to determine the heat resistance of their vegetative cells. Typically, *B. licheniformis* develops spores as a survival mechanism in nutrient depleted environments and these are very heat resistant to high temperature. The initial inoculum (8.0 ± 0.5 log CFU/mL) of tested *B. licheniformis* vegetative cells did not decrease even by 1 log reduction at the three temperatures tested; 72°C, 75°C and 80°C during 15 s, 30 s and 60 s heating intervals (Figure 5.3 – Figure 5.5). These results are in agreement with Yuan et al, (2012) in which they reported that the vegetative cells of 4 out of 5 *B. licheniformis* milk powder isolates cells were able to survive pasteurisation (72°C for 15s).

The *B. licheniformis* vegetative cells were heat resistant at these temperatures so D values could not be calculated. In addition, the *B. licheniformis* survival slightly increased after 30 s of heat treatment at both 72°C and 75°C. On the other hand, the 80°C temperature resulted in a slight decrease in *B. licheniformis* cell counts after 30 s up to 60 s (Figure 5.5). However, this temperature is out of the temperature range for the thermaliser (36°C – 77°C) used in the WPC80 manufacturing plant. Therefore, *B. licheniformis* vegetative cells are capable to survive this heat treatment providing opportunity to colonise the manufacturing plant and contaminate product.

5.5 CONCLUSIONS

B. licheniformis isolated from Mozzarella WPC80 powder is unable to ferment lactose but is able to grow in whey, a lactose rich medium. The fermentable carbon for the growth of *B. licheniformis* must come from either synergistic growth with lactose fermenting bacteria such as *Lactobacillus* species, also found in whey, or the use of protein as a source of carbon. Protease production by the *B. licheniformis* isolates suggests this use of protein as a substrate for growth is likely. Protease production will also influence product quality, in this instance, whey quality. Nitrate reduction by *B. licheniformis* is also likely to influence product quality. The *B. licheniformis* isolates from WPC grow as biofilms although the amount of EPS and biosurfactants that may influence biofilm formation does vary. The heat resistance of *B. licheniformis* vegetative cells isolated from WPC80 provides a new insight into the potential of these bacteria to survive conventional pasteurisation and contaminate the product.

CHAPTER 6

**BIOFILM AND SPORE FORMATION BY
B. licheniformis IN SINGLE AND CO-
CULTURE WITH *L. plantarum*
IN 1% RWPC80 WITH LACTOSE AND
MINERALS**

6.1 INTRODUCTION

Biofilm in most natural environments consist of multi species rather than a single species of microorganism (Flint, 1997; Costerton, 1995; Anand et al, 2014). One of the factors that may enhance biofilm formation is the synergistic activities in co-cultures. A study by Zhao et al, (2013) demonstrated the relationship of co-culture growth between *Geobacillus thermoglucosidans* and *A. flavithermus* whereby *Geobacillus thermoglucosidans* is unable to grow by itself. Abee et al, (2011) reported mixed species of *L. monocytogenes* and *L. plantarum* biofilms appeared to be more resistant to disinfectant treatments than single species biofilm or planktonic cells. Tang et al, (2009) showed an increase in biofilm formation where there was a combination of *Klebsiella oxytoca* isolates from dairy membrane on three types of UF membrane.

The 33 *B. licheniformis* isolated from Mozzarella WPC80 cannot ferment lactose (Chapter 5) and correlated to their API 50CHB kit test results negative for lactose utilisation (Table 3.8) so it is hypothesised that their growth must be supported by protein breakdown or synergistic activity with other microflora such as *Lactobacillus* species that can break down lactose to galactose and glucose for *B. licheniformis* use. This could be an important factor the growth of some strains of *B. licheniformis* in a whey product manufacturing plant.

One *L. plantarum* isolate (B30C21) was selected among three *L. plantarum* isolates from Mozzarella WPC80 (Table 3.9) for further investigation into a possible synergistic effect on selected *B. licheniformis* strains (E30C11 and F30C02), forming biofilm on SS. This B30C21 strain tested positive for lactose fermentation using 1% lactose broth (containing purple dye and reconstituted lactose) and API 50CHL kit.

6.2 MATERIALS AND METHODS

6.2.1 Source of isolates

For this chapter and onwards, two *B. licheniformis* and one *L. plantarum* (B30C21) were selected to investigate their synergistic study in forming biofilms on SS using 1% RWPC80 medium at 37°C. The properties of two selected *B. licheniformis* are described in Table 6.1

Table 6.1 The summary of the two selected *B. licheniformis* properties used in the synergistic study.

Isolate	Biofilm on SS coupon			Enzyme production		Lactose breakdown	Lichenysin	
	TSB	1% RWPC80	1% RWPC80 +lactose + minerals	Protease	Lipase	1% lactose broth	Lch AA gene	β-haemolysis
E30C11	+	+	+	+	-	-	+	+
F30C02	-	-	+	+	-	-	+	+

6.2.2 *B. licheniformis* E30C11 and F30C02 biofilm formation at 1, 2, 4, 8, 12 and 24 h in 1% RWPC80 with lactose and minerals at 37°C

In order to determine *B. licheniformis* biofilm formation within 24 h at their optimum growth temperature (37°C), the method used in Chapter 4.2.4 was used with the addition of shaking at 100 rpm to provide some kinetic energy as would be found in a flowing system. Each strain was tested separately in this trial. The SS coupons were retrieved at 1, 2, 4, 8, 12 and 24 h to quantify the amount of biofilm using a bead beating recovery (Chapter 4.2.4), serial 10-fold dilution in 0.1% peptone and growth on MPCA in duplicate at 37°C for 24 - 48 h. The individual colonies forming between 30 to 300 were counted for CFU per cm⁻² quantification.

6.2.3 Co-culture study between *B. licheniformis* (E30C11 and F30C02) with *L. plantarum* (B30C21) biofilm growth 4, 8, 12 and 24 h in 1% RWPC80 with lactose and minerals

The same culture conditions, sampling and recovery of cells were used as for Section 6.2.2 but including 6 log CFU/mL *L. plantarum* strain (B30C21) with each selected 6 log CFU/mL *B. licheniformis* strain (E30C11 and F30C02). MPCA and de Man, Rogosa and Sharpe agar (MRSA) (Merck, BDH, Palmerston North, New Zealand) were used for plate counting *B. licheniformis* and *L. plantarum* respectively. Duplicate plates were prepared for each. MPCA plates were incubated at 37°C for 24 to 48 h. MRSA plates which are selective media for *L. plantarum* were incubated anaerobically in an anaerobic jar filled with gas pack (BBL™ GasPak™ Family, New Zealand) for 24 - 48 h at 30°C. The individual colonies on plates containing 30 to 300 were counted for CFU per cm⁻² quantification.

6.2.4 Spore formation by *B. licheniformis* within biofilm in single and mixed populations

It is important to know the role of biofilm in the formation of spores of *B. licheniformis* as spores survive in the final WPC80 product. In addition to the enumeration for vegetative cells in Section 6.2.3, following the bead beating recovery (Chapter 4.2.4) the remaining samples after plating for vegetative bacteria were heated in a water bath at 80°C for 12 min prior to pour plating and incubation at 37°C. This heat treatment will eliminate the *B. licheniformis* vegetative cells and germinate the endospores (if any). Dhakal, (2013) noted that although their *B. licheniformis* were thermophilic, their growth and spore production was better at 37°C than 55°C, hence this was the incubation temperature used for this trial.

6.3 RESULTS

6.3.1 *B. licheniformis* E30C11 and F30C02 biofilm growth at 1, 2, 4, 8, 12 and 24h in 1% RWPC80 with lactose and minerals at 37°C

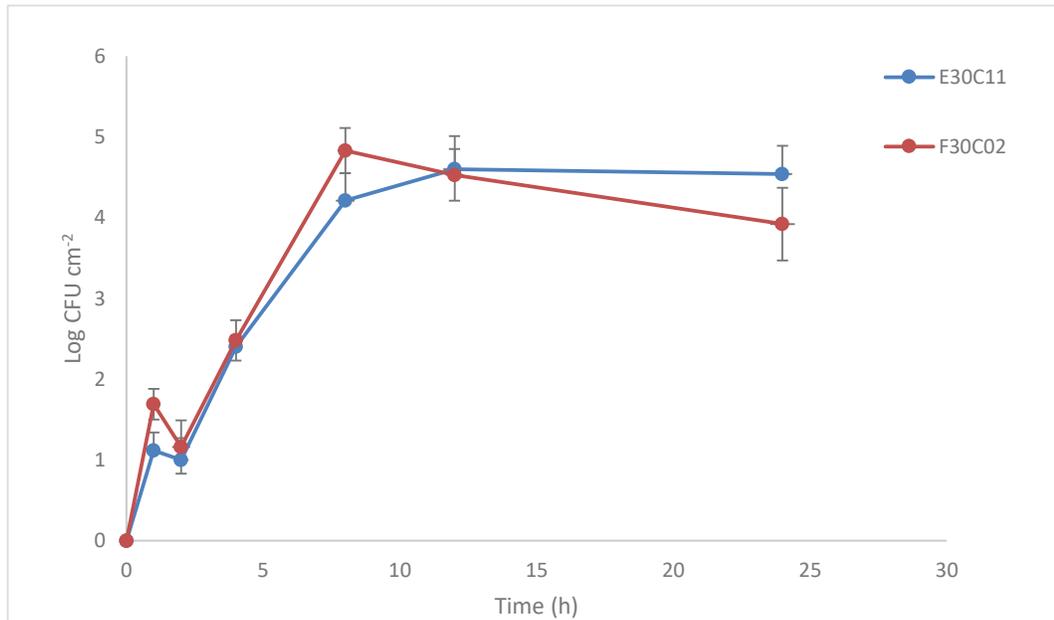


Figure 6.1 Biofilm formation by E30C11 and F30C02 plotted against time. The mean and standard deviation were from triplicate (N=3) independent experiments.

The biofilm growth by two selected *B. licheniformis* strains (E30C11 and F30C02) is shown in Figure 6.1. There was a steady increase starting 2 h until 8 h for both strains indicating a generation time in the biofilm of approximately 1.5 h. E30C11 continued to increase cell numbers from 8 h until 12 h but F30C02 started to decline until 24 h of incubation.

6.3.2 Biofilm of a co-culture of (*B. licheniformis* and *L. plantarum*) at 4, 8, 12 and 24 h in 1% RWPC80 with lactose and minerals at 37°C

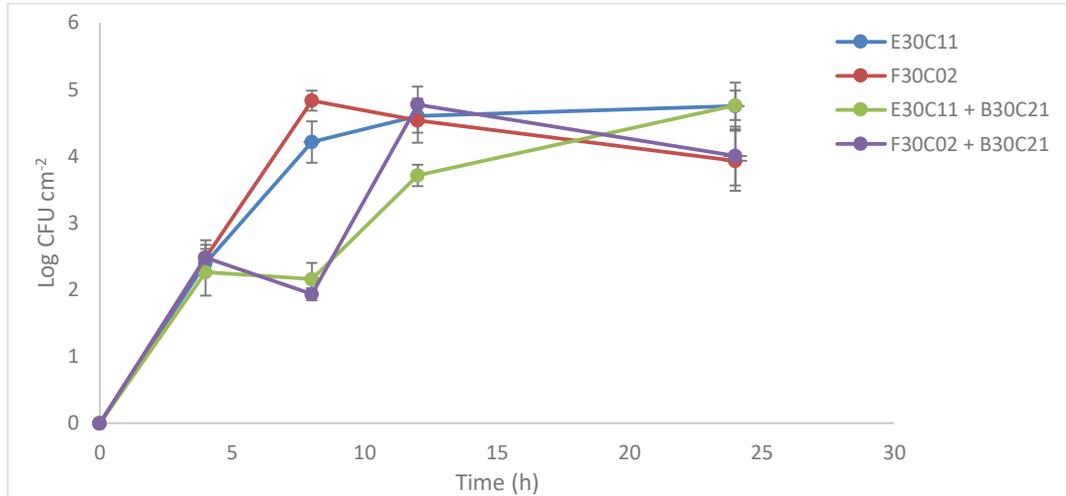


Figure 6.2 Counts of *B. licheniformis* E30C11 and F30C02 retrieved on MPCA from single species biofilm and mixed population biofilm with *L. plantarum* B30C21 in 1% RWPC80 with lactose and minerals. The mean and standard deviation were from triplicate (N=3) independent experiments.

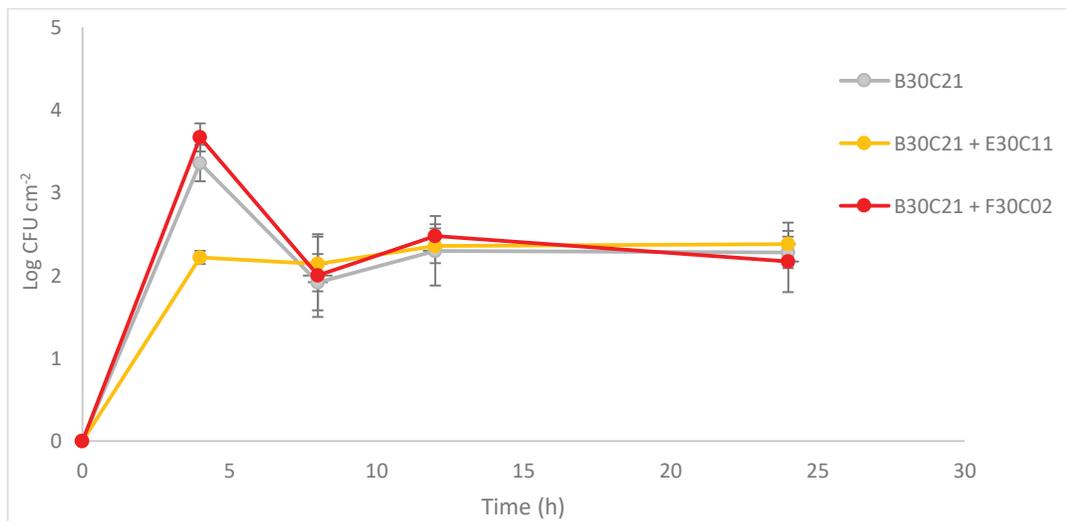


Figure 6.3 Counts of *L. plantarum* B30C21 retrieved on MRSA from single species biofilm and mixed population biofilm with *B. licheniformis* E30C11 and F30C02 in 1% RWPC80 with lactose and minerals. The mean and standard deviation were from triplicate (N=3) independent experiments.

Biofilm formation of *L. plantarum* was measured by the recovery of bacteria on selective MRSA, incubated anaerobically from samples taken at 4, 8, 12 and 48 h at 30°C. This method allowed the selective isolation of *L. plantarum* (B30C21) cells from a mixed population.

The first four hours showed the dominance of a single population of B30C21 and B30C21 with F30C02 on SS. The highest bacterial CFU count on MRS agar incubated anaerobically was $3.5 \log_{10}$ CFU cm⁻² formed from the co-culture between B30C21 and F30C02. This observation supports the hypothesis that *L. plantarum* can grow well in a medium containing lactose as the main carbon source and therefore may produce surplus glucose and galactose for use by other microorganisms in a co-culture environment. Meanwhile, *B. licheniformis* cells retrieved from MPCA at 4 h were about $2.5 \log_{10}$ CFU cm⁻² either in single or co-culture environments, showing no benefit to these bacteria in a co-culture.

At 8 h, there were sharp declines in biofilm formation by the co-cultures of E30C11 and F30C02 with B30C21. The decrease of approximately $1.5 \log_{10}$ CFU cm⁻² on MRSA may reflect the decrease in lactose concentration influencing the viability of the *L. plantarum* cells. A similar observation was noted on MPCA agar where there was a decrease of approximately $0.5 \log$ CFU cm⁻² possibly because of the production of lactic acid by the *L. plantarum* growth resulting in a reduction in the viability of *B. licheniformis* within the biofilm.

The biofilm formed from the co-culture of *B. licheniformis* and *L. plantarum*, measured on MRSA, reached stationary phase after 8 h at $2.5 \log_{10}$ CFU cm⁻² and remained at this level up to 24 h of incubation. Meanwhile, single and co-culture growth of *B. licheniformis* and *L. plantarum* measured on MPCA increased after 8 h reaching the highest biofilm formation at 12 h (approximately $5 \log_{10}$ CFU cm⁻²) for F30C02 and B30C21 as compared with E30C11 and B30C21 (an about $3.2 \log_{10}$ CFU cm⁻²). However, biofilm formation of the co-culture of F30C02 and B30C21 started to decrease 1-fold from 12 h until 24 h. In contrast, biofilm formation by co-culture of E30C11 and B30C21 still increased from 12 h until 24 h (approximately $5 \log_{10}$ CFU cm⁻²).

6.3.3 Spore formation within co-culture population of biofilm

No spore formation by *B. licheniformis* was detected from single, mixed cultures (grown together with *L. plantarum*) in the biofilm at every time interval tested. Dhakal, (2013) reported the incubation time for spore production is 10 - 11 days and its production by *B. licheniformis* was greater at 37°C than at 55°C. The incubation time for the present trial was therefore much less than that reported by Dhakal, (2013) for spore formation.

6.4 DISCUSSION

B. licheniformis is unable to ferment lactose (Chapter 5.3.1) and therefore will need to use other sources, including protein such as in whey (α -lactalbumin and β -lactoglobulin) breakdown to amino acids as source of energy (Chapter 5.3.2). In this co-culture study for *B. licheniformis* (E30C11 and F30C02) and *L. plantarum* (B30C21) it was postulated that this would enhance biofilm formation, by the action of *L. plantarum* breakdown of lactose in the whey to glucose and galactose and that *B. licheniformis* would use the glucose as a carbohydrate supply. Although there was a small increased biofilm formation of *B. licheniformis* in 1% RWPC80 with lactose and minerals at 12 h, this was insufficient to indicate enhanced biofilm formation in this co-culture environment.

A co-culture of *B. licheniformis* (E30C11 and F30C02) and *L. plantarum* B30C21 did not increase biofilm formation of *B. licheniformis* in 1% RWPC80 with lactose and minerals medium at 24 h. *B. licheniformis* will form biofilms in the presence of whey and minerals and this is the most likely source of product contamination.

Stanley et al, (2003) reported that high glucose concentrations inhibit biofilm formation by *B. subtilis* and therefore growth is better in the planktonic form. Although their study used *B. subtilis*, it is possible that we are seeing a similar effect of glucose inhibition with *B. licheniformis* in this present trial with a decline in *B. licheniformis* after 8 h in the co-culture (Figure 6.2). This observation followed the *L. plantarum* single biofilm formation which declined from 4 h to 8 h indicating lactose depletion and therefore possible high glucose in the medium.

On the other hand, Assaf et al, (2015) reported that lactose triggers biofilm formation by *Streptococcus mutans* and Duanis-Assaf et al, (2016) concluded that the LuxS based quorum sensing governs lactose induced biofilm formation by *B. subtilis*.

The contamination of *B. licheniformis* in milk and milk products is mainly due to its ability to form spores which allows them to survive heat processes (Dhakal, 2013; Stoeckel et al, 2016). Spores from different *Bacillus* species vary in their attachment to solid surfaces. Husmark, (1993) discovered that attachment was higher in the spore state than the vegetative state in five different *Bacillus* species and *B. cereus* is the

most hydrophobic species. The attachment process is quite rapid and within 1 h, and a maximum level of adhesion is reached for specific spore concentrations. However, according to the results from Chapter 5, most *B. licheniformis* vegetative cells are heat resistant up to 80°C for 60 s and unable to reach 1 log reduction thus reflecting their potential to survive the heat treatment.

In general, the attachment of microorganisms to surfaces is a very complex process with many variables affecting the outcome. Although spores when they are present, dominate in the attachment process, Faille et al, (2014) found no correlation between biofilm and sporulation within biofilms of *Bacillus* species. Spore surfaces are hydrophobic due to proteins on their outer surface (Wiencek et al, 1990). The high relative hydrophobicity of *Bacillus* spores is reflected in a high degree of attachment and the hydrophobic interactions increase proportionally with temperature.

Seale et al, (2015) noted that “spores of *B. licheniformis* can survive pasteurisation but don’t appear to germinate and in processing milk lines therefore, they are not viewed as so much of a concern as spores from *Geobacillus* spp or *A. flavithermus*”. Although this finding was noticed in a milk processing plant, a similar pattern of *B. licheniformis* sporulation was observed in the whey environment in this study, where there was no evidence of spores in a biofilm state within 24 h. The spores might not be produced within 24 h although if biofilm and foulant were not completely removed during CIP and continue over successive runs, this could result in spore formation. The low spore counts seen in this trial for 6 WPC80 samples where the spore counts were below 100 CFU/g, supports this hypothesis that the sporulation of *B. licheniformis* does not occur readily. The presence of *B. licheniformis* in the final product may be due to the resistance of the vegetative cells to heat treatment and dehydration (Chapter 5.3.3). It would be interesting to see if these dairy isolates vary in their heat resistance compared to isolates from other sources. To control these bacteria in a dairy environment, alternative strategies to the standard heat treatment need to be considered.

6.5 CONCLUSIONS

B. licheniformis form biofilm on SS surfaces in a whey environment in single or mixed populations. A co-culture of *B. licheniformis* with *L. plantarum* does not increase the biofilm formation. Spore production by *B. licheniformis* within the biofilm at 4, 8, 12 and 24 h of incubation on SS at 37°C in whey does not occur over this time period. The heat resistance of the vegetative cells of *B. licheniformis* emphasises the importance of these bacteria in dairy manufacture through their ability to survive heat treatments such thermalisation/pasteurisation.

Locating the zone in the manufacturing plant where these biofilms grow will be important for their control. We know that *B. licheniformis* cannot grow at 10°C, the temperature used in the UF plant, therefore this cannot be a site of biofilm development for these bacteria. The most likely source of whey product contamination during whey processing is before UF process during clarification or in the cool zone of the thermalisation heat exchanger and separator where temperatures are approximately 37°C and the concentration of protein (0.6%) will support more biofilm growth than more concentrated solutions.

CHAPTER 7

**THE POTENTIAL SOURCE OF *B.*
licheniformis CONTAMINATION
DURING MOZZARELLA WPC80
MANUFACTURE**

7.1 INTRODUCTION

The steps involved in Mozzarella WPC80 samples manufacture are outlined in Figure 2.4. WPC80 manufacture includes chilling, clarifying, thermalisation (covering a wide temperature range) (Appendix 1), separation, UF/Diafiltration, evaporation, concentration and drying. The UF membrane plant provides a huge surface area as a potential for biofilm formation and bacterial growth. Tang et al, (2009) found *Klebsiella* strains isolated from cold UF membranes and some were shown to be good biofilm formers. However, they should be able to be controlled with normal cleaning procedures.

In Chapter 3, we reported that Mozzarella WPC80 powder samples contained high bacterial counts and showed the predominant microflora was the mesophilic thermotolerant bacterium *B. licheniformis*. *B. licheniformis* is unable to grow at the low temperatures (10°C) used in the UF membrane plants to concentrate the whey for WPC80 manufacture. Growth of *B. licheniformis*, if it occurs in the manufacturing plant, must occur earlier than the UF stage as it is these early stages where conditions (nutrient and temperature) are most suitable for the growth of *B. licheniformis*. Growth is most likely to occur as a biofilm.

Biofilm can cause blockages, reduce heat transfer and passing dairy liquid, such as milk, is contaminated with cells and enzymes released from the biofilm into the passing milk. This chapter aimed to determine the potential source of *B. licheniformis* contamination in Mozzarella WPC80 manufacture and the ability to form biofilm and cause protein damage and product contamination.

7.2 MATERIALS AND METHODS

7.2.1 Source of strains

Thirty-three *B. licheniformis* strains isolated from whey protein concentrate 80 (WPC80) samples as reported by Zain et al, (2016) were used in this study. The isolates were maintained on nutrient agar slants at 4°C and in glycerol beads stock at -80°C.

7.2.2 Liquid whey samples from pre-UF sites

Five liquid whey samples from different points in the whey processing plant, pre-UF (raw whey balance tank, clarifier, thermaliser and separator) (Table 7.1) were provided by the same whey processing plant reported in Chapter 3.2.1.

Table 7.1 Liquid whey samples from pre-UF process of WPC80 powder.

Pre-UF liquid whey samples
1. Raw whey balance tank (A)
2. Clarifier (B)
3. Balance tank/thermaliser (C)
4. Post module/thermaliser (D)
5. Separator (E)

7.2.3 Isolation, identification, characterisation and biofilm formation of *B. licheniformis* isolates from pre-UF liquid whey samples

One mL of each liquid whey samples was diluted in 90 mL of sterile 0.1% peptone water (pH 7.0) and mixed using a vortex mixer. Samples were tested for microbial content using milk plate count agar (MPCA) (Merck, BDH, Palmerston North, New Zealand) and incubated at 37°C. The pure cultures grown on MPCA as single isolated colonies were screened using basic colony morphology and Gram staining as presumptive *B. licheniformis* (lichen-like colonies). Identification was confirmed by biochemical testing and partial 16S rDNA gene sequencing (Flint et al, 1999b).

Characterisation of presumptive *B. licheniformis* from pre-UF liquid whey samples were done on lactose fermentation, protease, lipase, nitrate, lichenysin and pellicles production as described in Chapter 5.2.

The confirmed *B. licheniformis* isolates were tested for biofilm formation on microtitre plates using TSB (Oh et al, 2007) (Figure 4.2.2) and subsequently on SS as described in section 4.2.4.

7.3 RESULTS

7.3.1 Isolation, identification and characterisation of B. licheniformis isolates from pre-UF liquid whey samples

The optimum temperature for *B. licheniformis* was 37°C based on microtitre plate results (Chapter 4) and our findings showed that they formed biofilm best with 1% RWPC80 with lactose and minerals (Chapter 4). This information indicates that the source of *B. licheniformis* contamination in whey manufacture is most likely before the UF process where the whey protein before concentration is about 0.6% and lactose concentration 4.5%.

To test our hypothesis, and identify the most likely zones in the manufacturing plant for biofilm growth of *B. licheniformis*, five liquid whey samples from different sites; raw whey balance tank, clarifier, thermaliser and separator were tested for *B. licheniformis*. Seven *B. licheniformis* isolates were found (2 isolates from the raw whey balance tank; A2 and A3), (1 isolate from clarifier; B2), (1 isolate from the thermaliser; D2) and 3 isolates from separator E1, E3 and E4) and identified by Gram-staining, colony and pellicle formation, API 50CHB testing and 16S rDNA gene sequencing as described in Chapter 3.

Further tests on their characteristics were done as described in Chapter 5.2. Figure 7.1 shows their colony formation on MPCA (A), β -haemolysis on Columbia blood agar after 48 h (B) (as indication of lichenysin production) and pellicle formation (C). Table 7.3 tabulated the summary of their characteristics as compared to E30C11 and F30C02 derived from Mozzarella WPC80 powder samples.

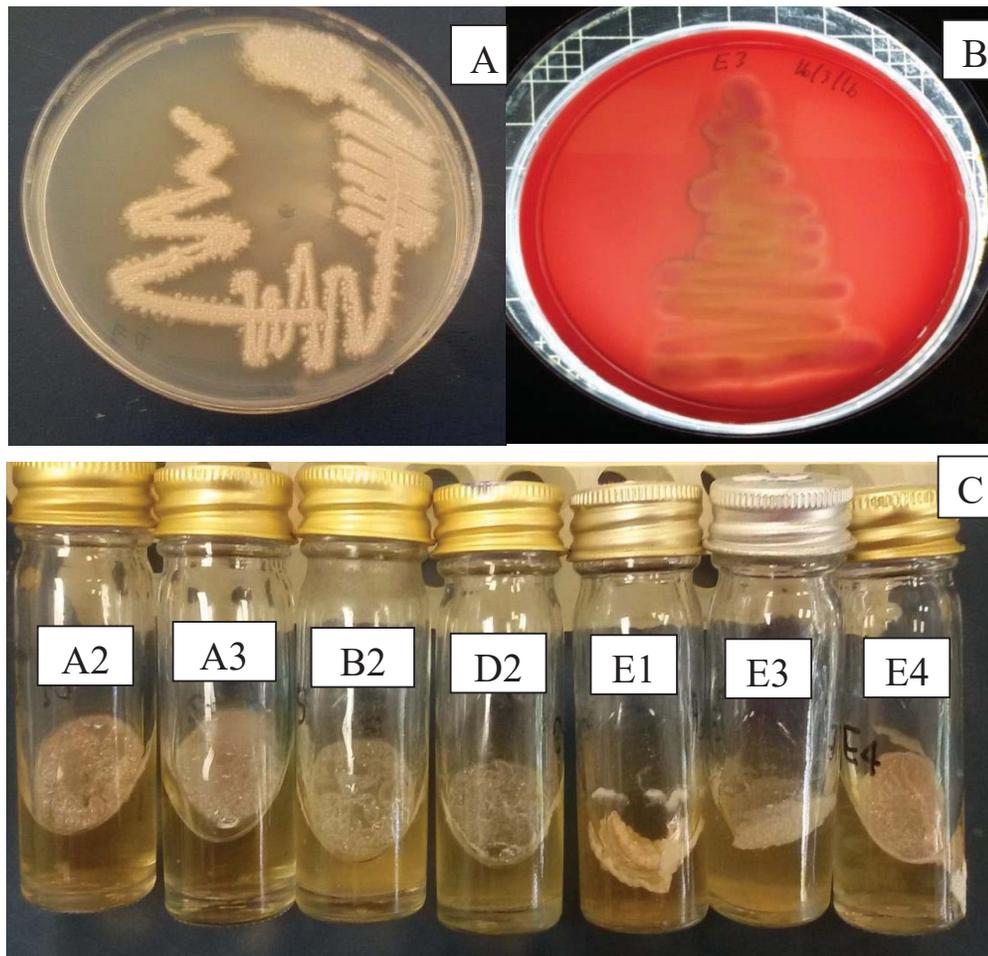


Figure 7.1 Phenotypic characteristic of pre-UF *B. licheniformis* A) colony formation on MPCA, B) β -haemolysis on Columbia with sheep blood agar, C) pellicle formation of A2, A3, B2, D2, E1, E3, and E4 strains after 2 days of incubation at 37°C in TSB.

A summary of the characteristics of the *B. licheniformis* isolates recovered from the pre-UF part of the manufacturing plant are listed in Table 7.2 and compared with E30C11 and F30C02 isolates from WPC80 powder samples.

Table 7.2 Summary of *B. licheniformis* characteristic from pre-UF sites compared to E30C11 and F30C02 from the WPC80 powder samples.

Characteristic	E30C11	F30C02	A2	A3	B2	D2	E1	E3	E4
Colony morphology	Dry Opaque Irregular edges Rod Gram positive	Mucoid Opaque Irregular edges Rod Gram positive							
Cell morphology									
Gram staining									
Growth temperature									
Growth at 10°C	-	-	-	-	-	-	-	-	-
Growth at 30°C	+	+	+	+	+	+	+	+	+
Growth at 37°C	+	+	+	+	+	+	+	+	+
Growth at 55°C	+	+	+	+	+	+	+	+	+
Lactose fermentation	-	-	-	-	-	-	-	-	-
Pellicles formation	+	+	+	+	+	+	+	+	+
Enzyme production									
Protease	+	+	+	+	+	+	+	+	+
Lipase	-	-	-	-	-	-	-	-	-
Lipopeptide: Lichenysin	+	+	+	+	+	+	+	+	+
API 50CHB	99%	99%	99%	99%	99%	99%	99%	99%	99%

7.3.2 Biofilm formation by pre-UF *B. licheniformis* isolates on plastic and SS

A preliminary biofilm study using the microtitre plate assay in TSB showed A2 and D2 formed strong biofilm. The remaining five *B. licheniformis* isolates did not form biofilm and their absorbance in the microtitre plate assay were similar to the negative control (sterile TSB without inoculum) (Figure 7.2).

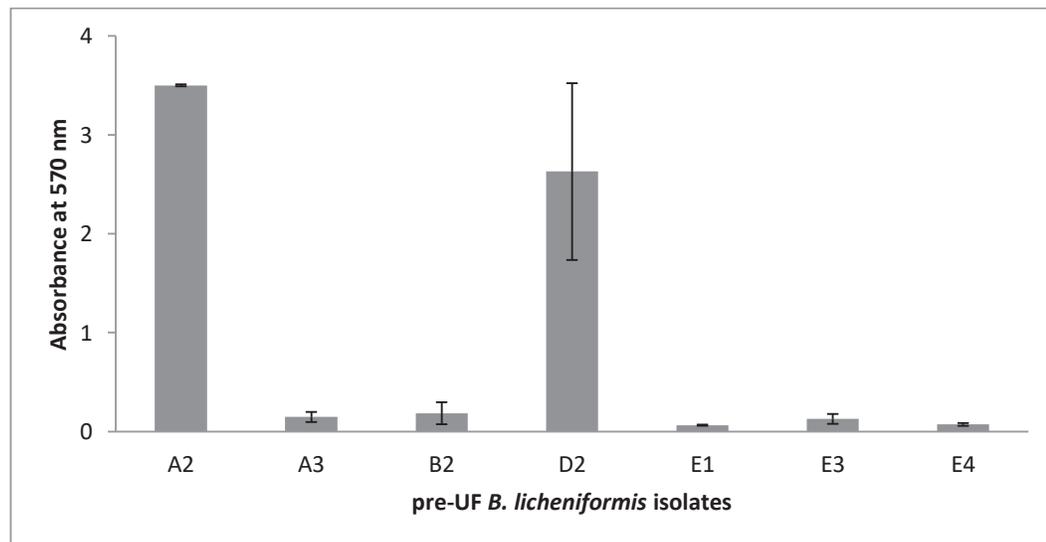


Figure 7.2 The microtitre plate assay results for biofilm formation in TSB by 7 *B. licheniformis* isolates taken before UF process. A2 and A3 isolates were derived from the raw whey balance tank (A), B2 isolates from clarifier (B), D2 from thermaliser and E1, E3 and E4 from separator. Only A2 and D2 showed strong biofilm formation on the polystyrene microtitre plate surface. The mean and standard deviation were from triplicate (N=3) independent experiments.

Further tests were done on the seven *B. licheniformis* isolates to examine their biofilm formation using three different media on SS and the results showed that they formed good biofilm in all 3 different media between 2.16 – 4.78 log₁₀ CFU cm⁻² (Figure 7. 3). The potential source of *B. licheniformis* contamination in the Mozzarella WPC80 powder is shown in the Figure 7.4.

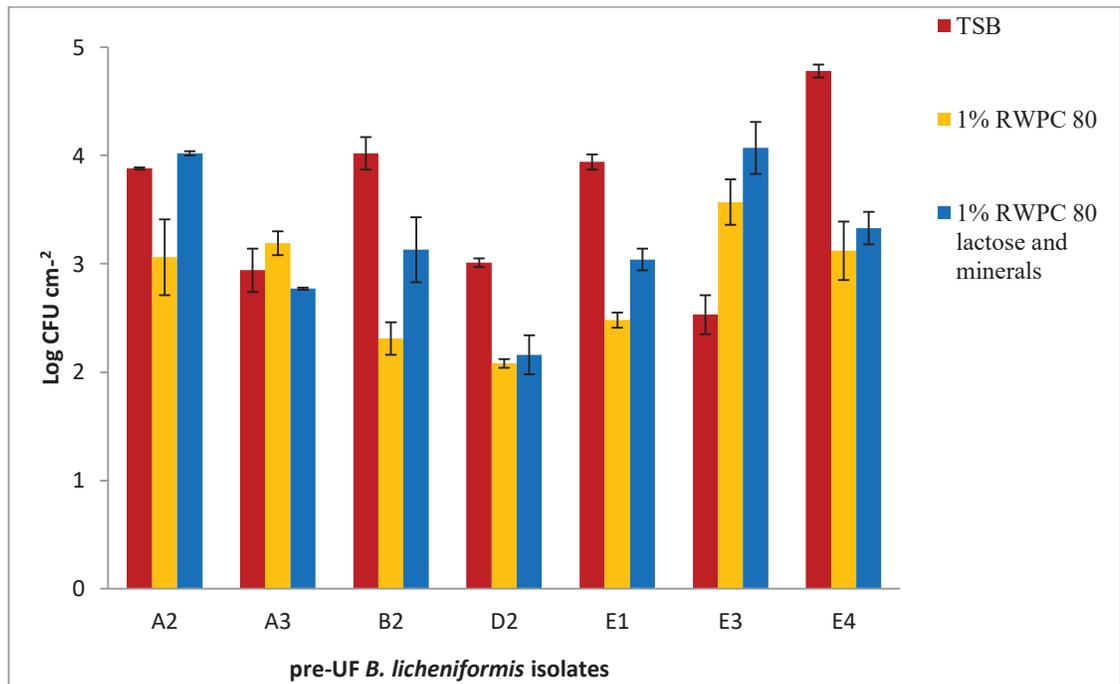


Figure 7.3 Biofilm formation on SS in 3 different media by 7 *B. licheniformis* isolates. A2 and A3 isolates were derived from the raw whey balance tank (A), B2 isolates from clarifier (B), D2 isolate from thermaliser (C, D) and E1, E3 and E4 from separator (E). The mean and standard deviation were from triplicate (N=3) independent experiments.

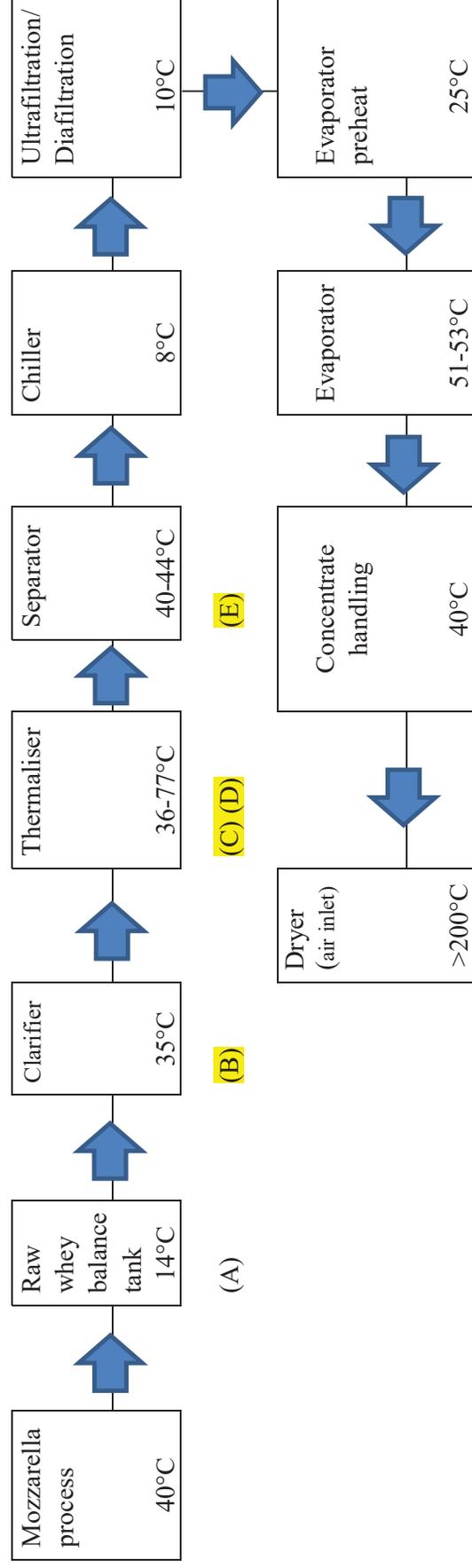


Figure 7.4 The diagram showed the potential source of contamination of *B. licheniformis* in the Mozzarella WPC80 powder; Raw whey balance tank (A), Clarifier (B), Thermaliser (C, D) and Separator (E). The highlighted letters with yellow colour indicates the most likely zone for biofilm growth and *B. licheniformis* contamination.

7.4 DISCUSSION

The objective of this chapter was to determine the possible source of predominant *B. licheniformis* contamination in a Mozzarella WPC80 manufacturing plant. Traditionally, microbial contaminants were believed to grow on the membrane surfaces of the UF plant as this represents the largest surface area in the plant as some plants operate their UF processes at 40°C (Lehmann, 1995). Changes from hot to cold UF have reduced the growth potential for bacteria on the membrane surfaces.

Seven *B. licheniformis* isolates were isolated from pre-UF liquid whey samples. This was confirmed by microscopy, colony and pellicle formation, biochemical and bio-molecular tests and compared with information from Chapters 3 and 5 (Zain et al, 2017). Their characteristics were in agreement with the *B. licheniformis* isolates from WPC80 powder (Logan & Vos, 2015). Their ability to attach and form biofilm was screened on microtitre plates and 2 (A2 and D2) isolates from 7 showed strong biofilm formation while the remaining 5 were non biofilm former (ODc value at 0.5). Further investigation using three different media; TSB, 1% RWPC80, 1% RWPC80 with lactose and minerals revealed that the 7 *B. licheniformis* were able to form biofilm on SS and the results showed that they formed good biofilm in all 3 different media between 2.16 – 4.78 log₁₀ CFU cm⁻² (Figure 7.3) (Aulikki, 2017). *B. licheniformis* biofilm formations were studied using the microtitre plate assay (CV staining) and cell enumeration on SS in this study. These results followed those in Chapter 4 for the WPC80 powder isolates that biofilm formed better on SS than the plastic surface of microtitre plate. The entire manufacturing plant containing SS surfaces provides potential sites for biofilm formation of *B. licheniformis* and this represents a much larger surface area than any other parts, such as rubber seals. However, contributing factors such as medium concentration, minerals content and temperature will affect the biofilm growth on SS.

In Chapter 4, it was shown that the predominant microflora *B. licheniformis* would not grow in the membrane plant because of the low temperature (10°C) and must be growing elsewhere. In addition, the effect of whey concentration on growth, water activity of concentrated product and the importance of cations further limited the opportunity for growth on the UF membrane.

Contamination of dairy products is mostly due to bacteria being released from biofilm in the processing plant rather from the farm itself. Crielly et al, (1994) identified *B. licheniformis* as the common species isolated in raw milk and contaminating the dairy production chains. *B. licheniformis* was also the predominant bacteria colonising UF plant operated at 40°C used for standardising milk used in cheese manufacture (Lehmann, 1995). The source of biofilms of thermotolerant bacteria such as *B. licheniformis* is believed to be before the UF zone where the whey protein concentration is about 0.6%. This bacterium is thermotolerant and capable of growing in sections of dairy manufacturing plant where temperatures reach 40°C–65°C (Burgess et al, 2010). Furthermore, because it is a spore former it is difficult to eliminate using food processing techniques. Spore forming bacteria such as *Bacillus* species can survive and colonise dairy manufacturing plant as a biofilm, releasing enzymes and bacteria to contaminate milk products (Burgess et al, 2010; Yuan et al, 2012).

Miller et al, (2015) managed to isolate *Bacillus* species spores in sweet whey, non-fat dry milk, acid whey and WPC80 and the highest frequency of *B. licheniformis* spores were detected in all whey products. It is important to locate the zone in the whey manufacturing plant where this bacterium survives and forms biofilm in order to be able to develop strategies for the control of this bacterium in a whey processing plant.

However, very little information is available on the source of contamination of these ubiquitous bacteria in whey processing plant. A study by Sadiq et al (2016a); Cook & Sandeman (2000) and Dhakal, (2013) reported *B. licheniformis* was the dominant thermophilic spore former in dairy products where the latter concluded that *B. licheniformis* survives in manufacturing plants in various forms or genotypes. This heterogeneity may assist in the survival in dairy environments and dairy products. The safety and quality of dairy products relies on heat treatments such as pasteurisation.

7.5 CONCLUSIONS

Biofilm formation by *B. licheniformis* isolates from WPC80 varies between strains and is dependent on the substrate surface with SS supporting more growth than polystyrene. TSB, whey concentration of 1% RWPC80 with and without lactose and minerals supports biofilm formation of 7 *B. licheniformis* from pre-UF sites unlike the *B. licheniformis* isolates from WPC80 powder where some of the isolates grew a better biofilm in 1% RWPC80 with lactose and minerals.

The most likely source of whey product contamination during whey processing in this case study is likely to be before UF (Figure 7.4) where the concentration of protein is 0.6%. Likely areas include, during clarification, in the cool zone of the thermalisation heat exchanger where temperatures are approximately 37°C and the separator where the temperatures around 40°C - 44°C. This combination will support more biofilm growth than more concentrated solutions. This suggestion is supported with the results from isolation, identification, characterisation and biofilm formation of 7 *B. licheniformis* isolates from liquid pre-UF samples that have been presented and discussed in this chapter.

CHAPTER 8

FINAL DISCUSSION AND CONCLUSIONS

8.1 DISCUSSION

In this study, microorganisms that contaminated six Mozzarella WPC80 powders come from one WPC80 manufacturing plant. The six Mozzarella WPC80 powders used for this study contained 4-5 log₁₀ CFU/g total aerobic mesophiles and < 100 CFU/g mesophilic aerobic spores. A study by Okuku et al, (2017) noted that aerobic mesophiles were the dominant contaminants in WPC80 powder after 18 months' storage compared to WPC34 which contained higher yeast and mould counts. However, the different whey sources, manufacturing process, storage conditions are likely to have contributed to the different results in study.

In this study, bacteria were identified using biochemical and molecular techniques. The predominant contaminants were *B. licheniformis*. The culturable population only was considered in this study as these are responsible for the quality grading of WPC resulting in consumer rejection of product and a cost to the dairy manufacturing company. Dairy product contamination is believed to be enhanced by the growth of biofilms in the manufacturing plant resulting in high microbial numbers from bacteria being released from the biofilm into the product. Biofilms may develop on any surface where the conditions are suitable for bacterial growth (Costerton 1995; Donlan 2002; Flint et al, 2001). These conditions include the availability of nutrients and suitable temperature and physical conditions such as a suitable substrate and flow conditions to allow the attachment and growth of microorganisms. In the dairy industry, biofilms develop on SS surfaces from which they detach and contaminate product. For example, *S. thermophilus* biofilm on UF membranes and SS pipework of a standard whey processing plant resulted in whey powders containing bacteria at > 4 log CFU/g (Flint, 1998).

The bacteria contaminating dairy manufacturing plant and dairy product are generally assumed to originate from the raw milk. Gram positive bacteria dominate the population in fresh raw milk but Gram negative bacteria become dominant as the milk is stored under refrigeration before entering a dairy manufacturing plant. Dairy manufacture selects for the more heat resistant Gram positive bacteria and propagates these through the development of biofilms in the manufacturing plant (Raats et al, 2011). Each Gram positive genera will vary in the requirements for growth within a

manufacturing plant. The conditions leading to *B. licheniformis* biofilm growth in a modern WPC manufacturing plant have not been determined until this study.

Biofilm formation by *B. licheniformis*, the dominant microflora in the WPC80 product analysed in this trial, was initially evaluated at three different temperatures; 30°C, 37°C and 55°C using a microtitre plate assay. The degree of attachment and biofilm formation of *B. licheniformis* isolates was determined after 24 h incubation in TSB medium in static conditions. The results were divided into 3 categories; none, moderate and strong biofilm formation. *B. licheniformis* biofilm formation occurred preferentially at 37°C with 9/33 isolates formed strong biofilm. Meanwhile, 24/33 *B. licheniformis* isolates were identified as unable to form biofilm at the three different temperatures.

Dairy practices using 30°C and 55°C do encourage the growth of some *Bacillus* species however Zhao et al, (2013) reported that incubation at 65°C is favoured by thermophilic bacteria such as *Geobacillus* species and *A. flavithermus*. Dhakal, (2013) noted that their *B. licheniformis* strain isolated from milk was reluctant to attach microtitre plates using TSB medium. The microtitre plate assay is a convenient rapid method to screen microbial isolates for biofilm formation. However, the suitability of this test has been questioned for some bacterial species (Peeters et al. 2008). It appears that from the present study that the microtitre plate assay is not suitable for *B. licheniformis*. This agrees with Dhakal, (2013).

In the present trial, there were two factors to consider in the assessment of the ability of the *B. licheniformis* isolates to form biofilm. One was the microtitre plate surfaces which Dhakal, (2013) had reported were unsuitable for *B. licheniformis* biofilm testing and the second was the lab medium compared with whey. Therefore, the assessment of biofilm formation was done on SS surfaces in whey medium and this was compared with SS surfaces in TSB.

Reconstituted whey medium (Tang, 2011) was used at three different concentrations (1%, 5% and 20%) to mimic the composition at three different stages in WPC manufacture. In this study, most biofilm formed at the 1% concentration with lactose and minerals (removed later in the manufacturing process) added. This eliminates a large section of the manufacturing plant (UF and evaporation) as sites to

support biofilm formation of *B. licheniformis*. Some strains were unable to form biofilm in 1% WPC without minerals. Minerals are removed later in the WPC manufacturing process, therefore any *B. licheniformis* growth is most likely to occur before dialysis.

The inability *B. licheniformis* to grow at 10°C (the temperature of the UF process) as discussed in Chapter 5 is further evidence to eliminate UF as a section of plant where these bacteria may propagate. This is important as the surface area of an UF plant is large and potentially a site for microbial colonisation.

The most likely sites for *B. licheniformis* growth in a WPC manufacturing plant are believed to be the clarifier (to remove particles), thermaliser (to reduce microbial load in the cheese whey) and separator (to remove fat). All these unit operations have zones at suitable temperatures for the growth of *B. licheniformis*. This was supported with the isolation of 7 *B. licheniformis* isolates retrieved from these areas of the manufacturing plant. Three from 7 isolates were from the separator which operates at 45°C. CIP and other control measures such as altering temperatures, to reduce biofilm formation and reduce contamination of WPC with *B. licheniformis* need to focus on these areas.

Dhakal, (2013) concluded that an important consideration in the survival and dispersal of *B. licheniformis* in milk and milk products is the ability to produce spores which survive heat treatment and can germinate in suitable zones of a dairy manufacturing plant. Spore counts of *B. licheniformis* in raw milk are usually < 50 CFU per mL (Crielly et al, 1994). *B. licheniformis* was reported as the dominant thermophilic spore forming bacteria in dairy products (Cook & Sandemann, 2000; Dhakal, 2013). Ronimus et al, (1997) revealed interesting results on thermophilic spores of *B. licheniformis* and *Geobacillus* species in that they survived over 90 years in New Zealand milk powder.

In the present study, no spore production was detected in biofilm grown in 1% RWPC80 with minerals on SS at 4, 8, 12 and 24 h of incubation. This observation may be because the spore formation of *B. licheniformis* requires more than 24 h. As manufacturing plants are cleaned on a regular basis, well before 24 h, it is curious that spores can form in the manufacturing plant. The most likely explanation is that

residual bacteria and spores may persist in a mature biofilm after cleaning and continue to propagate in successive manufacturing runs, allowing them time to produce spores (Witthuhn et al, 2011). The *B. licheniformis* vegetative cells were heat resistant when tested at 72°C, 75°C and 80°C with < 1 log reduction from the initial ($\sim 8 \pm 0.5$ log CFU/mL) population (Chapter 5).

The characterisation of the isolates of *B. licheniformis* surprisingly showed that they did not ferment lactose. This was not expected for bacteria isolated from a dairy system, especially as they were shown to need lactose to support the best biofilm growth. The bacteria must obtain carbohydrate and one possibility is the use of protein in the whey (α -lactalbumin and β -lactoglobulin).

All isolates in this trial produced protease enzyme (not lipase), which is a concern in terms of spoilage of whey. Enzymes like protease and lipase do influence the quality of milk and milk products by altering the sensory qualities such as texture, taste and aroma. They will also affect the nutritional value (Chen et al, 2003; Teh et al, 2012; Teh et al, 2013). Several other *Bacillus* and *Paenibacillus* species produce a variety of proteases, lipases and phospholipases that affect the quality of dairy products (Lücking et al, 2013) although it is often the psychrotrophic population that is responsible for much of the enzyme deterioration of dairy products. A study by Raats et al, (2011) concluded that an estimated 10% loss of protein and fat in milk was because of enzymes produced by psychotropic bacteria. Psychrotrophs produce enzymes that cause defects in cheese by lowering the yields and producing off flavours, especially rancidity via lipase production (Champagne et al, 1994). Another factor that potentially influences product quality and acceptability is the ability *B. licheniformis* to convert nitrate and nitrite. Nitrate and nitrite are permitted at low levels (50 mg/kg calculated as nitrate ion) in food products (Food Safety Australia and New Zealand). Elevated levels of nitrite in the WPC manufacturing might be experienced by dairy manufacture and are a cause for concern by some dairy product customers.

Although not the main focus of this study, *P. glucanolyticus* was isolated from the WPC80 powder samples and is a potential spoilage bacterium. *Paenibacillus* species grow at psychrotrophic temperatures and are therefore able to grow during whey UF which operates at 10°C (Vithanage et al, 2014). A study by Huck et al,

(2007) identified *Paenibacillus* as a bacterium responsible for significant spoilage that limited HTST-pasteurised milk product shelf life. This was supported by a study by Ivy et al, (2012) on *Paenibacillus* as the predominant psychrotolerant spore former in fluid milk. Driehuis et al, (2016) showed *Paenibacillus polymyxa* spores to be present in dairy cow milk, faeces and silage.

The focus of the present study was on *B. licheniformis* as this was the predominant isolate in the WPC tested. As mentioned earlier, one of the surprising outcomes from this study was the inability of *B. licheniformis* to utilise lactose. One possible source of carbohydrate could be the glucose released from lactose utilisation by other bacteria in WPC. Synergistic interplay between *B. licheniformis* with *L. plantarum* was hypothesised to increase the magnitude of biofilm formation as the latter able to convert lactose to glucose. However, the biofilm in co-culture environment with these two species did not increase biofilm formation by either bacterium.

There are other aspects that were considered in understanding the formation of biofilm in a WPC manufacturing plant. These include the ability to produce EPS (an essential component of a biofilm) and the production of biosurfactants (which have been reported for *B. licheniformis*).

EPS production was detected in all 33 *B. licheniformis* isolates including 7 pre-UF *B. licheniformis* isolates. However, there was no correlation between the amount of EPS produced during the Congo red assay and biofilm formation. It can be concluded that polysaccharide production is not the only factor responsible for biofilm formation of *B. licheniformis* isolated from WPC80. *B. licheniformis* is reported to produce a biosurfactant, lichenysin. The 33 *B. licheniformis* including 7 pre-UF *B. licheniformis* isolates from this study were all shown to contain the gene for lichenysin production. The haemolysis of sheep blood, shown for the *B. licheniformis* isolates in this study, is an indicator of lichenysin production. However, attempts to isolate lichenysin were unsuccessful. This may be due to difficulties with the method used or the haemolysis results may not be reflecting lichenysin production. If that is true, we assume that under our experimental conditions, the gene for lichenysin production was not expressed and future work needs to look at conditions that may result in the expression of this gene. Further experiments on different extraction methods from *B.*

licheniformis within biofilm in whey environments need to be addressed. This is important as biosurfactants have been shown to help in the dispersal of *B. subtilis* from a mature biofilm (Branda et al, 2001) and therefore may be able to be used to control *B. licheniformis*.

This study differs from a processing facility in that batch experiments rather than a continuous process was used. Continuous experiments with a continuous supply of fresh nutrient would be more representative of a WPC processing plant. To confirm the most likely sources of contamination with *B. licheniformis* in WPC processing, an intensive swabbing/sampling at different zones within manufacturing plants (plate heat exchanger of thermaliser and clarifier/separator) would provide useful information to help in targeting sites for controlling contamination. For routine monitoring, testing samples such as separator sludge for testing at 37°C rather than the current currently only 30°C and 55°C, would have a better chance of isolating *B. licheniformis*.

The strategies to control *B. licheniformis* in WPC processing include more frequent cleaning of the thermaliser or running two thermaliser, each for short periods so the growth of *B. licheniformis* is limited. The flow rate (turbulent flow) could be controlled as many studies reports that increased flow enhances the release of bacteria from biofilms. Surface modification of stainless steel in terms of hydrophobicity and surface charges, silver impregnation and antimicrobial coatings (bacteriocin) might help reduce the attachment of microorganisms. Modifications to the CIP practices including a greater use of surfactants, enzymes and chelating agents may assist in controlling biofilms of *B. licheniformis* (Anand et al, 2014).

8.2 CONCLUSIONS

B. licheniformis has the potential to be a major contaminant of WPC. Biofilm growth is the most likely source of product contamination and this can only occur in some very specific parts of the manufacturing plant where conditions such as temperature and salt content are suitable. These parts of a WPC manufacturing process (clarifier, thermaliser and separator) need to be the focus for any modifications to the manufacturing process to control the biofilm growth of these bacteria. Protease production makes these bacteria a concern for spoilage of the WPC and any consumer products manufactured from this product. The conditions resulting in spore production, biosurfactant release and EPS production need further investigating.

8.3 HIGHLIGHTS OF THE STUDY

1. The best temperature for biofilm formation of *B. licheniformis* was at 37°C.
2. SS was the best surface for biofilm formation compared to plastic for *B. licheniformis*.
3. 1% reconstituted WPC80 with salts, supports biofilm formation by *B. licheniformis*.
4. *B. licheniformis* is unable to grow under the conditions present in a whey UF plant operating at 10°C.
5. Vegetative cells of *B. licheniformis* isolated from WPC80 survive pasteurisation temperatures.
6. The source of *B. licheniformis* contamination in WPC80 processing plant is postulated at thermaliser and separator regions.
7. *B. licheniformis* isolates were unable to ferment lactose but able to grow in a dairy environment.

8.4 FUTURE RECOMMENDATIONS

1. Synergistic studies with *Paenibacillus* species and thermophilic bacteria such as *A. flavithermus* to determine if *Paenibacillus* species can provide a fermentable carbohydrate from lactose for *B. licheniformis* growth.
2. Exploring the conditions for lichenysin production within biofilm and how this may influence biofilm growth.
3. Determining the conditions that result in spore formation in biofilms of *B. licheniformis*.
4. An in-depth study on CIP practise around the critical areas (before UF process) and its effects on *B. licheniformis* cell counts.
5. Develop ways to control biofilm formation and growth of spore-formers in WPC production.

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APPENDICES

APPENDIX 1



Tetra Pak (New Zealand) Ltd
 5 Pacific Rise, Mt Wellington
 P O Box 76 037, Manukau City
 Ph: +64 9 573 5588; Fax: +64 9 573 5599

30102-67234, 67235, 67309 Edition 3

Manufacturing no. **30102-67234, 67235, 67309** Date **12/4/2005**
 Plate Heat Exchanger type **C10-SR** Quantity **3**

Supplier **Tetra Pak (New Zealand) Ltd**
 Ph. (09) 573 5588 Fax. (09) 573 5599

Installed at **Fonterra Clandeboye**

Assembled by **Tetra Pak (New Zealand) Ltd**

- Plates are parallel flow.
- The plate pack is tightened to **1994 mm** (the "A" dimension).
- The plates are assembled with gasket facing the pressure plate as per attached plate hanging list.
- The performance of the plate heat exchanger is as shown under:

Section I is closest to the frame plate.

Section	Flowrate (kg/h)	Media	Temperature progr. (°C)	dP (kPa)	Grouping	IN	OUT
I	60920	Whey	70 → 77	74	1*12MH	A11V	S2
	72862	Hot Water	75 ← 80	94	1*13ML	S3	A14
II	61700	Whey	36 → 72	101	3*34H	A22	A11
	60769	Whey	40 ← 77	98	3*34H	A14V	A23V
III	61680	Whey	16 → 36	46	2*42H	T2	A22V
	61607	Whey	20 ← 40	46	2*42H	A23	T3

Dimensions:

Carry Bar LC	mm	3600	Connection RJT	mm	101
Tightening Bolt LTB	mm	3450	Tightening Bolt LLB	mm	3450
Dimension "A"	mm	1994	Dimension "C"	mm	3845
Dimension A _A (Sec1)	mm	116	Dimension A _B (Sec2)	mm	914
Dimension A _C (Sec3)	mm	752			

Future extension plates 64

Minimum CIP Flowrate Required 51,000L/h

Overall dimensions: LxWxH	mm	3802 x 850 x 2167
Liquid volume (total):	litre	927.9
Net weight, empty / operating	kg	2670 / 3620
Design / Test Pressure	bar	10 / 13
Design Temperature	°C	110

Attachment :

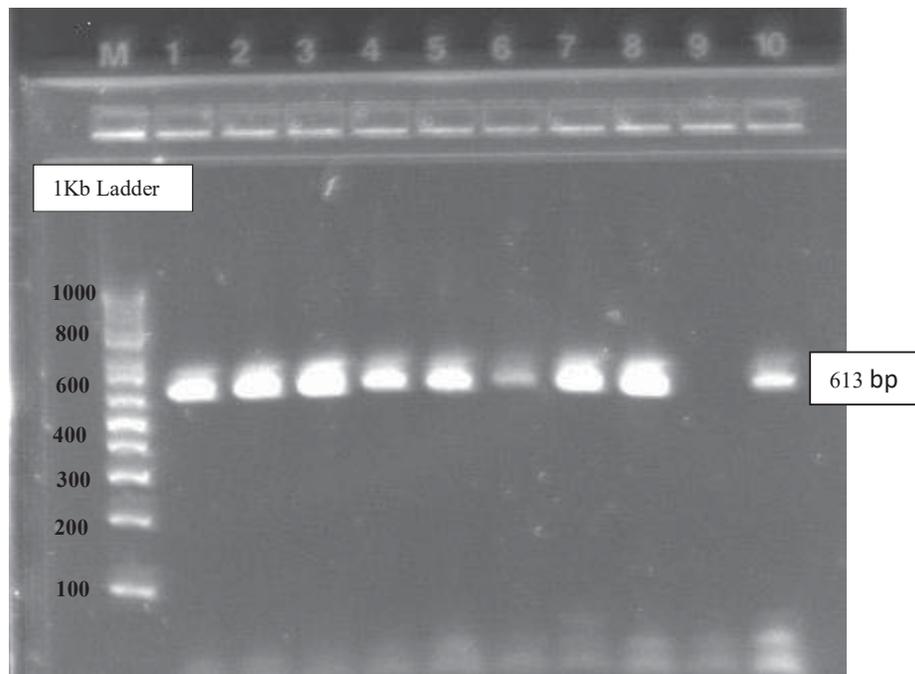
- Plate Hanging List
- General Arrangement Drawing

APPENDIX 2



Colony formation by one pure single isolate suspected to be *B. cereus* on Mannitol yolk polymyxin agar after 24 h incubation at 30°C.

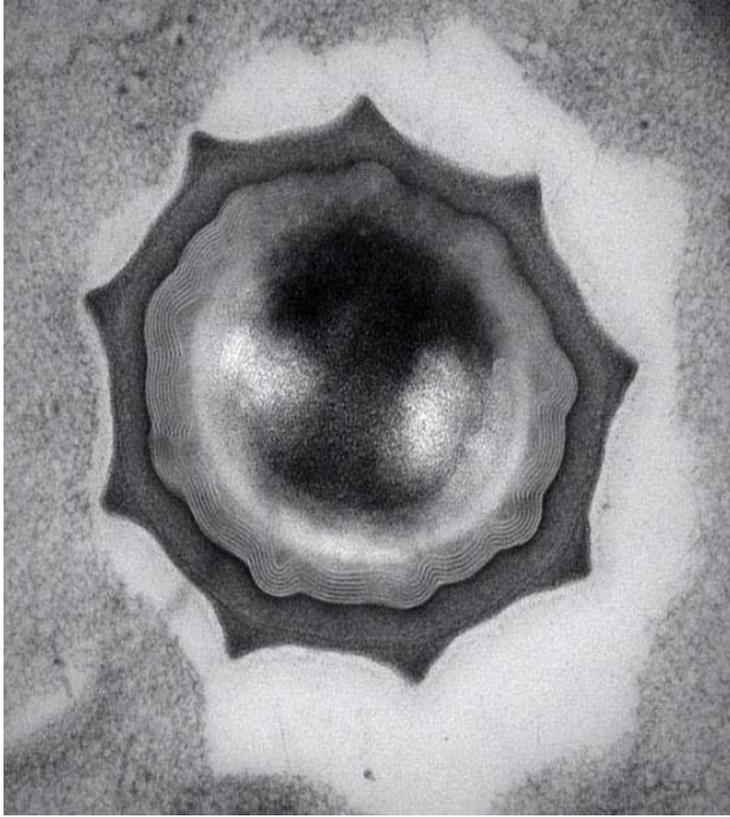
APPENDIX 3



Positive PCR results for several *B. licheniformis* isolates

Well No.	Details		Result
	Bacteria Isolates	Primers	
M	1 Kb ladder	-	-
1	D55C01	B-Lich F-1 B-Lich R-1 **targeting <i>Gyr B</i> gene	+
2	D55C02		+
3	D55C03		+
4	D55C04		+
5	D55C11		+
6	E55C02		+
7	F55C01		+
8	F55C02		+
9	F55C11		-
10	F55C12		+

APPENDIX 4



 American Society for Microbiology
Page Liked · 7 March 2015 · 

Pic of the Day: *Paenibacillus glucanolyticus* spore

Paenibacillus species have been isolated from a wide variety of sources including soil, water, the plant rhizosphere, plant materials, food, fodder, faeces and diseased insect larvae. They are facultative anaerobes or strict aerobes and mostly motile, endospore forming rods. This is the first time that this bacterium has been isolated from WPC 80 samples in New Zealand and identified as *Paenibacillus glucanolyticus* based on 16S rDNA sequence analysis. Interestingly, their spores portrayed a very unique shape, different from spores of *Bacillus* species under TEM observation.

Created by: Siti Norbaizura Md Zain. PhD student. IFNHH, Massey University, New Zealand

Credits to : Jordan Taylor. Manawatu Microscopy and Imaging Centre. New Zealand

This photo was taken from our site www.microbeworld.org. Submitted by: Baizura Zain
Thanks to the author(s): Jordan Taylor, Manawatu Microscopy Imaging Centre | Steve Flint, IFNHH, Massey University, New Zealand | Siti Norbaizura Md Zain, IFNHH, Massey University, New Zealand

Submit your pics to #ASM via www.microbeworld.org

*The spore image of *P. glucanolyticus* was selected as photo of the day in American Society for Microbiology (ASM) Facebook page (7 March 2015)*

APPENDIX 5

HOME » WHAT WE DO » AGRICULTURE AND FOOD SECURITY » FOOD ASSISTANCE » RESOURCES » WHEY PROTEIN CONCENTRATE COMMODITY FACT SHEET

WHEY PROTEIN CONCENTRATE COMMODITY FACT SHEET

WBSCM Material Description and Number

Not yet available in WBSCM

General Information

Whey protein concentrate (WPC) is a family of dry dairy ingredients used to add concentrated whey protein to food products. WPC is produced by removing a certain percentage of non-protein constituents from pasteurized whey derived from cheese processing. The finished WPC products available for food aid are WPC34, which contains more than 34 percent whey protein, and WPC80, which contains more than 80 percent protein. WPC is processed by physical separation techniques such as precipitation, filtration or dialysis. WPC must be pasteurized and manufactured in the U.S. from cow's milk that was produced in the U.S. For more information on nutrition, processing, packaging and shelf life, refer to the [United States Dairy Export Council Reference Manual](#)¹. It is recommended that WPC be stored in cool, dry environments with temperatures of less than 80 degrees Fahrenheit and relative humidity of less than 65 percent. WPC should be used within 9 to 12 months. It is typically packaged in air tight bags containing an inner film lining including pinch top, block bottom bags, or pinch top, pinch bottom, flat tube bags. For full product specifications refer to the USDA Commodity Requirements Document for dried dairy ingredients. The appropriate Web Based Supply Chain Management (WBSCM) code should be used to order WPC34 or WPC80.

Programming Guidance

WPC is a dairy ingredient that can be used to enrich the protein content and quality of processed food products including emergency food products A-20, A-28 and A-29, ready-to-use supplementary and therapeutic foods (RUSF, RUTF) and other nutritional supplements. It can be used for applications involving food product development to enhance protein content.

Nutrition/Preparation Information

WPC is a nutrient-dense, high-quality animal-based protein used for supplementation or fortification. WPC improves texture, enhances flavor and color, emulsifies and stabilizes dry mixes, extends shelf-life, and improves the quality of processed dairy and meats products, bakery products, snack foods, beverages, cereal products and is used in special sports and nutritional products. WPC34 and WPC80 are nutritionally similar in fat content, but differ in lactose and protein concentrations. WPC34 contains 34 to 36 percent protein and 48 to 52 percent lactose, while WPC80 contains 80 to 82 percent protein and 4 to 8 percent lactose. WPC34 provides similar concentration of lactose, protein and minerals as non-fat dry milk and can be used in comparable applications.

Nutritional Content

The table below includes values for WPC34 and WPC80 taken from the [U.S. Dairy Export Council Reference Manual](#)¹.

Nutrient	WPC34 (100g)	WPC80 (100g)
Water (g)	3.93	4.11
Energy (kcal)	369.00	412.00
Protein (g)	34.36	80.00
Total Lipid (fat) (g)	3.93	6.60
Carbohydrate	50.80	5.31
Fiber, total dietary (g)	0.0	0.0
Sugars, total (g)	0.0	0.0
Minerals		
Calcium (mg)	569.00	423.00
Iron (total mg)	0.89	1.20
Magnesium (mg)	104.00	50.00
Phosphorous (mg)	547.00	0.00
Potassium (mg)	1680.00	517.00
Sodium (mg)	630.00	255.00
Zinc (mg)	0.21	n/a
Iodine (mg)	0.0	0.0
Vitamins		
Vitamin C, total ascorbic acid (mg)	2.00	0.0
Thiamin (mg)	0.36	0.0
Riboflavin (mg)	1.80	0.0
Niacin (mg)	0.37	0.0

Vitamin B5 (mg)	0.0	0.0
Vitamin B6 (mg)	0.0	0.0
Folate, DFE (µg)	0.0	0.0
Vitamin B12 (µg)	0.0	0.0
Vitamin A (IU)	100.00	0.0
Vitamin E (alpha-tocopherol) (mg)	0.0	0.0
Vitamin D (D3) (µg)	0.0	0.0
Vitamin K (phylloquinone) (µg)	0.0	0.0
Lipids		
Fatty acids, total saturated (g)	0.0	0.0
Fatty acids, total monounsaturated (g)	0.0	0.0
Fatty acids, total polyunsaturated (g)	0.0	0.0
Cholesterol (mg)	97.00	n/a
Other		
Caffeine	0.0	0.0

USDA Commodity Requirements Document

http://www.fsa.usda.gov/Internet/FSA_File/ddi2.pdf



MASSEY UNIVERSITY
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**STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS**

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: **SITI NORBAIZURA MD ZAIN**

Name/Title of Principal Supervisor: **PROF STEVE FLINT**

Name of Published Research Output and full reference:

Tang Xuemel, Steve Flint, Rod Bennett, John Brooks and Siti Norbaizura Md Zain (2015). Biofilm in the dairy industry. Chapter 8: Biofilm contamination of ultrafiltration and reverse osmosis plants. John Wiley & Sons, Oxford.

In which Chapter is the Published Work: **Chapter 2 & 3**

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: and / or
- Describe the contribution that the candidate has made to the Published Work:

The candidate did the preparation of the manuscript for publication

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Name of Candidate: SITI NORBAIZURA MD ZAIN

Name/Title of Principal Supervisor: PROF STEVE FLINT

Name of Published Research Output and full reference:

Siti Norbaizura Md Zain, Steve Flint, Rod Bennett and Tay Hong Soon (2016). Characterisation and biofilm screening of the predominant bacteria isolated from whey protein concentrate. *Dairy Science & Technology*, 96(3), 285-295. doi: 10.1007/s13594-015-0264-z

In which Chapter is the Published Work: Chapter 3 & 4

Please indicate either:

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The candidate did the necessary laboratory work and prepared the manuscript for publication.

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**STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS**

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: **SITI NORBAIZURA MD ZAIN**

Name/Title of Principal Supervisor: **PROF STEVE FLINT**

Name of Published Research Output and full reference:

Siti Norbaizura Md Zain, Rod Bennett and Steve Flint (2017). The Potential Source of *B. licheniformis* Contamination During Whey Protein Concentrate 80 Manufacture. *Journal of Food Science*. 82(3), 751-756. doi: 10.1111/1750-3841.13833

In which Chapter is the Published Work: **Chapter 5 & 7**

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