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TESTING FOR TOTAL BACTERIA IN DAIRY POWDER- COMPARISON OF TEST INCUBATION TEMPERATURES (A CASE STUDY)

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ABSTRACT

The objective of this study is to identify any deficiencies in the incubation temperatures currently used in the dairy industry for the microbiological assessment of dairy samples. In New Zealand, dairy industries use the Aerobic Plate Count (APC) to enumerate mesophiles at 30°C and thermophiles at 55°C. However, there are potentially some microorganisms in dairy samples with optimal growth temperatures outside the current temperature range used by the industry for microbiological testing. Therefore, in this study, 70 milk powder samples were tested for the APC at 30°C, 37°C, 55°C and 65°C. The results showed no significant difference ($p > 0.05$) between the number of bacteria capable of growth at 30°C and 37°C in all samples. The average number of isolates capable of growth at 30°C and 37° was 2.27 and 2.26 $\log_{10}$ CFU/g respectively. However, bacterial growth at 55°C (1.78 $\log_{10}$ CFU/g) was significantly higher ($p < 0.05$) than growth at 65°C (1.54 $\log_{10}$ CFU/g). *B. licheniformis* was found to be the dominant bacteria in the dairy powder samples when testing was done at 30°C and 37°C. *G. stearothermophilus* and *A. flavithermus* were found in dairy powder samples when tested at 55°C and 65°C. These results indicate that the current testing temperatures (30°C and 55°C) used in the dairy industry are satisfactory.

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LIST OF ACRONYMS AND ABBREVIATIONS

APHA	American Public Health Associations
ADPI	American Dairy Products Institute
BMP	Butter Milk Powder
CFU	Colony Forming Unit
DCANZ	Dairy Companies Association of New Zealand
FAO	Food and Agriculture Organisation
FSANZ	Food Standards Australia New Zealand
g	gram
h	Hour
IDF	International Dairy Federation
IFP	Infant Formula Powder
ISO	International Organisation For Standardisation
min	Minute
mL	Milliliter
MPC	Milk Protein Concentrate
MPCA	Milk Plate Count Agar
MPI	Ministry For Primary Industries, New Zealand
NFDM	Non Fat Dry Milk
PCA	Plate Count Agar
rpm	Revolutions per minute
s	Seconds
SMA	Standard Method Agar
SMP	Skim Milk Powder
SPC	Standard Plate Count
TSB	Tryptic Soy Broth
USDA	United Stated Department of Agriculture
VNC	Viable but Nonculturable
WPC	Whey Protein Concentrate
WMP	Whole Milk Powder

1. INTRODUCTION

Dairy industry is the backbone for the economy of many countries in the world, including New Zealand. Almost 3% of the world's milk is produced in New Zealand, and about 95% of New Zealand's milk is exported to other countries. The five main countries that import milk from New Zealand are China, Australia, America, United Arab Emirates and Japan. Whole milk and skim milk powder are the top two dairy products that are important to the New Zealand economy (Dairy Companies Association of New Zealand, 2018). However, the New Zealand dairy industry loses millions of dollars every year because of contamination of milk powder with thermophilic bacterial spores (Scott, 2006). Thermophilic and mesophilic spore forming bacteria are capable of surviving pasteurisation and growing during the manufacture of powdered milk products resulting in the contamination of products.

Contamination of milk powder with spore formers is a concern mainly due to the potential for spoilage and the customer perception of product quality. It is important to ensure that all milk powders produced are free from both spoilage and pathogenic bacteria, and this is achieved through heat treatment and maintaining plant hygiene. Standard laboratory tests are used to determine the quality of the products and to ensure the cleanliness of the processing lines (Tabit, 2016).

Bacteria that are capable of contaminating milk have a wide temperature range in which they can grow. For example, *Bacillus cereus* (*B.cereus*) can grow between 4°C to 50°C, and its optimal growth occurs at 30°C–37°C (MPI, 2015). Currently, milk is tested at 30°C and 55°C (IDF/ISO); however, the optimum growth of some bacteria in milk powder is not 30°C or 55°C. Even though most bacteria can grow within a wide range of temperature, bacterial growth is slower at temperatures outside the optimal growth temperature. It is possible that some of these slow growing bacteria are not detected at the temperatures used for routine testing. The possibility of the growth of a high number of thermophiles during the milk evaporation between 65°C to 75°C is possible. Under these conditions, incubation of Aerobic plate count (APC) tests at 65°C might reveal more bacteria than testing at 55°C. A dairy company had found that high numbers of thermophiles were growing during the evaporation process of milk between 65°C to 75°C

and incubation of the APC tests at 65°C revealed thermophile numbers 1000 times (log 3) greater than testing at 55°C (Personal Communication). There is some justification to investigate whether the incubation temperatures used for the testing of standard product are appropriate.

2. LITERATURE REVIEW

2.1. Raw Milk

Raw milk is a valuable food material which can be used for the manufacture of many food products including cheese, yoghurt, butter, milk powder, and whey products (Chandan, 2011; Spreer, 2017). The primary composition of raw milk is water (87.4%), lactose (4.9%), fat (3.6%), protein (3.4 %), and minerals (0.7%) (Chandan, 2011; Spreer, 2017). Before raw milk can be processed, its quality is checked in order to meet company and regulatory standards. Raw milk should meet the standard of fat and protein content with low levels of microbial and somatic cell counts, low freezing point as well as low amounts of inhibitors such as the antibiotics-Penicillin (Murphy et al., 2016; Spreer, 2017)

High quality raw milk has been found to have a good level of nutrients, taste, flavour and is low in microbial count. According to the United States Food and Drugs Administration (USFDA), a high quality milk or grade A has 100,000 CFU/mL in a Standard Plate Count (SPC) test (Murphy et al., 2016). However, in New Zealand the standard for microbiological limits for top quality raw milk is at 50000 CFU/mL higher than USFDA regulation (MPI, 2016a). In terms of nutrition, based on Food Standards Australia and New Zealand (FSANZ), milk must contain at least 32g/kg fat and 30 g/kg protein (Food Standards Australia New Zealand, 2015).

Many investigations looking at the correlation between raw milk quality and the quality of final dairy products have reported that high quality raw milk results in high quality dairy product (Murphy et al., 2016). For example, a meta-analysis study conducted by Geary et al. (2014) assessed the effect of high somatic cell count in raw milk samples which were used to make cheese had concluded that high somatic cell count (SCC) will produce high moisture cheese and a low level of fat and protein, which affects the yields. Another example from Paludetti et al. (2019) assessed the effect of two different raw milk samples which were used to make Skim Milk Powder (SMP). The raw milk had total bacteria counts of 3.60 ± 0.55 and 4.37± 0.62 $\log_{10}$ CFU/mL, respectively. Evaluation of the milk powder made from the raw milk identified bacterial levels of 2.36±0.09, and 3.55± 0.13 $\log_{10}$ CFU/g in the SMP. The presence of a high microbial load in raw milk

can also produce enzymes such as protease and lipase that will breakdown proteins in raw milk which will eventually cause spoilage (Murphy et al., 2016; Spreer, 2017).

2.2. Types of Dairy Powders

Milk powder is the dairy product formed when majority of the water in milk is evaporated and then dried to a moisture content of 2.5-4% w/w (Skanderby et al., 2009). Milk in a powdered form has a longer shelf life because of its low moisture content (Walstra, 1999). Milk powder can be used in many products such as baked goods, infant formula, confectionery products, ice cream and many others (Wehr & Frank, 2004). Milk powder can be easily transported from one place to the other resulting in low transportation cost compared with liquid milk. In this thesis, five types of dairy powders were subjected to tests. They were whole milk powder (WMP), skim milk powder (SMP), butter milk powder (BMP), whey protein concentrate (WPC), milk protein concentrate (MPC).

2.2.1. Whole Milk Powder (WMP)

WMP is dried milk that contains milk fat between 26% to 42% w/w with no more than 5% w/w water and no less than 34% w/w milk protein (Food and Agriculture Organization of the United Nations, 2011). This meets the requirements of the Australian and New Zealand food standards which state that the milk fat content in WMP should not be less than 26% w/w and not be more than 5% w/w water (Food Standards Australia New Zealand, 2015). Figure 1 shows the approximate content of WMP (Ann Augustin & Clarke, 2011).

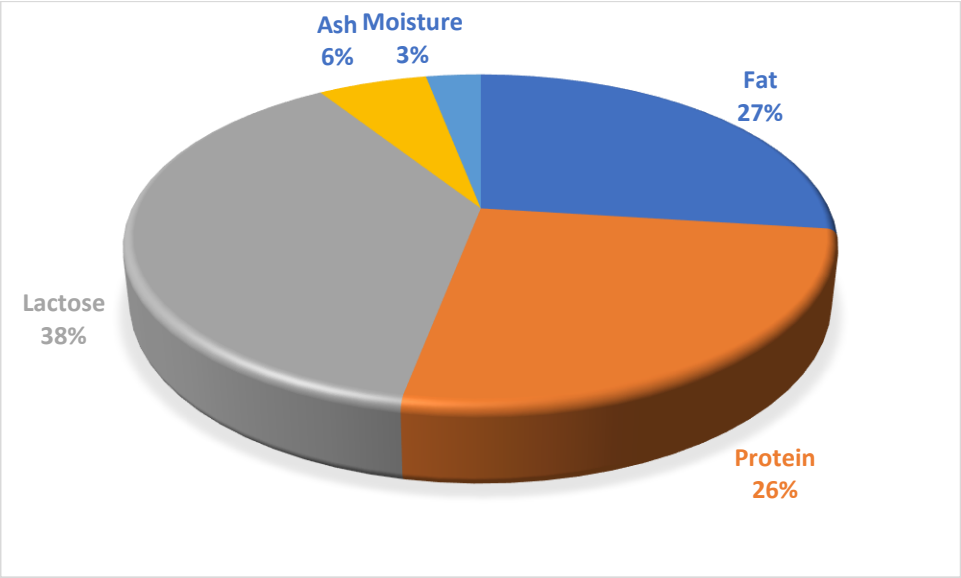

Figure 1. Approximate percentage of the components in WMP.

2.2.2. Skim Milk Powder (SMP)

Based on Food and Agriculture Organization of the United Nations (2011), SMP has maximum milk fat content of 1.5% w/w with no more than 5% of w/w water and no less than 34% w/w milk protein. This meets the requirements of the Australian and New Zealand food standards where not more than 1.5% w/w milk fat is allowed and not more than 5% w/w water is permitted in SMP (Food Standards Australia New Zealand, 2015). The approximate composition of a typical SMP can be seen in Figure 2 (Ann Augustin & Clarke, 2011).

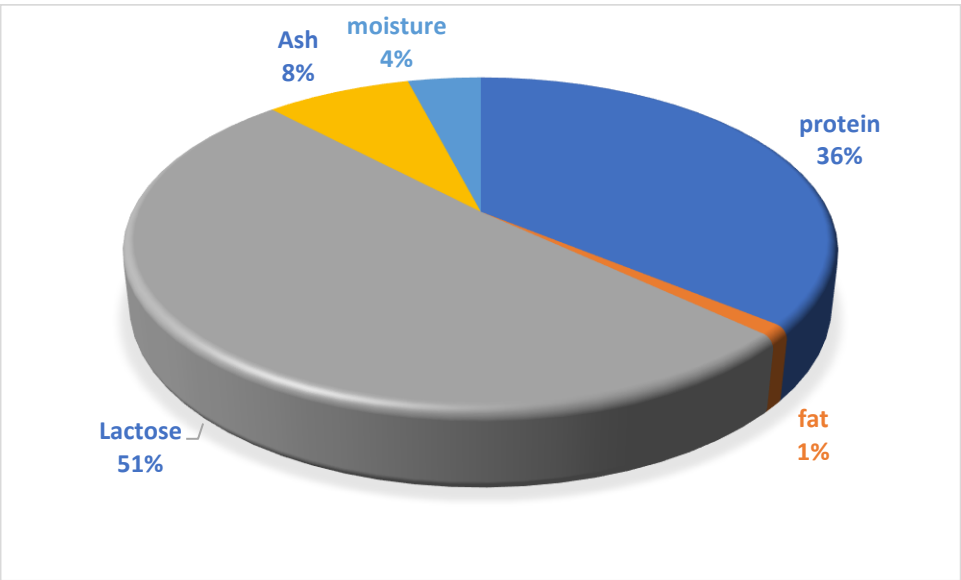

Figure 2. Approximate percentage of the components in SMP

2.2.3. Milk Protein Concentrate (MPC)

MPC contains both casein and whey protein (Ann Augustin et al., 2011). The protein content in MPC varies between 40% and 85% (Ann Augustin & Clarke, 2011). An example of MPC composition can be seen in Figure 3 (Ann Augustin et al., 2011).

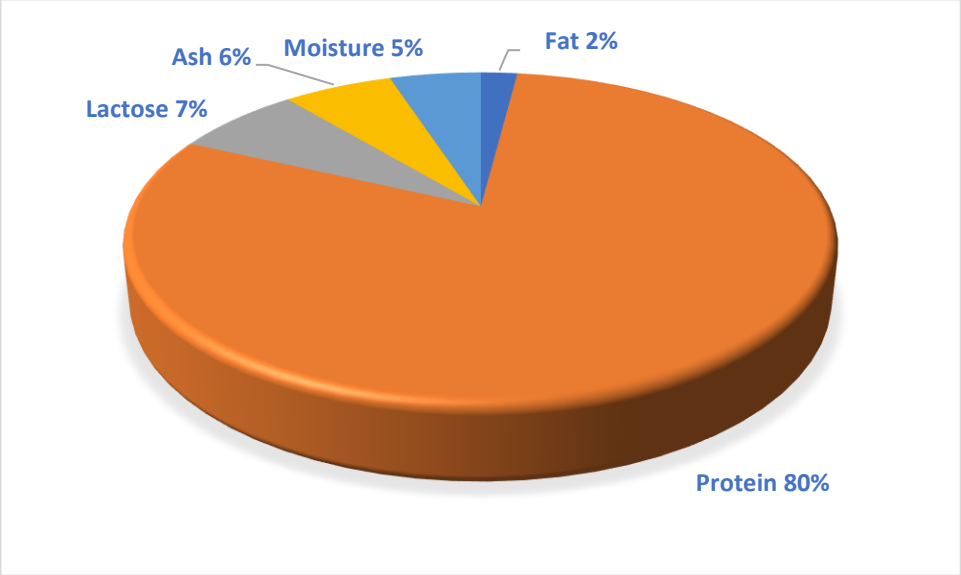

Figure 3. Approximate composition of a typical MPC.

2.2.4. Whey Protein Concentrate (WPC)

WPC is a by-product from cheese making after separation of casein and fat during milk coagulation (Spreer, 2017). WPC usually contains 65 to 80% w/w protein, 4.0 to 21.0% w/w lactose and 3.0 to 5.0% w/w minerals (Jelen, 2009). Generally, there are two types of whey; acid whey and sweet whey. Sweet whey is produced from cheese manufacturing while acid whey is produced from destabilisation of the milk casein colloid by acidification of milk to a pH under 5.0 (Jelen, 2009).

Both wheys have similar amounts of whey protein (approximately 8 g/L) and lactose (approximately 46 g/L); however they are quite different in the amounts of calcium (0.4 to 0.6 g/L for sweet whey and 1.2 to 1.6 g/L for acid whey) and lactic acid (2.0 g/L for sweet whey and 6.4 g/L for acid whey) (Jelen, 2009). Based on the percentage of the protein, there are several types of WPC available commercially; they are WPC 35, 55, 65, and 80. WPC 35 is usually used as a replacement for skim milk powder (Huffman &

Ferreira, 2011). It also can be used in products like yoghurt, bakery mixes, dietetic foods, and confectionary. WPC 55, 65 and 80 are usually used in the production of food that requires a protein boost such as nutritional drinks, tube feeding, sports and nutritional bars, soups, protein fortified beverages, bakery products, meat and animal feeding. WPC 80 also has good water-binding and thickening properties (Huffman & Ferreira, 2011). The approximate composition (% w/w) of WPC 80 can be seen in the Figure 4 (Chandan, 2011).

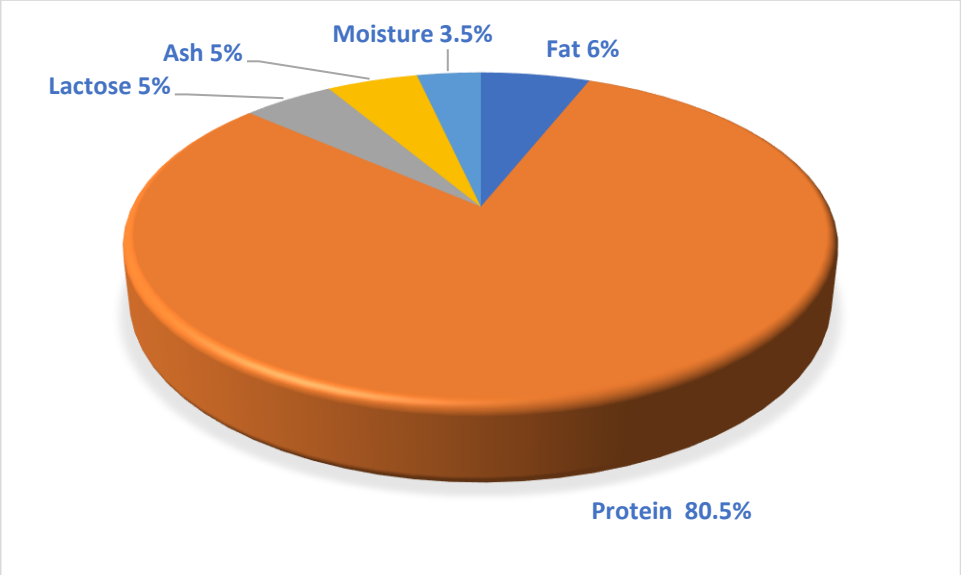

Figure 4. Approximate percentage of the components in WPC 80.

2.2.5. Buttermilk Powder (BMP)

BMP is a dairy product that should not have more than 7.0% w/w of moisture and highest fat content of 15.0% (Spreer, 2017). This product is a by-product from butter manufacture. BMP contains high level of phospholipids that causes this product to have a shorter shelf life compared to other dairy powders. This is because phospholipids can easily degrade causing off-odour and off-flavour such as SMP and WPC (Chandan, 2011). The United States Department of Agriculture (USDA) has a much stricter requirements for the buttermilk powder; it should contain at least 4.5% milk fat, less than 5% moisture and at least 30% protein (United States Department of Agriculture, 2001). BMP can be used in the production of ice cream, bakery products, dry mixes and confectionary (Chandan, 2011). The approximate composition of BMP can be seen in Figure 5 (Chandan, 2011).

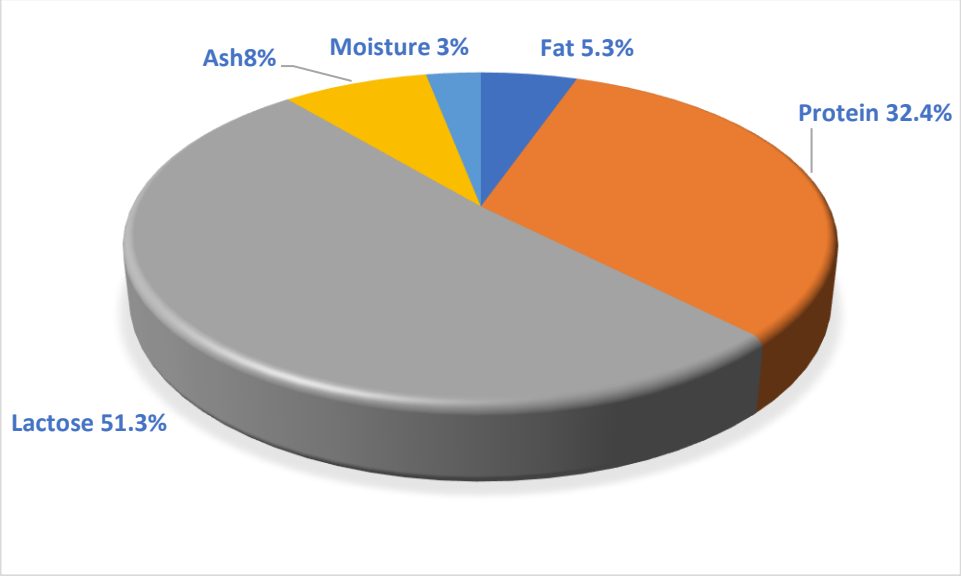

Figure 5. Approximate percentage composition in BMP.

2.3 Dairy Powder Manufacture

2.3.1 Milk Powders

The manufacture of milk powder is a simple process but carried out at a large scale. It is simply the removal of moisture from milk while ensuring that all the desired natural

properties of milk such as colour, nutrients, taste, and solubility are maintained. It is important that this process is done in a cost-effective way and under strict hygienic conditions. During the manufacture of milk powder, total solids are increased by boiling milk at low pressure and at low temperatures in a process known as evaporation. The concentrated milk is then spray dried to further remove moisture and produce powder. The skim milk and whole milk powder manufacturing process is shown in Figure 6 (Ann Augustin & Clarke, 2011; Augustin & Margetts, 2003; McHugh et al., 2017; Skanderby et al., 2009).

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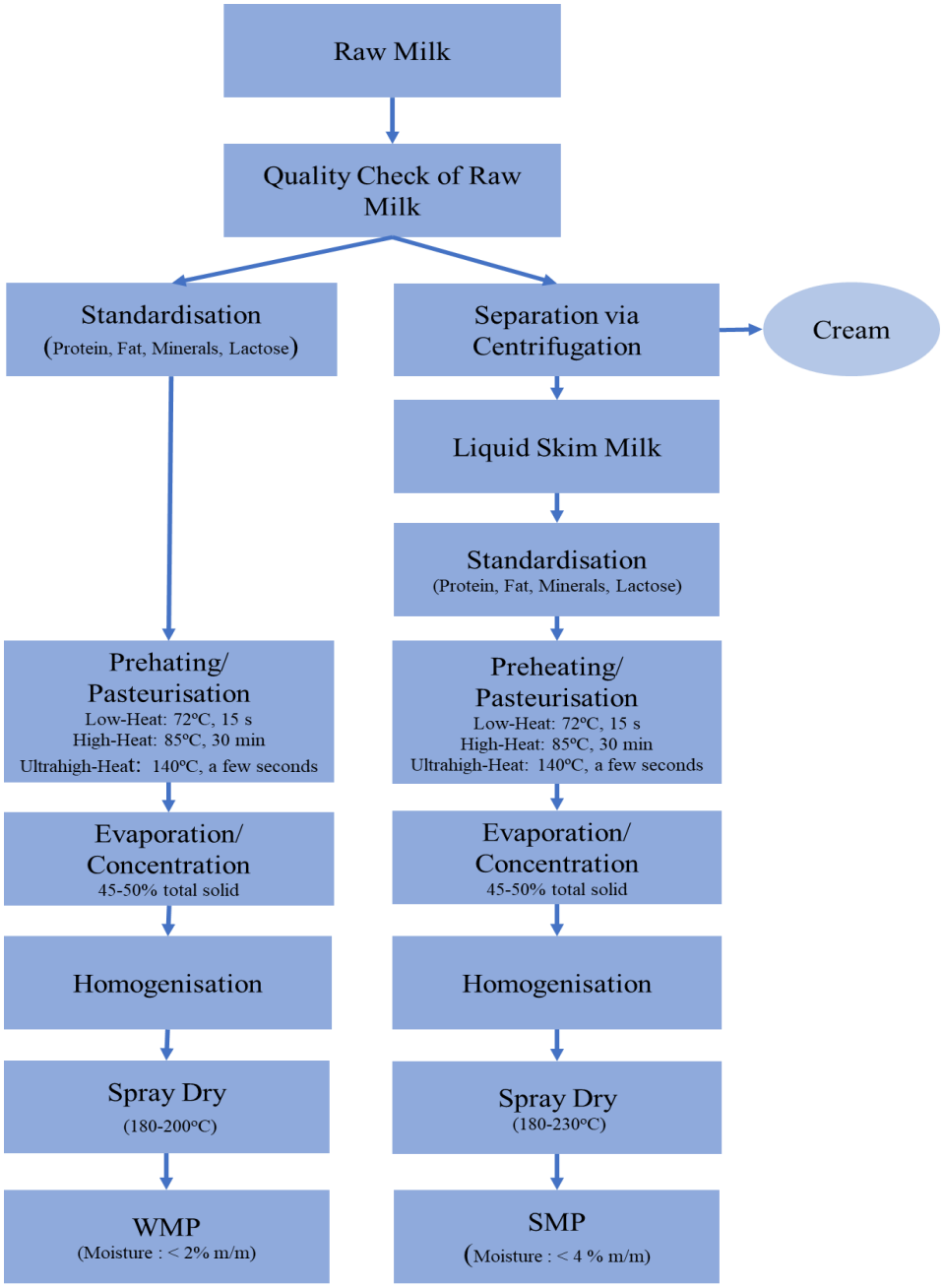

Figure 6. Flow chart about the production of SMP and WMP.

Raw milk must be transported from the farm to the factory at a low temperature in clean milk tankers. Refrigeration to keep milk temperature to 6°C or below 6°C must be maintained throughout transportation for a maximum of six hours to prevent bacterial growth (MPI, 2017). Standardisation is done to add or remove fat and or protein, to meet regulatory standards according to the product type that needs to be manufactured (Skanderby et al., 2009). If SMP is to be produced, liquid skim milk and cream will be separated using a centrifugal separator. For skim milk powder production, retentate is added to the skimmed milk in order to achieve low fat and high protein content. Surplus cream is used to make butter or anhydrous milk fat (Skanderby et al., 2009). The next step is preheating/pasteurisastion at 72^{o}C for 15 s. This step is very important to kill almost all pathogenic bacteria, psychrotropic and spoilage microorganisms, even though thermophilic and spore forming bacteria such as *Bacillus* spp. remain (Skanderby et al., 2009). This heating process also denatures the whey protein, and decreases the lipid oxidation rate, maintaining the milk quality (Skanderby et al., 2009). If the product is required to contain very low counts of spore forming bacteria, then high heat treatment is used (110-120°C for 4-12 s) (Skanderby et al., 2009). The next process of evaporation/concentration involves removing approximately 50% of the moisture using heat for more efficient spray drying. The evaporation process is very important to minimise energy consumption during the drying step. In the evaporator, heated milk is concentrated from a total solids of 13% (whole milk) and 9% (skim milk) to a higher total solids between 40 and 50% (Ann Augustin et al., 2011; Spreer, 2017). The milk then undergoes homogenisastion to reduce the surface free fat in milk powder to increase flowability, wettability and stability of milk powder during storage (Ann Augustin & Clarke, 2011).

The next step is drying. One of the most common drying methods is spray drying. Spray drying involves atomising (making small droplets of liquid) concentrated milk from the evaporator into fine granules. This is done in a large drying chamber in a hot air stream (up to 200^{o}C) using either a rotating disk atomiser or series of high-pressure lines (Walstra et al., 2006). The dryer will remove water from milk concentrate to produce long shel-life product (Ann Augustin & Clarke, 2011). Milk droplets are cooled by evaporation and they never reach air temperature. Concentrates may be heated before atomization to reduce viscosity and to increase the energy available for drying. Most of the remaining

water is evaporated in the drying chamber, leaving a fine powder of about 6% water content with an average particle size usually of <0.1 mm in diameter. Final or "secondary" drying takes place in a fluid bed, or in a series of such beds, where hot air blows through a fluidised powder layer, removing water to give the product a 2-4% final moisture content (Spreer, 2017). The amount of moisture which should be removed from liquid milk depends on the type of product the industry wants to produce. For example, in SMP and low fat milk powder, moisture must be less than 4% w/w. However for WMP and any high in fat milk powder, moisture must be less than 2% w/w to prevent fat oxidation during storage. The last step is packing to protect milk powder from moisture, oxygen, light and heat to maintain its quality and shelf life. Milk powder is prone to moisture absorption from the air, which causes rapid loss of quality and caking. Milk powder is packaged into either multi-layered plastic bags (25 kg) or bulk bins (600 kg) (Pearce, 2017). WMPs are often packaged with nitrogen gas to protect from oxidation and maintain their flavour. Bags generally consist of several layers to provide the necessary strength and barrier properties (Walstra et al., 2006).

2.3.2 Protein Powders

Other samples used in this project are milk protein concentrat (MPC) and whey protein concentrate (WPC). The manufacturing process for WPC is quite similar to SMP (Walstra et al., 2006): pasteurisation, separation, evaporation and spray drying (McHugh et al., 2017). The additional process in MPC and WPC processing is the membrane separation using ultrafiltration. The flow chart for the production of MPC and WPC can be seen in Figure 7 (McHugh et al., 2017; Md Zain, 2018; Mistry & Maubois, 2004; O'Kennedy, 2009).

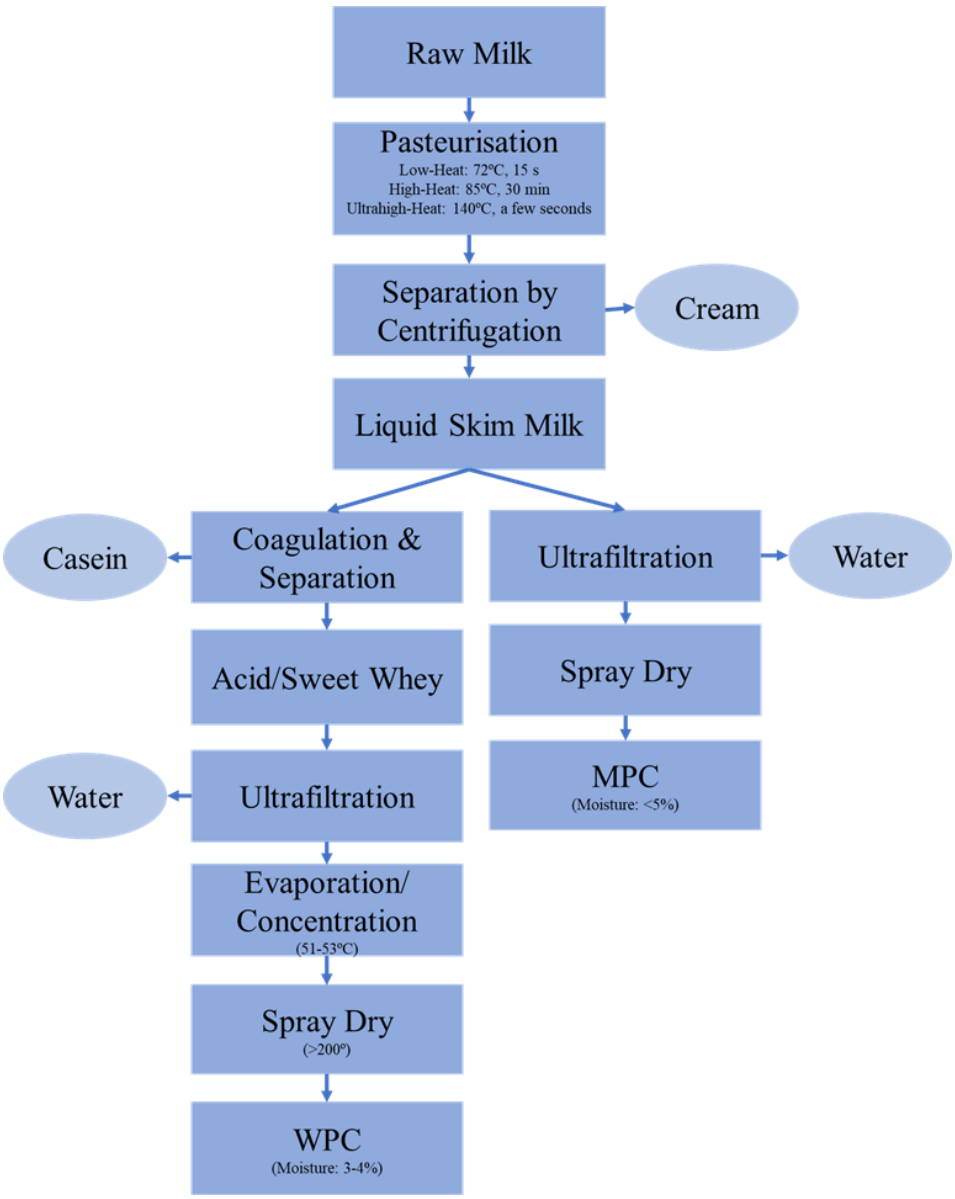

Figure 7. Flowchart about WPC and MPC production

2.3.3 BMP

BMP is another product used in this study. This product is obtained by drying buttermilk, a by-product of butter manufacture. The flow chart for the production of BMP can be seen in Figure 8 (Ann Augustin & Clarke, 2011; Augustin & Margetts, 2003).

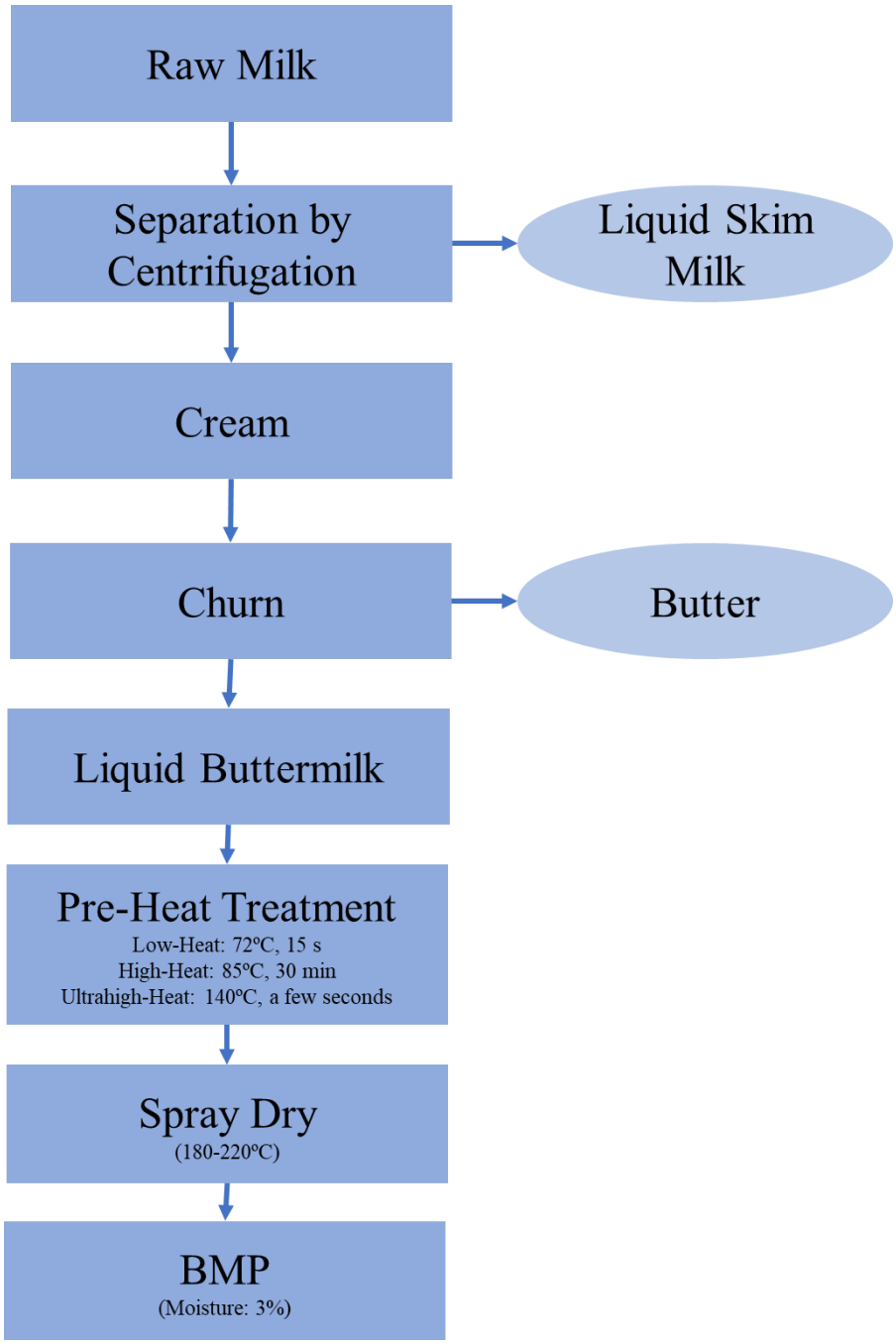

Figure 8. Flow chart about the production of BMP.

2.4. Standard Testing Methods for Dairy Powders

2.4.1 Overview of the Current Standard Testing Methods Used in the Dairy Industry

There are different standard testing methods used in the dairy industry in different countries. For example, the European Union has a standard testing method which is used by the dairy inductries in the union (European Union Reference Laboratory, 2015). Similarly, the American Public Health Association (APHA) has their standard methods for testing dairy products. Also, countries which are under the International Dairy Federation must comply to the testing methods set by the federation and hence there are some similarities and differences in the testing methods used by different countries. Some of the testing methods used by all the different agencies are the same such as testing for thermophilic spores (Wehr & Frank, 2004., International Dairy Fedaration/International Standard Organisations, 2013). This review focuses on the standard testing methods used by APHA and that of the International Dairy Federation (IDF) to test for milk and milk products.

The American Public Health Association has physical, chemical and microbiological test methods for dairy products. The physical and chemical tests include pH, acidity, density and freezing point (Wehr & Frank, 2004). For the microbiological tests, total aerobic plate count is used for mesophiles (bacteria that grow in moderate temperatures), thermophiles (bacteria that grow at high temperatures), and spore counts. A milk sample is plated standard methods agar (SMA), then incubated at 32ºC for 48 hours for total aerobic count and incubated at 55ºC for 48 hours for total thermophilic bacteria. This gives an estimate for the total mesophilic bacteria and total thermophilic bacteria that can grow in that condition (Wehr & Frank, 2004). Total spore count is tested by heating milk sample to 80ºC for 12 minutes. The high temperature (80ºC) is able to kill all vegetative cells and activate bacterial spores. After they are allowed to cool down, samples are diluted and plated on Standard Plate Count Agar (SPCA) containing 0.1% starch to enhance spore germination) and finally incubated at 32ºC for mesophilic spores (Wehr & Frank, 2004). There is no APHA method for thermophilic spores.

The IDF/ISO test method for the total aerobic plate count is done at 30ºC for 72 hours and uses Milk Plate Count Agar (MPCA), which is PCA/SMA containing 0.1% milk powder (International Dairy Fedaration/International Standard Organisations, 2004). The IDF/ISO method thermophilic bacteria is the same as that used by APHA. The standard testing methods used in both APHA and IDF/ISO are compared in Table 1.

Table 1. The comparison between the standard testing methods used by IDF/ISO and APHA for testing milk and milk products.

Standard	**Temperature for Aerobic Plate Count**	**Temperature for Total Thermophilic Count**	**Media**	**Reference**
Raw Milk				
IDF/ISO	30^{o}C, 72 hours	55^{o}C, 48 hours	MPCA	(International Dairy Fedaration/International Standard Organisations, 2013)
APHA	32^{o}C, 48 hours	55^{o}C, 48 hours	SPCA	(Wehr & Frank, 2004)
Milk Powder				
IDF/ISO	30^{o}C, 72 hours	55^{o}C, 48 hours	MPCA	(International Dairy Fedaration/International Standard Organisations, 2013)
APHA	32^{o}C, 72 hours	55^{o}C, 48 hours	SPCA	(Wehr & Frank, 2004)

2.4.2 Problems Associated with the Standard Testing Methods

The most commonly used microbiologial count method for counting bacteria in dairy products is the standard plate count (SPC). The SPC has been reported as a reliable and accurate method even though it is time consuming (Wehr & Frank, 2004). The international dairy organizations such as IDF and APHA accept and employ the use of SPC for bacteria enumeration in dairy products (Angelidis, 2015).

The ability of bacteria to grow in a wide range of temperatures is one major problem which makes bacterial testing difficult. Testing for just 30°C -32°C or 55°C may not give the exact microbial count in the sample (McHugh et al., 2017). The assessment of results is based on microbial limits set by the manufacturers, customers or regulatory authorities (National Research Council, 1985). The limit of some common bacteria associated with milk powder products is given in Table 2 (American Dairy Products Institute, 2020). During testing, the presence of certain bacteria especially pathogenic bacteria such as *B. cereus* in a dairy product makes testers classify the product as unsafe (MPI, 2016b). Bacteria are able to thrive in stress conditions produced by temperature, pH, water activity, salt stress etc. One adaptation to withstand stress is the formation of biofilms and spores (Lindsay et al., 2006; Majed et al., 2016). Some bacteria form a viable, non-culturable (VNC) state where they are not detected by standard testing methods (Fakruddin et al., 2013; Gunasekera et al., 2002). During favourable conditions, the VNC bacteria can become viable and can potentially cause spoilage or food borne illness.

Table 2. Microbiological specification for dairy powders.

Parameter	**Type of powder**				
	WMP	**SMP**	**MPC**	**WPC**	**BMP**
Standard Plate Count (CFU/g)	≤ 30,000	≤ 30,000	≤ 30,000	≤ 30,000	≤ 20,000
Coliform (CFU/g)	≤ 10	≤ 10	≤ 10	≤ 10	≤10
Listeria (CFU/g)	Absent	Absent	Absent	Absent	Absent
Salmonella spp (CFU/100 g)	Absent	Absent	Absent	Absent	Absent
Yeast and Molds (CFU/g)	<100	<100	<100	<100	<100

2.4.3. Techniques to Improve the Efficiency of Testing Methods

2.4.3.1. Techniques Used in Bacteria Enumeration

The inadequacy of using just the standard cultural testing methods to test for the microbial diversity in milk and milk products has been reported (Quigley et al., 2013). For example, Paszyn'ska-Wesołowska & Bartoszcze (2009); Yeung (2012) suggest that the use of standard cultural testing methods is not enough to determine the diverse microbial life that grows in milk and milk products. More efficient techniques have been developed to overcome the problems associated with standard testing methods. The flow cytometer is an instrument that can be used to enumerate bacterial cells based on optical detection and fluorescence (Ou et al., 2017). Its main advantage over plate count methods is the ability to enumerate single cells in large sample set (Gunasekera et al., 2000). Another microbial enumeration instrument is the Bactrac. The Bactrac enumerates bacteria growth using impedance analysis performed using calibrations established with standard plating methods (Faraji et al., 2014). The principle behind impedance microbiology is tracing the bacterial growth by measuring changes in the electrical conductivity (Bancalari et al., 2016). During bacterial growth, metabolic processes produce changes in the growth medium due to the metabolism of the nutrients in the growth medium resulting in the production of charged ionic components (Bancalari et al., 2016). The charged ionic components increase the electrical conductivity of the medium and this change is proportional to the number of bacteria, representing bacteria growth (Bancalari et al., 2016).

2.4.3.2. Techniques used in bacteria identification

Other new techniques include Polymerase Chain Reaction (PCR) and the 16S rDNA sequencing which are useful in the identification of bacterial species. PCR and 16S sequencing are able to identify culturable bacteria in dairy samples. For a more comprehensive test to identify both culturable and unculturable bacteria, a complete genome sequencing can be done by the use of high throughput sequencing machines (Fu et al., 2020).

The Polymerase Chain Reaction (PCR) is a technique that can be used to identify bacteria. The principle behind this technique is targeting the 'housekeeping genes'. These genes are highly conserved among different bacteria and easily help in identification of that bacteria (Woo et al., 2008). Specific primers are designed to target the 16S rDNA genes and they are amplified by PCR. There have been several uses of 16S rDNA in the dairy industry. The main disadvantage of 16S rDNA is its inability to differentiate between closely related species (Woo et al., 2008). There have been numerous applications of 16S rDNA sequencing in dairy research. Flint et al. (2001b) used 16S rDNA to identify *Anoxybacillus flavithermus. (A. flavithermus)* in milk powder samples. Also, Zain et al. (2016) and Li et al. (2019) used 16S rDNA to identify bacteria in WPC 80 and SMP.

The principle of the PCR technique is the amplification of the bacterial DNA encoding the 16S genome using primer pairs and DNA polymerase (Domingues, 2017). This method is rapid and provides a good, but not perfect, indication of the identification of bacteria (Harkanwaldeep et al., 2011; Kim et al., 2014). Real-time PCR enables enumeration of bacteria and may produce higher results than culturing/conventional methods as this method detects both culturable and non-culturable forms (Farhoudi et al., 2019). The disadvantage of PCR is that, it is more expensive than the traditional methods. Moreover, the use of the PCR requires trained personnel with the required skills to operate.

Another new and useful technique for bacterial identification is the matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). In this technique, bacterial DNA samples are fixed in a crystalline matrix and then bombarded with a laser. This leads to bacterial DNA absorbing the wavelength of the laser with the help of a detector which produces an output which is based on the mass to charge ratio value of ions in the sample's DNA (Hosseini & Martínez Chapa, 2016). MALDI-TOF has been found to be cheaper than next generation sequencing. Moreover, its sensitivity and rapid detection make it the choicest method for many institutes (Hosseini & Martínez Chapa, 2016., Singhal et al., 2015). The disadvantage of this technique is that identification of new isolates is feasible only if the spectral data base has peptide mass fingerprints of the type bacterial strains of specific species (Singhal et al., 2015).

2.5 Bacteria in Raw Milk and Milk Powder

2.5.1. Bacteria in Raw Milk

The high water content in raw milk results in a high water activity and an ideal environment for the growth of many bacteria. Bacteria found in raw milk include psychotrophs (eg.*Pseudomonas, Aeromonas, Seraratia, Acinetobacter, Hafnia, B. cereus, Microbacterium, Staphylococcus* and *Carnobacterium*), mesophiles (eg. *Bacillus subtilis* (*B. subtilis*)) and thermophiles (eg.*Geobacillus stearothermophillus* (*G. stearothermophillus*)) (Yuan et al., 2019). The number of bacteria in raw milk is an indicator of the quality of the milk which indicates the hygiene and cleanliness of the milking process, maintaining chilled temperatures during storage and transportation and the health of the cow udder (Spreer, 2017). In raw milk, we normally find Streptococci, Lactobacilli, Micrococci, Coliform, spore forming Bacilli and Clostridia (Spreer, 2017). Most pathogenic bacteria die in heat treatment/pasteurisation at 72°C for 15 s or equivalent except for some bacteria such as *B. cereus* and *Clostridum spp* (Skanderby et al., 2009). For example, a study by (Hill et al., 2012) found *Staphylococcus aurerus* (*S. aureus*), non pathogenic *Escherichia coli (E. coli)*, *Listeria monocytogenes* (*L. monocytogenes*), *Listeria innocua* (*L. innocua)*, and *Campylobacter* in New Zealand's raw milk. Table 3 gives some characteristics of some common bacteria associated with raw milk.

Table 3. The optimum and range of growth temperature of some bacteria found in raw milk.

Bacteria Name	**Optimum Growth Temperature (°C)**	**Range of Growth Temperature (°C)**	**Reference**
Pseudomonas	37	4 – 42	(Msalya, 2017; Zhang et al., 2019)
L. monocytogenes	30-37	-1.5 – 45	(Hill et al., 2012)
L. innocua,	30-37	-1.5 – 45	(Hill et al., 2012)

Listeria spp	30-37	-1.5 – 45	(Hill et al., 2012; MPI, 2013)
Klebsiella spp	35-37	15 – 40	(Msalya, 2017)
Proteus spp	37	-	(Msalya, 2017)
Campylobacter spp	42	30-47	(Hill et al., 2012; Marshall et al., 2016; Skanderby et al., 2009)
E. coli O157:H7	37	4-45	(Hill et al., 2012; Skanderby et al., 2009)
Non- STEC O157	37	-	(MPI, 2013)
Salmonella spp	37	5-46	(MPI, 2013)
S. aureus	37	4-46	(Ikeda et al., 2005; Walstra, 1999; Zhang et al., 2015)
B. licheniformis	37	31-76	(Kent et al., 2016; Sadiq et al., 2016)
Bacillus pumilus	37	31-76	(Ivy et al., 2012; Sadiq et al., 2016)
Geobacillus	55-65	37-75	(Kent et al., 2016)
Aeribacillus	50-65	30-70	(Kent et al., 2016)
Paenibacillus	30-40	<15-55	(Kent et al., 2016; Scheldeman et al., 2004)
Brevibacillus	30	-	(Kent et al., 2016)
Lysinibacillus	30-37	15-45	(Kent et al., 2016)
Anoxybacillus	50-65	40-68	(Kent et al., 2016)

2.5.2. Bacteria in Dairy Powder

During the manufacture of milk powder, the water content in raw milk is evaporated. The evaporation process involves heat treatment and this leads to reduction in the number of psychotrophs and other mesophiles. However, this condition is still favorable for the

growth of spore forming bacteria and thermophiles. This is evident in various studies which have been conducted on bacteria in milk powder (Gopal et al., 2015). For example, a study by VanderKelen et al. (2016) found that *B.licheniformis* was found in both raw milk and milk powder. The presence of *B. licheniformis* on milk powder processing surfaces has been attributed to its ability to form biofilms (Md Zain, 2018). *B. licheniformis* can contaminate in subsequent manufacturing steps on equipment such as cream separators, heat exchangers, preheaters and evaporators in dairy powder manufacturing plant (Md Zain, 2018).

Ronimus et al. (2003) identified *A. flavithermus*, *B. licheniformis*, *G. stearothermophilus* and *B. subtilis* in New Zealand's milk powders. A recent study from Li et al. (2019), showed that 68% of isolates from skim milk powder were *B. licheniformis*, 17% *Bacillus paralicheniformis* (*B. paralicheniformis*), and 13% bacillus species. H15-1. From these studies above, the majority of the bacteria in milk powders are spore formers and thermophiles.

Dairy powder is a product that is produced using high temperatures. Only, some spore forming bacteria and thermophilic bacteria are able to exist in dairy powder. Table 4 records a summary of published research on bacteria found in dairy powder.

Table 4. Summary of some bacteria associated with dairy powder

Name of Bacteria	**Type of Milk Powder**	**Growth Temperature (°C)**			**References**
		Min.	**Opt.**	**Max.**	
B. licheniformis	SMP, WMP WPC, IFP	15	30-45	50-55	(Li et al., 2019; Ronimus et al., 2003; Rückert et al., 2004; Sadiq et al., 2016; Skanderby et al., 2009; Zain et al., 2016)
B. cereus	SMP, WPC	5-20	30-37	45-48	(Li et al., 2019; Skanderby et al., 2009; Zain et al., 2016)

G. stearothermophillus	SMP, WMP, IFP	30-45	55-60	60-70	(Ronimus et al., 2003; Rückert et al., 2004; Sadiq et al., 2016; Skanderby et al., 2009)
A. flavithermus	SMP, WMP, IFP	30-38	55	65-72	(Burgess et al., 2010; Flint, et al., 2001; Ronimus et al., 2003; Rückert et al., 2004; Sadiq et al., 2016)
B. subtilis	SMP, WMP, WPC	6-20	30-40	45-55	(Ronimus et al., 2003; Rückert et al., 2004; Skanderby et al., 2009; Zain et al., 2016)
B. pumilus	SMP, WMP, WPC 80	5-15	30-37	50-55	(Burgess et al., 2010; Rückert et al., 2004; Zain et al., 2016)
Paenibacillus glucanolyticus	WPC 80	-	37	-	(Mathews et al., 2016; Zain et al., 2016)
B. coagulans	SMP, WMP	15-25	35-50	55-60	(Li et al., 2019; Rückert et al., 2004; Skanderby et al., 2009)
Bacillus thuringensis	WPC	-	30	-	(Zain et al., 2016)
Brevibacillus spp	SMP	-	30	-	(Li et al., 2019)
Lysinibacillus spp	SMP	15	30-37	45	(Li et al., 2019)
Bacillus sporothermodurans	-	20	-	45-55	(Burgess et al., 2010)
B. circulans	SMP, WMP	5-20	30-37	35-50	(Rückert et al., 2004)
Ureibacillus thermosphaericus	SMP, WMP	-	-	-	(Rückert et al., 2004)
C. botulinum	-	3	25-40	48	(Skanderby et al., 2009)
C. perfringens	-	8-20	-	50	Skanderby et al., 2009)

Clostridia *spp.*	SMP	3.3	25-40	80	(Brasca et al., 2020; Li et al., 2019)
B. circulans	SMP, WMP	5-20	30-37	35-50	(De Souza & Leal Martins, 2001; Rückert et al., 2004)
Enterococcus	SMP	10	35	45	(Araújo & Ferreira, 2013; Li et al., 2019)
Thermoactinomyces vulgaris	SMP, WMP, IFP	30	-	65	(Lacey, 1978; Sadiq et al., 2016)

From the table above, it can be seen that the most prevalent genus in dairy powder is *Bacillu*s. *Bacillus* is are ubiquitous genus and very diverse. The majority of the members of the *Bacillus* genus are non-pathogenic. However, *B. cereus* and *B. anthracis* are known pathogens. Some species produce toxins capable of causing foodborne illness (Gopal et al., 2015). The most prevalent *Bacillus* species in milk powder are *B. cereus* and *B. licheniformis*.

B. cereus is pathogenic bacteria that has been found in dairy powder (Becker et al., 1994). Some *B. cereus* strains produce toxins which can cause illness (Tewari & Abdullah, 2015), and some other *B. cereus* strains produce lipolytic and proteolytic enzymes that result in spoilage (Cressey et al., 2016). Most of them are mesophilic, and some strains can be psychotropic making them important in the spoilage of milk (Durak et al., 2006; Šimun et al., 2012).

B. licheniformis is the most frequently isolated bacterium found in milk powder or raw milk (Buehner et al., 2015; Kent et al., 2016). The high prevalence of *B. licheniformis* has been attributed to its ubiquitous nature. The prevalence of *B. lichineformis* in milk powder samples can be seen in table 5 below.

Table 5. Prevalence of *B. licheniformis* in different dairy powder.

Survey Sample	**Prevalence of Bacteria**	**Country**	**Reference**
IFP, WMP	36.8%	China	(Yuan et al., 2019)

NFDM	63%	US	(Buehner et al., 2015)
SMP	68%	Ireland	(Li et al., 2019)
SMP, WMP, IFP	43%	China	(Sadiq et al., 2016)
WPC	67%	New Zealand	(Zain et al., 2016)

In addition to *B. cereus* and *B. licheniformis*, three other genera are frequently associated with milk powder. These include *Geobacillus*, *Anoxybacillus* and *Paenibacillus.* The genus *Geobacillus* gets its name from high-temperature environments such as geothermal features (Marchant & Banat, 2010). They are common contaminants of milk due to their ability to survive pasteurisation and grow rapidly as biofilms at tempertures found in dairy manufacturing plant (Flint, et al., 2001a; Murphy et al., 1999). They are capable of producing spores and are good biofilm formers (Seale et al., 2012). One member of the *Geobacillus* genus which has become an important spore forming bacterium in whole and skim milk powder is *G. stearothermophilus (*Rückert *et al.,* 2004*).* According to a study by Scott et al. (2007), *Geobacillus* sp were found in all sites in a manufacturing plant. 21% of milk powder samples in China were identified as *G. stearothermophilus* (Sadiq et al., 2016).

The genus *Paenibacillus* is another important bacterium in the milk powder industry. *Paenibacillus* have been isolated from the soil, plants, water and food products. It has however been reported that, the most likely sources of contamination by *Paenibacillus* on dairy farms are silage and feed concentrates (te Giffel et al., 2002; Vaerewijck et al., 2001). *Paenibacillus* spores are found in both raw and pasteurized milk. Some common species of the genus *Paenibacilus* which have been isolated from UHT milk are *Penibacillus polymyxa* and *Paenibacillus lactis* (Heyndrickx et al., 2012). A distinguishing feature about *Paenibacillus* is their ability to survive high temperature short time (HTST) pasteurization as well as surviving refrigeration (Durak et al., 2006)

Anoxybacillus flavithermus, an isolate originally from the hot springs in New Zealand (Heinen et al., 1982) has also been associated with milk powders (Flint, et al., 2001b). *A. flavithermus* is a facultatively anaerobic thermophile which can grow within the range of 37°C - 65°C. The optimal growth has been found to be 62°C. Ronimus et al. (2003) reported that *A. flavithermus* are capable of thriving in aerobic habitats. According to Rückert et al. (2004), *A. flavithermus* is one of the three most common contaminants in

milk processing. The 8.5% of of isolates in milk powder samples incubated at 55°C in China were *A. flavithermus* (Sadiq et al., 2016). *Anoxybacillus* has the ability to form biofilms and contaminate the milk powder manufacturing plant.

The ability of aerobic spore forming bacteria to produce heat resistant spores and enzymes make them important in the dairy industry. The enzymes produced are capable of withstanding all heat treatments used in the dairy industry causing final product quality defects leading to spoilage and reduced shelf life (Sadiq et al., 2016). For example, spores produced by *G. stearothermophilus* reduced by only 25% after undergoing a heat treatment of 125°C for 30 minutes (Sadiq et al., 2016). Enzymes produced by *B. licheniformis* such as lipase and esterase cause spoilage in evaporated milk (Kalogridou-Vassiliadou, 1992).

Some taxa other than *Bacillu*s have also been implicated in the contamination of milk powder. The prominent among them is the genus *Clostridium*. Some members of the genus *Clostridium* which have been reported to be contaminants of dairy products include *Clostridium halophilum*, *Clostridium perfringens*, *Clostridium septicum* and *Clostridium botulinum* (Barash et al., 2010; Brett et al., 2005; Buehner et al., 2015). Members of the genus *Clostridium* are capable of producing spores and are strictly anaerobic. Among the members in this genus, the most important of them in terms of dairy powder safety is *C. botulinum* due to its ability to produce botulinium toxin. A study by Brett et al. (2005) reported that contamination of infant formula milk powder by *C. botulinium* caused infant botulism in a 5-month old baby.

The importance of incubating microbiological tests on milk powders at 37°C has been demonstrated. In research conducted by Zain et al. (2016), the biofilm formation of several strains of *B. licheniformis* in WPC 80 was compared at 30°C, 37°C and 55°C. They observed that, the majority of *B. licheniformis* strains produced strong biofilms during incubation at 37°C. This indicates that, testing for bacteria at 37°C might provide further vital information about the state of the bacteria which would not be revealed when incubated at 30°C (Zain et al., 2016)

According to Li et al. (2019), it is important to use more than one incubation temperature in the enumeration of bacterial populations in milk powder. This is because reliance on

only one temperature (30°C) failed to give an accurate identification of bacteria and the total population in skim milk powder. Using an incubation temperature of only 30°C will detect only a certain number of bacteria capable of growing at that temperature and therefore might give a false total bacterial count (Li et al., 2019).

In the assessment of mesophilic and thermophilic spores in Chinese milk powders, Sadiq et al. (2016) enumerated the bacteria numbers by incubating at 37°C for 24 hours on tryptic soy agar. In addition to the spore counts on plates, they also used 16S sequencing and Randomly Amplified Polymorphic DNA Analysis (RAPD) to analyse the diversity of bacteria species in milk powder. Their results indicate that, testing milk powder at 37°C when used in combination with molecular methods is useful in determining the bacterial composition in milk powders.

Despite varying, the optimum growth of thermophiles is generally between 40°C and 65°C (Scott et al., 2007). Dairy powders are subjected to high temperatures during manufacture and, therefore, bacteria that can survive until the end of the production are spore formers, including thermophiles such as *A. flavithermus* and *G. stearothermophilus*, and mesophiles such as *B. licheniformis*.

Based on the studies above, some bacteria that can be found in milk powders have the ability to grow at temperatures outside 30ºC and 55ºC. It is therefore important to test the growth of bacteria in different temperatures which are not included in the standard testing procedures. This study evaluated the effects of different incubation temperatures (30°C, 37°C, 55°C and 65°C) on the growth of bacteria in different dairy powders (WMP, SMP, MPC, WPC, BMP).

3. AIMS/OBJECTIVES AND HYPOTHESIS

3.1. Aims and Objectives

1. To compare the effect of different incubation temperatures (30°C, 37°C, 55°C, 65°C) used in enumerating total bacteria indifferent dairy powder.
2. To assess the feasibility of adding 37°C and 65°C incubation temperatures to standard testing methods used in counting bacteria in dairy powder.
3. To identify bacterial strains that grow at 30°C, 37°C, 55°C and 65°C

3.2. Research Question

Are the current standard methods used for bacteria enumeration using 30°C and 55°C incubation temperature sufficient to give an accurate count of total bacteria in dairy powder?

3.3. Hypothesis

The current methods used in testing for microorganisms (incubation temperature at 30°C and 55°C) in milk powder do NOT give an accurate number of the total microbial count in dairy powder.

4. METHODOLOGY

4.1. Samples

Five types of milk powders, WMP (30 samples), SMP (13 samples), MPC (11 samples), WPC (9 samples) and BMP (7 samples), were obtained from one dairy's manufacturing company in New Zealand. The samples in powdered form were received in foil-lined paper pouches. The manufacturing years of the samples were between 2016 and 2019.

4.2. Aerobic Plate Count

Enumeration of total bacteria for each sample was done at different incubation temperatures (30°C, 37°C, 55 °C and 65°C). Ten grams of each milk powder sample (WMP, SMP, MPC, WPC and BMP) was homogenised with 90 mL of 0.1 % warm (45°C +/- 0.1°C) buffered peptone water (GranuCult®, Merck, KGaA, Germany) using a Smasher™ Lab Blender (AES-Chemunix, USA) for 120 s at a speed of 250 rpm (International Dairy Fedaration/International Standard Organisations, 2001). Homogenates were serially diluted with 0.1% sterile buffered peptone water. One mL of the dilutions was transferred into a sterile petri dish and 15 mL of milk plate count agar (MPCA) (Oxioid LTD, Hampshire, England) (50°C-53°C) was added and gently shaken to ensure a uniform mix. Plating was done in triplicate. After the agar solidified, they were incubated in 30°C, 37°C, 55°C, and 65°C for 48 hours. For thermophilic counts, the agar needs an overlay so that the agar would not dehydrate at high temeprature especially at 65°C. After incubation, colonies formed on the agar were counted with a colony counter (aCOLade^2, Synbiosis, Synoptics LTD, UK) as the total number of aerobic bacteria. Colony counts between 25-250 per plate (Wehr & Frank, 2004) were calculated as colony forming units per gram (CFU/g) and converted to $\log_{10}$ CFU/g. The closest figure to 25 was chosen for the plates counts which were below 25.

To ensure confidence in our testing procedure, three samples with the same codes were each tested by two different researchers at the same time and the results obtained were compared with each other. The results can be seen in appendix B.

4.3. Bacteria DNA Isolation and Polymerase Chain Reaction (PCR)

Three to five colonies from each plate (Appendix G) was incubated in 10 mL Tryptic Soy Broth (TSB) (Bacto^{TM}, Becton, Dickinson and Company, USA) for 18-24 h at 30°C, 37°C, 55°C, and 65°C. After incubation, three µL of the culture was used as template DNA and was transferred into a Platinum Green Hot Start PCR 2X Master Mix (Invitrogen by Thermo Fisher Scientific, Lithuania). The PCR mix consisted of 25 µL of Platinum Green Hot Start PCR 2X Master Mix and 20 µL DNA free water (Invitrogen by life technologies UltraPure™ Distilled water, USA). The Universal primers used for the 16S rDNA PCR were Bac27F (5'- AGAGTTTGATCATGGCTCAG-3') and U1492R (5'-TACGGCTACCTTGTTACGACTT-3') (Christison et al., 2007; Flint et al., 1999).

One µL of both forward and reverse primers were used. The PCR master mix with DNA was run with the ProFlex PCR system (Applied Biosystems by life technologies, Singapore). The program of the PCR cycle was: Denaturation at 94ºC for 5 min, annealing at 96ºC for 25 s, 50ºC for 45 s, 72ºC for 2 mins for 30 cycles, and a final extension at 72ºC for 7 mins. The amplified PCR products were visualized with agarose gel electrophoresis. The gel electrophoresis was done by pipetting 7 µL aliquots of the amplified PCR product into wells of E-Gel EX Agarose 2%. Six µL of E-Gel Low Range Quantitive DNA for both ladder and buffer was used as the marker. The agarose gel was then slotted into the iBase of the E-Gel Pre-cast Agarose Electrophoresis System and was allowed to run for 11 mins. The amplified products were purified with a spin column. One hundred and fifty µL of DNA binding buffer was added to each 30 µl of amplified DNA products. The mixture was transferred to a spin column in a collection tube. It was centrifuged at 10 rpm for 1 min. The flow which came through was discarded and washed twice with 200 µL of wash buffer. Centrifugation at 10 rpm for 1min was done and 6 µL and 4 µL of sterile DNA elution buffer was added to the column matrix. Finally, they were centrifuged to obtain pure amplicons and purified products were checked with the nanodrop microvolume spectrometer (Colibri, Titertek-Berthold, Germany).

4.4 16S rDNA sequencing

Purified DNA was sent to the Massey Genome sequencing unit for sequencing. Forty-eight DNA from 3 milk powder samples tested at all temperatures used in this study were sent. Each had 2 different primers Bac27F (5'- AGAGTTTGATCATGGCTCAG-3') and U1492R (5'-TACGGCTACCTTGTTACGACTT-3') NCBI Blast and RDP seqmatch were used to manually check for the partial 16 S rDNA sequences and the corrected sequence used in BLAST was used to find their closest homologues.

4.5 Statistical Analysis

For the statistical analysis, the mean number of bacteria recovered from milk powder samples at the different incubation temperatures were analysed with Minitab 19 statistic programming (Minitab, LLC., USA) to test for T-test, One way analysis of variance (ANOVA) using Tukey's test at a 95% confidence ($p < 0.05$). The mean, standard deviations (SD), standard errors (SE) and all graphics were done in excel (Microsoft, USA).

5. RESULTS AND DISCUSSION

From a total of 70 milk powder samples tested (Appendix A), 35 of the dairy powder samples had higher aerobic plate counts when tested at 37°C as compared to 30°C although this was not statistically significant ($p > 0.05$) (Appendix C). The average bacterial count during testing at 30°C was found to be 2.27 $\log_{10}$ CFU/g while testing at 37°C resulted in 2.26 $\log_{10}$ CFU/g. Similarly, from a total of 70 dairy powder samples, 26 had higher aerobic plate counts when tested at 65°C than at 55°C. Testing for total bacteria at 65°C supported the growth of certain bacteria; however, the number of bacteria capable of growth at 55°C was significantly higher ($p < 0.05$) than at 65°C (Appendix D). The average bacterial count during testing at 55°C was found to be 1.78 $\log_{10}$ CFU/g while testing at 65°C resulted in 1.54 $\log_{10}$ CFU/g (Appendix D). From the results, it can be seen that testing for aerobic plate counts in milk powders at 30°C and 55°C as its currently done in the dairy industry (IDF/ISO) are appropriate for enumerating total mesophilic and thermophilic bacteria.

However, the number of bacteria enumerated at 37°C and 65°C could have been higher if the sampling size was increased. This can be seen in the different milk powder samples which were tested. Out of the 30 WMP samples tested, 19 of them had higher aerobic plate count numbers at 37°C which represents about 63% of the WMP samples. For SMP, a total of 13 samples were tested and 5 of them had higher bacteria numbers at 37°C which is about 38%. Only 29 % of the BMP samples tested had bacteria numbers higher during testing at 37°C. This may be due to the low sampling size (7) of the BMP samples.

The comparison of the percentage of bacteria count tested at 30°C and 37°C can be seen in the Figure 9 below.

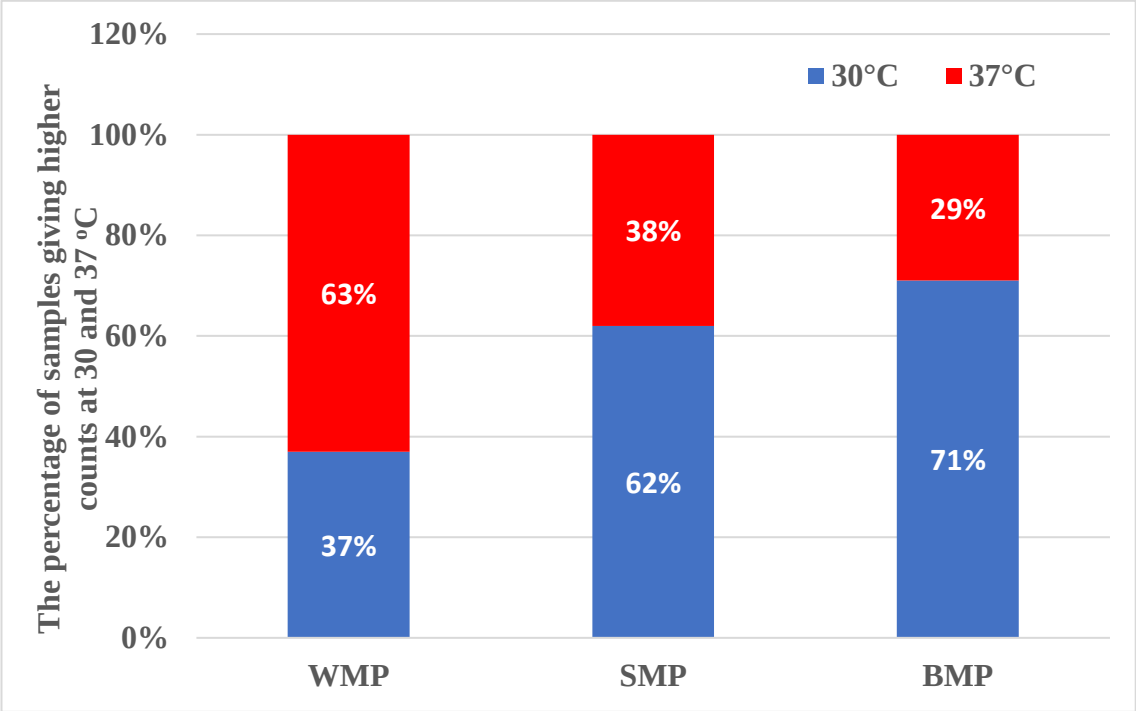

Figure 9. A comparison of bacteria counts in WMP, SMP and BMP tested at 30°C and 37°C.

The average total bacteria count found in all dairy powder samples tested for all temperatures ranged from 0.86 to 2.80 $\log_{10}$ CFU/g. For WMP, SMP, MPC and WPC, bacteria counts at 30°C and 37°C were higher than 55°C and 65°C. This indicates that most of the bacteria in WMP, SMP, MPC and WPC were mesophilic and possibilities of thermotolerant bacteria. These results are similar to Zain et al. (2016) work, which found that the total mesophilic bacteria (incubation at 30°C) in WPC was higher than thermophilic bacteria (incubation at 55°C). The number of bacteria isolated from BMP at 30°C and 37°C were almost the same at 55°C and 65°C suggesting that BMP contained equal numbers of both mesophiles and thermophiles. An alternative explanation is that the bacteria present were capable of growth at both mesophilic and thermophilic temperatures (thermotolerant). MPC had the highest average total number of bacteria growing at 30°C and 37°C with 2.80 and 2.76 $\log_{10}$ CFU/g respectively. The lowest average total number of bacteria was seen in WPC at 65°C with 0.86 $\log_{10}$ CFU/g. The

average number of bacteria counted at each temperature for each product can be seen in Figure 10 below.

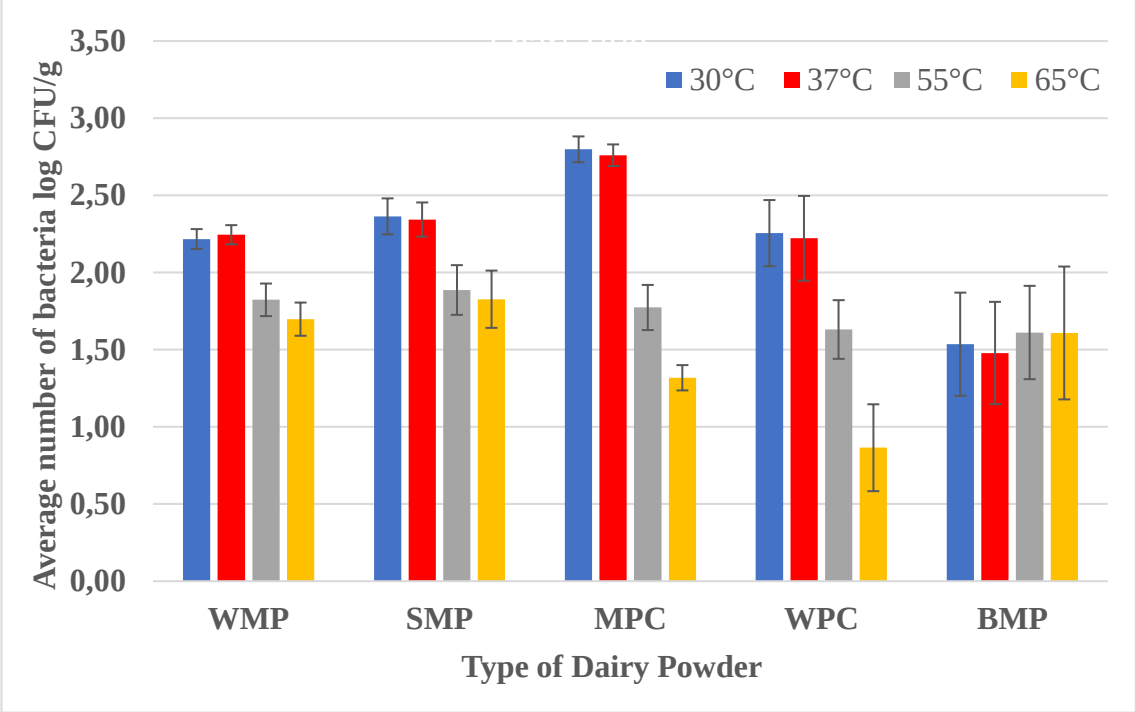

Figure 10. The mean and SE of total number of bacteria in WMP, SMP, MPC, WPC and BMP.

Statistical analysis revealed that the type of dairy powder sample can have an effect on the number of bacteria. Using One Way Anova and Tukey pairwise comparison test, there were significant differences ($p < 0.05$) in the number of bacteria found in the different types of dairy powder at 30°C, 37°C, and 65°C. For example at 30°C and 37°C , BMP was significantly different from the rest of the dairy powder samples. On the other hand, there were no significant differences in all dairy powder samples at 55°C ($p > 0.05$). This may be due to the fact that only thermophiles are capable of growth at that temperature. The results indicate that there was no significant difference in the number of bacteria in SMP and WMP at all temperatures (30°C, 37°C, 55°C, 65°C) (Appendix F).

5.1. Comparison of the Number of Bacteria in Dairy Powders Found in the Present Study with Other Studies

5.1.1. Testing for Bacteria at 30°C

Some authors have reported total bacteria count in dairy powder samples. Li et al. (2019) recorded a range from <1 to 3.15 $\log_{10}$ CFU/mL bacteria in their SMP samples at 30°C. This data is quite similar to the results in the present study with the number of bacteria in SMP samples at 30°C from 1.3 to 2.92 $\log_{10}$ CFU/g. On the other hand, Zain et al. (2016) recorded a range from 4-5.77 $\log_{10}$ CFU/g from in their WPC 80 samples. These results are much higher than the present study (0.7-3.16 $\log_{10}$ CFU/g) but were selected for investigation based on unusually high counts.

5.1.2. Testing for Bacteria at 37°C

Several authors have reported a higher bacterial count when milk powder was tested at 37°C compared with 30°C. A study conducted by Yuan et al. (2012) reported that some strains of thermophilic bacillus were able to grow on plate count agar (PCA) at 37°C after 48 hours. They suggested that high aerobic plate counts at 37°C have a strong correlation to a high thermophile count at 55°C. Unfortunately, the authors did not report the exact number of bacteria they counted during incubation at 37°C (Yuan et al., 2012). In a similar research, Sadiq et al (2016) found an average of 2 $\log_{10}$ CFU/g of mesophilic spore count in Chinese milk powder samples during testing at 37°C. Likewise, Zain et al. (2016) also did not count the total number of total bacteria in WPC at 37°C. However, they compared the biofilm formation of several *B. licheniformis* isolates at 30°C, 37°C and 55°C. They observed that the highest number of *B. licheniformis* isolates (33 isolates) formed biofilms at 37°C on microtiter plates in TSB medium (Zain et al., 2016).

5.1.3 Testing for Bacteria at 55°C

Several studies tested total thermophilic bacteria at 55°C since milk powder is an ideal medium for the growth of thermophile (Wehr & Frank, 2004). Many thermophilic bacteria grow at 55°C (Wehr & Frank, 2004). From the present work, the average number

of thermophiles tested at 55°C found in WMP and SMP was 1.82 and 1.89 $\log_{10}$ CFU/g respectively. This present study agrees with other studies which have been reported. For example, in a study done by Sadiq et al. (2016), the average number of thermophiles in WMP and SMP that were manufactured in Heilongjiang, China was found to be 1.81 and 2.65 $\log_{10}$ CFU/g respectively.

However, there are other studies which have found much higher results than in this present study. Reginensi et al. (2011) found the average number of thermophiles in Uruguayan skim and whole milk powders tested at 55°C to be approximately 3 $\log_{10}$ CFU/g. Rajput et al. (2009) found 2.04-2.69 $\log_{10}$ CFU/g thermophiles in SMP and 2.59-2.77 $\log_{10}$ CFU/g in WMP from Pakistan.

Rückert et al. (2004) compared thermophilic Bacilli in milk powders from 18 different countries. The number of thermophiles in WMP and SMP obtained from New Zealand was 2.85 $\log_{10}$ CFU/g and 3.04 $\log_{10}$ CFU/g respectively. There were higher number of thermophiles in milk powders from France, Great Britain and USA. WMP sample from France had 4.54 $\log_{10}$ CFU/g thermophiles and SMP sample from Great Britain and USA had higher counts of 4.60 $\log_{10}$ CFU/g and 5.34 log CFU/g respectively (Rückert et al., 2004). The findings from Rucket et al. (2004) showed the numbers of thermophiles counts from France, Great Britain, and USA exceeded the average acceptable limits for thermophiles (Table 2). Possibilities of biofilms formation due to insufficient cleaning practices or poor quality of raw milk. This suggests that the number of thermophiles in milk powder varies between countries and this may be due to different microbial limits in dairy products or variations in manufacturing processes.

5.1.4 Testing for Bacteria at 65°C

To the best of my knowledge, there is no research work done on the total thermophilic bacteria testing at 65°C. However, based on the results, some isolates from 26 dairy powder samples had better growth when tested at 65°C than at 55°C, and the difference is significant (p<0.05) (Appendix E). This indicates that, even though a relatively low number of bacteria are capable of growth at 65°C, it is still important for dairy companies to consider testing at 65°C. There is a likelihood that the neglect of testing at this

temperature may prevent the dairy industry from identifying certain strains of bacteria capable of growth at 65°C. In a research by Ronimus et al. (2003), it was shown that *A. flavithermus* and *G. stearothermophilus* found in milk powder grew strongly at 65ºC. In another study by Karaca et al. (2019), *A. flavithermus* was able to form higher quantity of biofilms on stainless steel coupons at 65°C than 55°C.

5.2 PCR and 16S rDNA sequencing

The results (Table 6) of the 16S rDNA sequencing of bacterial isolates from milk powder samples showed *B. licheniformis* as the most dominant bacterial isolate when testing was done at 30°C and 37°C.

Table 6. 16S rDNA sequencing results of isolates obtained from dairy powder samples

Dairy Powder Sample	Incubation Temperature Used to Isolate Bacteria	No. of Colonies Sampled	Sequencing Result
WMP (Sample No. 23)	30°C	5	All are *B. licheniformis*
	37°C	4	All are *B. licheniformis*
	55°C	4	All are *G. stearothermophilus*
	65°C	3	All are *G. stearothermophilus*
WPC (Sample No 59)	30°C	4	All are *B. licheniformis*
	37°C	4	All are *B. licheniformis*
	55°C	4	All are *A. flavithermus*
	65°C	4	All are *A. flavithermus*
BMP (Sample No. 65)	30°C	4	All are *B. licheniformis*
	37°C	5	Four of them are *B. licheniformis*
			One of them *is B. pumilus*
	55°C	4	All are *A. flavithermus*
	65°C	3	All are *A. flavithermus*

B. licheniformis represented 52% of the isolates, followed by *A. flavithermus* accounting for 31% of the isolates while *G. stearothermophilus* and *B. pumilus* were found to be 15% and 2% respectively (Figure 11). Interestingly, the dominant bacteria detected at both 30°C and 37°C was *B. licheniformis*. The dominant bacteria detected at both 55°C and 65°C for each milk powder sample was found to be the same. In the case of BMP and WPC, *A. flavithermus* whereas for WMP, *G. stearothermophilus*. It is interesting Another interesting observation from this study is the consistency in the number of bacteria of bacteria isolates from milk powder when tested at 30°C and 37°C. For example, the number of *B. licheniformis* isolated from WMP at both 30°C and 37°C were about 2.2 $\log_{10}$ CFU/g whilst testing at both 55°C and 65°C resulted in 1.7 $\log_{10}$ CFU/g of *G. stearothermophilus*.

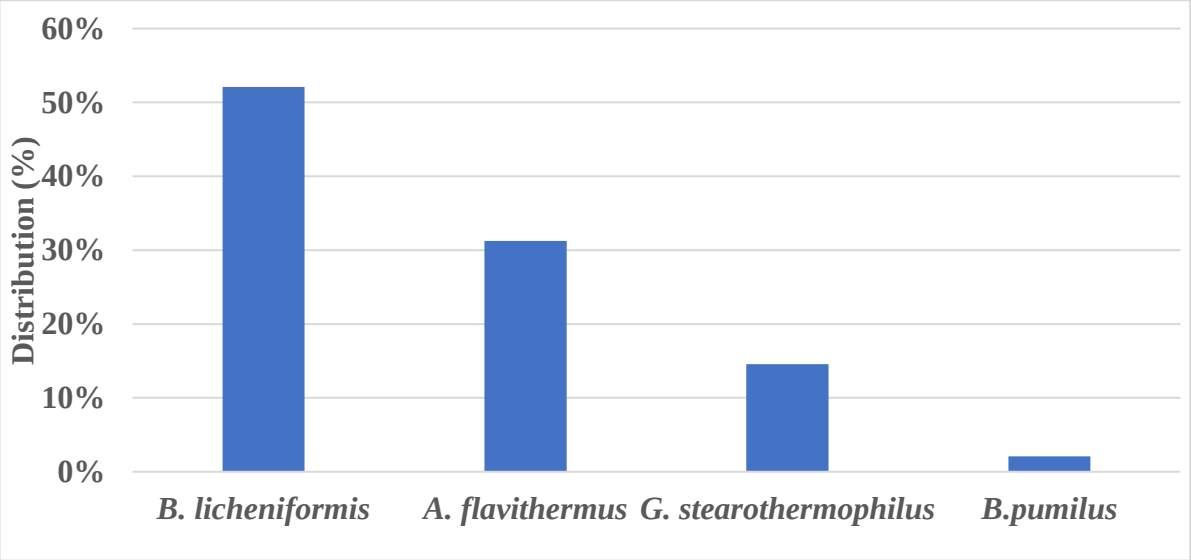

Figure 11. Species distribution of 48 isolates selected for 16S sequencing.

The identity of samples tested at 30 and 37 °C were confirmed as *B. licheniformis*. Other researchers Li et al. (2019); Zain et al. (2016) have also found *B. licheniformis* from 16S rDNA sequencing of isolates from dairy powder samples tested at 30°C. For example, Li et al. (2019) found *B. licheniformis* as the most dominant bacteria in Irish dairy powder tested at 30°C. Similarly, Zain et al. (2016) found *B. licheniformis* as the dominant bacteria in WPC tested at 30°C. Due to the ability of *B. licheniformis* to persist in dairy products as well as surviving in high temperatures (range of growth 15°C-55°C), they are considered as the most prevalent bacteria species in dairy powders leading to their high

prevalence in dairy products. The growth of *B. licheniformis* at 37°C has also been reported by other authors (Ronimus et al., 2003; Sadiq et al., 2016; Zain et al., 2016). Ronimus et al. (2003) reported that *B.licheniformis* showed strong growth at 37^{o}C. Sadiq et al. (2016) found *B.licheniformis* spores in milk powder samples when testing was done at 37°C. From this study, *B. licheniformis* was confirmed at both 30°C and 37°C.

Other researchers have also isolated *B. pumilus* in dairy powder samples (Sadiq et al., 2016; VanderKelen et al., 2016; Zain et al., 2016). In this current study, one isolate from milk powder tested at 37°C was found to be *B. pumilus*. *B.licheniformis* and *B.pumilus* are capable of causing spoilage in milk powder samples. Most of the studies about bacteria causing spoilage in dairy products have focused on *B. cereus* (Yoo et al., 2014). However, many authors have also confirmed *B. licheniformis* and *B. pumilus* as important spoilage bacteria due to their ability to produce proteolytic enzymes (Reginensi et al., 2011). In addition, the thermo-tolerant ability of *B. licheniformis* enables it to survive pasteurisation. *B. licheniformis* is also a strong biofilm former and therefore can attach and survive on equipment surfaces. These abilities make it extremely difficult for the dairy industry to control or reduce *B. licheniformis* contamination (Gopal et al., 2015).

Sequencing of isolates during testing at 55°C and 65°C revealed *G. stearothermophilus* and *A. flavithermus* as the dominant bacterial species. *G. stearothermophilus* and *A. flavithermius* are the two main thermophilic bacteria routinely found in milk powder making them important contaminants in milk powder (Burgess et al., 2010). The prevalence of both *G. stearothermophilus* and *A. flavithermus* in dairy powder during testing at 55°C has been reported by several authors (Ronimus et al., 2003; Rückert et al., 2004; Sadiq et al., 2016; Scott et al., 2007). Ronimus et al. (2003) found the ability of both bacteria to grow strongly at 65°C. Even though they are not pathogens, their occurrence in milk powder samples is an indication of bacterial contamination which might be due to unhygienic processing or long manufacturing runs. Excessive numbers of these bacteria in dairy products have been reported as undesirable for consumption since they have ability to form biofilm and cause spoilage, making the product exceed the specifications of customers and regulatory agencies (Md Zain, 2018).

Interestingly, in this study, WMP was dominated by *G. stearothermophilus* whilst WPC and BMP were dominated by *A. flavithermus*. Some of the findings from this study in agreement with the findings of Karaca et al. (2019) who reported that *Geobacillus* formed strong biofilms and a high spore count in whole milk. On the other hand, *Anoxybacillus* formed a strong biofilm and had a high spore count in skim milk. A possible reason for this observation might be due to the composition of the milk. It seems that the fat content in whole milk prevent the growth of *A. flavithermus*. Further research to determine the relationship between the fat content of different milk powders and the growth of *A. flavithermus* will be interesting to investigate. In addition, research to understand the correlation between the milk powder type and the number of bacteria, also the bacteria species capable of forming high biofilms and high spore count will be important to the dairy industry.

6. CONCLUSION

This research investigated different incubation temperatures (30°C, 37°C, 55°C and 65°C) used for testing of total bacteria in dairy powder. The number of bacteria identified during 30°C was not significantly different from that of 37°C. Even though testing at 65°C supported the growth of some bacteria, the number of bacteria identified when testing was done at 55°C was significantly higher than 65°C. Some of the dairy powder samples had isolates which had better growth at 37°C and 65°C. The results revealed *B. licheniformis* as the dominant isolate (52%) from the dairy powder samples used in this study when tested at 30°C and 37°C. *A. flavithermus* (31%) and *G. stearothermophilus* (15%) were other isolates identified in this study when tested at 55°C and 65°C. The results of this study indicated that the current testing temperatures (30°C and 55°C) used in the dairy industry are satisfactory; however testing for total bacteria at 37°C and 65°C might be an added advantage since these temperatures have been found to favour the growth of specific species such as *B. licheniformis*, *A .flavithermus* and *G. stearothermophilus*. The limititions in this study include the uneven number of samples in the different types of dairy powder. Further research to use the same number of the different types of dairy powder as well as including the sample size will be important to the dairy industry. In addition, research to determine the relationship between the fat content of different milk powders and the growth of *A. flavithermus* will be interesting to investigate. Lastly, research to understand the correlation between the milk powder type and the number of bacteria, also the bacteria species capable of forming high biofilms and high spore count will be important to the dairy industry.

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8. APPENDICES

Appendix A. Number of bacteria in WMP, SMP, MPC, WPC and BMP samples.

No	Sample Types	AverageNumber of Bacteria (log CFU/g ± SD)			
		30ºC	**37ºC**	**55 ºC**	**65 ºC**
1	WMP	1.70±0.00	1.72±0.12	1.35±0.16	1.71±0.15
2	WMP	2.30±0.15	2.38±0.11	1.99±0.21	1.19±0.20
3	WMP	2.39±0.10	2.65±0.14	2.43±0.17	1.29±0.16
4	WMP	3.12±0.02	2.97±0.09	2.79±0.24	2.08±0.25
5	WMP	2.39±0.05	2.19±0.10	1.61±0.23	0.47±0.40
6	WMP	2.10±0.05	2.15±0.05	1.57±0.26	1.83±0.05
7	WMP	1.46±0.28	1.54±0.24	1.73±0.14	1.55±0.13
8	WMP	2.37±0.07	2.32±0.04	0.90±0.17	2.23±0.34
9	WMP	2.57±0.12	2.58±0.13	2.30±0.06	2.33±0.21
10	WMP	2.26±0.07	2.27±0.05	1.30±0.00	1.00±0.00
11	WMP	2.61±0.10	2.77±0.05	2.62±0.05	1.97±0.12
12	WMP	2.38±0.20	2.41±0.11	2.11±0.13	2.08±0.07
13	WMP	2.32±0.06	2.30±0.07	1.40±0.46	1.63±0.06
14	WMP	1.98±0.07	2.21±0.03	2.75±0.01	2.69±0.03
15	WMP	2.35±0.08	2.29±0.01	1.79±0.20	1.81±0.13
16	WMP	1.84±0.06	1.98±0.03	1.30±0.00	1.00±0.00
17	WMP	2.28±0.12	2.45±0.02	2.39±0.07	1.85±0.13
18	WMP	2.26±0.04	2.33±0.07	1.97±0.19	1.62±0.28
19	WMP	1.36±0.10	1.40±0.17	1.56±0.24	1.94±0.19
20	WMP	2.26±0.05	2.12±0.13	1.94±0.12	1.98±0.14
21	WMP	1.74±0.13	1.64±0.30	1.30±0.30	1.10±0.17
22	WMP	2.09±0.02	2.08±0.06	1.37±0.19	1.24±0.09
23	WMP	2.42±0.08	2.32±0.11	3.50±0.08	3.66±0.09
24	WMP	2.20±0.05	2.24±0.03	1.64±0.12	1.49±0.28
25	WMP	2.50±0.05	2.48±0.11	1.78±0.00	1.82±0.07
26	WMP	1.93±0.07	2.05±0.05	1.73±0.15	1.84±0.03
27	WMP	2.59±0.07	2.49±0.04	1.44±0.28	1.48±0.18

28	WMP	2.35±0.07	2.38±0.07	1.21±0.45	1.42±0.17
29	WMP	2.21±0.19	2.25±0.05	1.68±0.07	1.39±0.09
30	WMP	2.17±0.07	2.39±0.02	1.25±0.13	1.25±0.13
31	SMP	2.16±0.11	2.17±0.09	1.37±0.44	1.30±0.43
32	SMP	2.25±0.25	2.39±0.09	2.08±0.11	1.77±0.07
33	SMP	2.34±0.12	2.23±0.20	1.51±0.47	1.29±0.53
34	SMP	1.97±0.01	1.63±0.06	1.38±0.14	1.06±0.10
35	SMP	2.86±0.06	2.84±0.03	2.54±0.17	2.19±0.07
36	SMP	2.39±0.06	2.42±0.03	1.72±0.36	1.85±0.05
37	SMP	2.82±0.03	2.81±0.07	2.06±0.08	2.57±0.06
38	SMP	2.38±0.10	2.45±0.07	1.93±0.25	1.99±0.29
39	SMP	2.46±0.08	2.37±0.07	1.20±0.35	1.00±0.00
40	SMP	2.43±0.06	2.34±0.02	1.62±0.15	1.40±0.17
41	SMP	2.45±0.07	2.40±0.06	2.59±0.14	2.66±0.02
42	SMP	2.92±0.05	2.86±0.04	1.39±0.36	1.49±0.43
43	SMP	1.30±0.00	1.56±0.24	3.14±0.09	3.18±0.12
44	MPC	3.00±0.04	3.02±0.05	2.38±0,07	1.36±0,22
45	MPC	3.01±0.02	2.92±0.03	2.25±0.01	1.39±0,09
46	MPC	3.04±0.10	2.88±0.02	2.31±0.20	1.60±0,00
47	MPC	2.74±0.07	2.62±0.06	1.30±0.00	1.45±0,13
48	MPC	3.12±0.05	2.99±0.03	1.63±0,26	0.70±0,00
49	MPC	2.64±0.09	2.60±0.07	2.31±0,15	1.72±0,10
50	MPC	2.72±0.07	2.77±0.03	2.03±0,38	1.30±0,00
51	MPC	2.72±0.10	2.76±0.10	1.38±0,43	1.13±0,38
52	MPC	3.03±0.06	2.98±0,10	1.46±0,28	1.10±0,17
53	MPC	2.56±0.04	2.55±0,04	1.36±0,39	1.39±0,36
54	MPC	2.19±0.13	2.27±0,13	1.10±0,17	1.36±0,39
55	WPC	2.16±0.22	2.19±0.10	1.16±0.15	No count
56	WPC	2.49±0.22	2.43±0.09	0.96±0.24	0.70±0.00
57	WPC	2.24±0.12	2.30±0.04	1.19±0.20	No count
58	WPC	2.08±0.00	2.04±0.04	0.80±0.17	0.23±0.40
59	WPC	2.39±0.17	2.45±0.11	2.29±0.03	2.19±0.04
60	WPC	3.06±0.10	3.07±0.10	2.37±0.20	1.00±0.00

61	WPC	2.03±0.05	2.39±0.02	2.22±0.31	No count
62	WPC	3.16±0.04	3.12±0.04	1.52±0.07	1.26±0.24
63	WPC	0.70±0,00	No count	2.17±0.05	2.40±0.13
64	BMP	2.21±0.06	2.17±0.08	1.69±0.17	2.25±0.00
65	BMP	2.04±0.04	2.13 ±0.08	2.80 ±0.06	2.98±0.06
66	BMP	2.31±0.20	2.18 ± 0.03	2.40 ± 0.07	2.67±0.04
67	BMP	No count	No count	0.70 ±0.00	No count
68	BMP	2.09±0.08	2.07 ± 0.04	1.73 ±0.23	1.96±0.05
69	BMP	1.40±0.35	1.00 ±0.00	0.70 ±0.00	0.70±0.00
70	BMP	0.70±0.00	0.80 ±0.17	1.26 ±0.24	0.70±0.00

Appendix B. The comparison results between researcher and laboratory staff (Dr. Baizura Zain).

No		**Number of bacteria (CFU/g ± SD)**			
		30°C	**37°C**	**55 °C**	**65 °C**
1	Lab staff	2.36	2.38	2.18	1.90
	Researcher	2.38	2.45	1.65	1.85
2	Lab staff	2.00	2.11	1.65	1.98
	Researcher	2.15	2.00	1.54	1.90
3	Lab staff	2.57	2.72	2.48	1.60
	Researcher	2.72	2.81	2.67	2.08

Appendix C. T-test fot the log number of bacteria at incubation 30 and 37ºC (Minitab 19).

Descriptive Statistics

Sample	N	Mean	StDev	SE Mean
30°C	70	2,2725	0,5687	0,0680
37°C	70	2,2647	0,5923	0,0708

Estimation for Paired Difference

Mean	StDev	SE Mean	95% CI for μ_difference
0,0078	0,1501	0,0179	(-0,0280; 0,0436)

μ_difference: mean of (30°C - 37°C)

Test

Null hypothesis H_0: μ_difference = 0

Alternative hypothesis H_1: μ_difference ≠ 0

T-Value	P-Value
0,43	0,667

Appendix D. T-test for the log number of bacteria at 55 and 65 ºC (Minitab 19).

Descriptive Statistics

Sample	N	Mean	StDev	SE Mean
55°C	70	1,7814	0,5862	0,0701
65°C	70	1,5463	0,7366	0,0880

Estimation for Paired Difference

Mean	StDev	SE Mean	95% CI for μ_difference
0,2351	0,5352	0,0640	(0,1075; 0,3628)

μ_difference: mean of (55°C - 65°C)

Test

Null hypothesis H_0: μ_difference = 0

Alternative hypothesis H_1: μ_difference ≠ 0

T-Value	P-Value
3,68	0,000

Appendix E. T-test for the log number of bacteria at 55 and 65ºC, which 65ºC has higher bacteria counts (Minitab 19).

Descriptive Statistics

Sample	N	Mean	StDev	SE Mean
55°C	26	1,864	0,626	0,123
65°C	26	2,086	0,590	0,116

Estimation for Paired Difference

Mean	StDev	SE Mean	95% CI for μ_difference
-0,2229	0,2696	0,0529	(-0,3318; -0,1140)

μ_difference: mean of (55°C - 65°C)

Test

Null hypothesis	H_0: μ_difference = 0
Alternative hypothesis	H_1: μ_difference ≠ 0

T-Value	P-Value
-4,22	0,000

Appendix F. Tukey Pairwase Comparisons for the number of bacteria at WMP, SMP, MPC, WPC, BMP (Minitab 19 results).

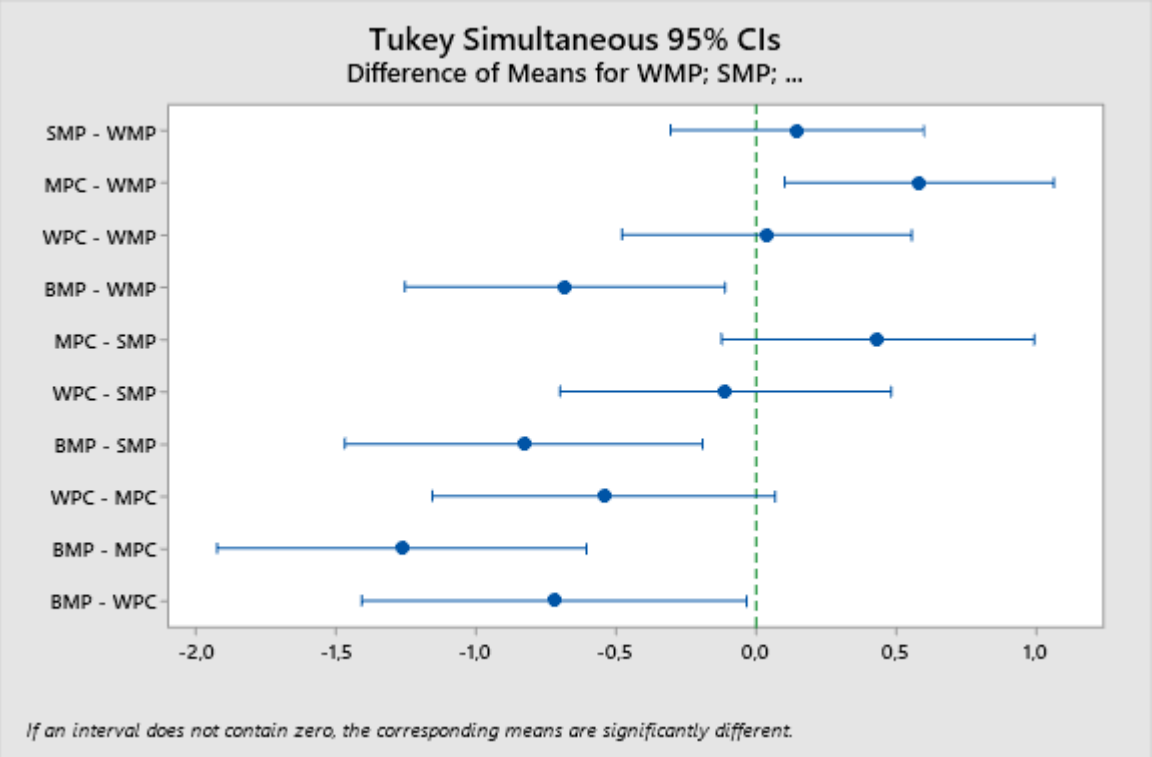
1. **30°C**

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping		
MPC	11	2,7987	A		
SMP	13	2,364	A	B	
WPC	9	2,256	A	B	
WMP	30	2,2169		B	
BMP	7	1,536			C

Means that do not share a letter are significantly different.



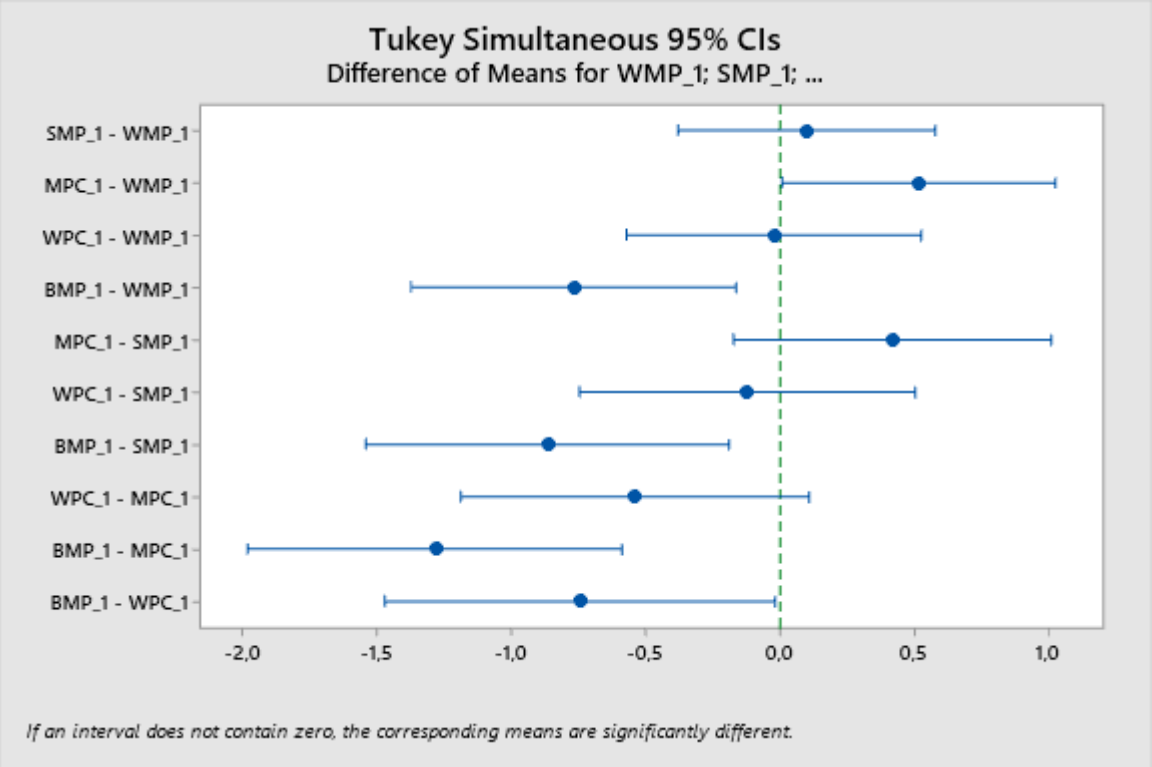
2. 37°C

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping		
MPC_1	11	2,7604	A		
SMP_1	13	2,343	A	B	
WMP_1	30	2,2452		B	
WPC_1	9	2,222	A	B	
BMP_1	7	1,479			C

Means that do not share a letter are significantly different.



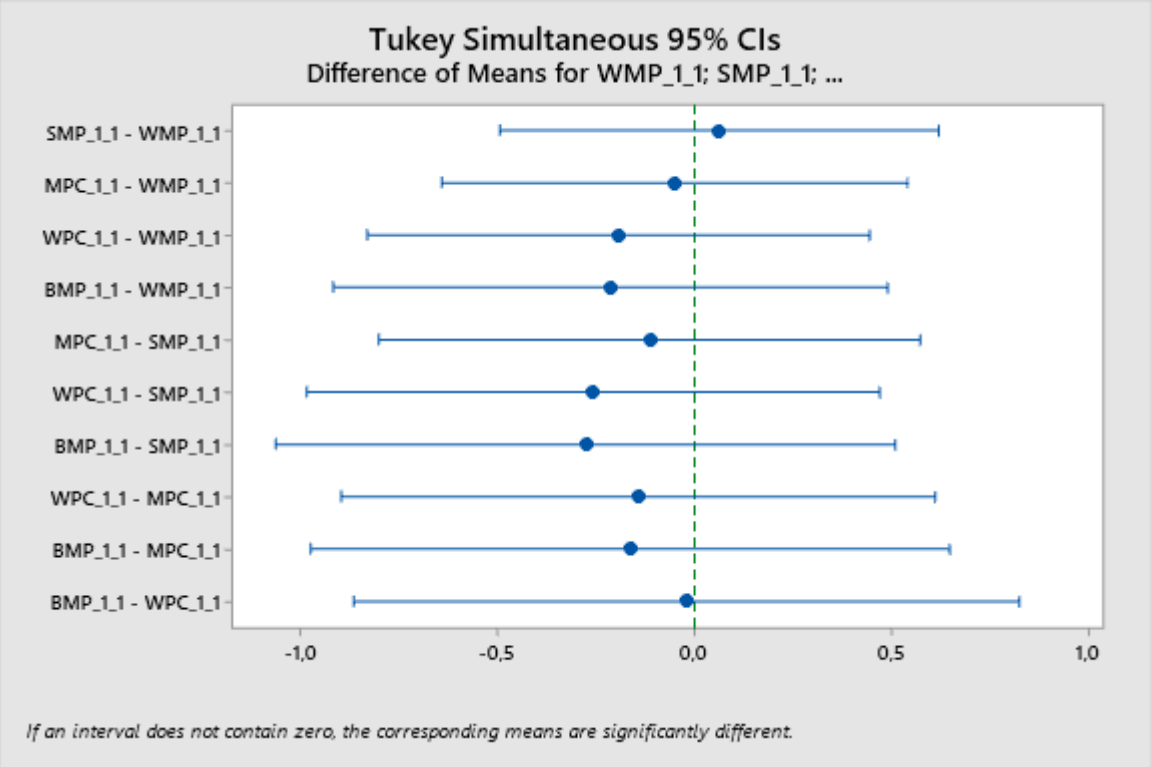
3. 55°C

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
SMP_1_1	13	1,887	A
WMP_1_1	30	1,823	A
MPC_1_1	11	1,773	A
WPC_1_1	9	1,631	A
BMP_1_1	7	1,611	A

Means that do not share a letter are significantly different.

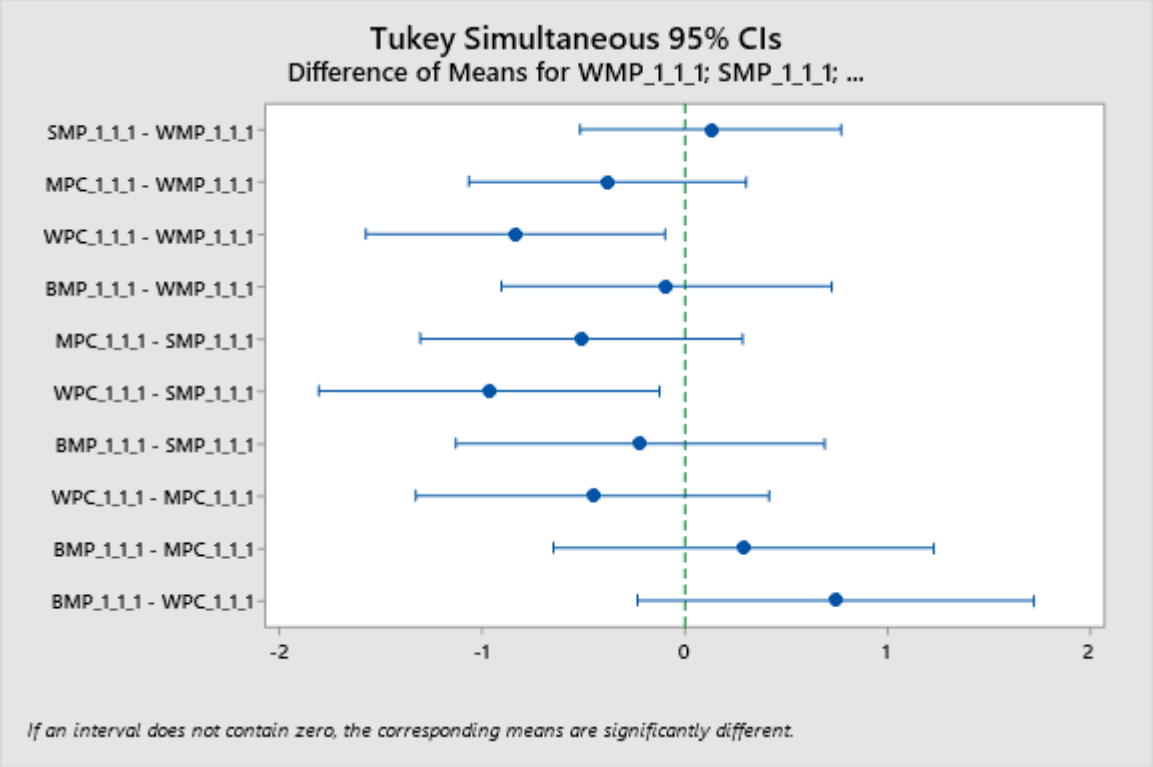


4. 65°C

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping	
SMP_1_1_1	13	1,827	A	
WMP_1_1_1	30	1,698	A	
BMP_1_1_1	7	1,609	A	B
MPC_1_1_1	11	1,3184	A	B
WPC_1_1_1	9	0,865		B

Means that do not share a letter are significantly different.



Appendix G. Examples of colonies that found in MPCA