PSYCHROTROPHIC BACTERIA AND BIOFILMS IN THE DAIRY INDUSTRY

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Executive summary

At a MilkSA DRDC committee meeting on 11 May 2020, Prof. Jooste, in conjunction with Dr Meissner and Mr. Du Plessis, were requested to compose a document about psychrotrophic bacteria and biofilms. This would include the effect of the psychrotrophs and their biofilms on milk quality and the methods to combat the contamination. The following information is submitted in accordance with this request.

Microbial contamination generally can occur from three main sources i) from within the udder, ii) from the exterior of the udder and iii) from the surface of milk handling and storage equipment. Psychrotrophic bacteria are not part of the normal udder microflora, so the numbers present in raw milk are related to sanitary conditions during production and to the length and temperature of storage before pasteurization. Psychrotrophic bacteria are those bacteria, which can grow at 7ºC or less regardless of their optimal growth temperature. The presence of psychrotrophs, have an economic impact on the global dairy industry, causing significantly negative effects on milk yield and limiting the shelf-life of dairy products. They are also notorious for being able to form biofilms on milk contact surfaces.

Biofilms are surface-associated bacterial communities embedded in a matrix of self-produced or extracellular polymeric substances. Biofilms develop in three critical stages namely, adherence to a food contact surface, proliferation, and eventual dispersion of surface cells. Owing to their resistance to heat treatments and to various cleaning agents, biofilms formed on dairy processing lines are difficult to remove. In nature biofilms can be composed of a single species, but more commonly they are comprised of a consortium of species. Bacteria in biofilms communicate with each other using chemical signalling molecules when specific cell densities are reached via a process termed quorum sensing. The complex and multi-layered structures of biofilms allow the bacterial communities to live in a sessile and protected environment.

In a historical perspective, it has been interesting to witness the transition of delivery of milk to processing dairies from cans in the mid 1960’s to bulk transport of refrigerated milk in insulated tankers two decades later. This transition had a dramatic effect on the emergence of psychrotrophic bacteria in South African milk supplies. This effect was not only evident in the bacterial count of these organisms in the milk, but also on the bacterial types and of the increase

of psychrotrophic bacteria percentagewise in relation to the so-called total bacterial count. What was even more surprising was the fact that a seasonal effect became evident, especially regarding the prevalence of bacterial types despite refrigeration on the farm and transport in insulated milk tankers. A transition took place in Bloemfontein milk from a relatively inert group of bacteria (*Acinetobacter*) dominating in the summer milk to domination by a psychrotroph (*Pseudomonas*) in the winter milk This pointed to contamination of, and growth in, the pipelines that were exposed to environmental temperatures and the possible presence of biofilms.

In a more recent study of South African refrigerated milk transported in bulk road tankers in 2006, additional evidence was found to strengthen the theory that the milk is contaminated by biofilms growing in the pipelines and equipment and that high counts in the refrigerated milk are not necessarily as a result of the growth and multiplication of the psychrotrophs in the milk during transport and storage. In this study 55% (11 out of 20) of the tanker milk samples analysed, had psychrotrophic counts exceeding the EU standard of $\log_{10}$ 3.7 cfu/mL (5000 cfu/mL) on arrival at the processing dairy. Conversely however, the finding that 45% of the milk samples were within the EU standard, showed that a psychrotrophic count standard of 5000 cfu/mL in bulk-transported refrigerated milk on arrival at the processing plant is not only feasible, but possible. No significant statistical relationship was found between transportation time or temperature of the milk and the microbial load in the raw tanker milk samples on arrival at the processing plant. Once again, the assumption can be made that contamination of equipment and pipelines and growth, most probably, in biofilms, but not in the milk during transportation, was the cause of the high counts encountered. Identification of Gram-negative isolates from the milk samples showed 65% of the isolates to be *Pseudomonas fluorescens* that is generally regarded to be the most harmful psychrotroph in terms of milk spoilage.

A recent study by Prof Celia Hugo at Free State University in 2017, supported by MilkSA, was aimed at the effect of psychrotrophic counts and bacterial types in milk on the flocculation of raw milk after refrigerated storage. Milk was sampled on 10 farms in the Free State over a four-week period. A range of standard and accelerated psychrotrophic tests were performed on the fresh milk. The milk was then incubated at 7^{o}C until the milk flocculated with the alizarol test. The range of counts were then repeated to determine the counts at the time of flocculation. Bacterial isolates were identified to determine which bacteria were prevalent at flocculation. The findings

in this study showed that the accelerated psychrotrophic count, the proteolytic psychrotrophic count and the *Pseudomonas* count had a significant influence on the time of the raw milk to flocculation after incubation at 7^{o}C. With the *Pseudomonas* plate count of milk samples from the ten farms as reference, it clearly showed how the other parameters correlate with the mean *Pseudomonas* count. As the *Pseudomonas* count decreased, the other two counts decreased accordingly and the time that the milk could be stored at 7^{o}C before flocculation increased. Tentative standards for the three bacterial counts were proposed. *Pseudomonas* spp. and particularly *Ps. fluorescens*, were found to be the prevalent organisms at the time of flocculation.

With reference to problems caused by biofilms on milk contact surfaces, the following can be kept in mind: i) Bacteria within biofilms are protected from sanitizers due to multispecies cooperation and the presence of extracellular polymeric substances, by which their survival and subsequent contamination of processed milk products is promoted; ii) Biofilms are large, complex, and organized bacterial ecosystems that are regulated by a variety of environmental and physiological triggers, such as quorum sensing, nutrient availability, and cellular stress; iii) Once established on milk contact surfaces, biofilms accelerate corrosion and material deterioration of the equipment; iv) Many biofilms produce heat-stable extracellular lipases, proteases, and lecithinases that contribute to milk spoilage; v) Bacteria in biofilms have intrinsic mechanisms that protect them from even the most aggressive environmental conditions, including the exposure to antimicrobials/sanitisers.

Effective monitoring for psychrotrophic bacteria and biofilms in the dairy industry remains an ongoing challenge. Regarding monitoring of psychrotrophic bacteria, the standard procedure for bacterial counting of psychrotrophs entails an incubation time of the agar plates for 10 days at 7^{o}C. This long period is just not practical for routine monitoring purposes. For this reason, accelerated tests have been proposed and evaluated. These tests include the Psychro-fast test; plate counts for the rapid enumeration of psychrotrophic bacteria and proteolytic psychrotrophs in milk and the *Pseudomonas* plate count. The potential also exists to detect and enumerate bacteria in real time using flow cytometry in conjunction with the fluorescence *in situ* hybridization (FISH) technique.

Regarding the monitoring for, or detection of, biofilms on milk contact surfaces, techniques are available for exposed surfaces. Examining pipelines and equipment without dismantling remains a challenge. The different methods employed for sampling and enumeration of bacteria in biofilms in a dairy plant are swabbing, rinsing and agar contact methods. Visually inspecting surfaces under good lighting, smelling for offensive odours and feeling for greasy or encrusted surfaces is always recommended to detect and rectify obvious shortfalls in sanitation.

Microbiological assessments, that follow on such an examination, are typically used to ensure compliance with microbial standards and to optimize sanitation procedures. Traditional microbiological culture methods are however time consuming and not always consistent. For immediate results, a spray-on technique such as Biofinder (INNOGIENE, Tania Garcia-Warner, tania@innogiene.co.za) could be useful. Advanced physical techniques that are available for examining biofilms on equipment surfaces include the application of Confocal laser scanning microscopy (CLSM) combined with several fluorescent stains. *In situ* techniques that have been developed for examining the intact equipment for the presence of biofilms are also described. Discussions held with factory managers and other professionals at South African UHT factories by Du Plessis (2016) imparted information that fluorescent techniques were used in some factories to detect biofilms in the equipment.

More specific approaches to monitoring contaminants and biofilms on milk contact surfaces, entail the identification of the bacteria involved. Identification involves the use of phenotypic systems such as API-20NE, BIOLOG Gen lll, MALDI-TOF-MS and BIOMERIEUX VITEK 2 identification systems. Molecular tools have an added advantage in that they could reveal the presence of psychrotrophic bacteria that are undetected using traditional culture-based approaches. Several DNA based molecular techniques for rapid, and possibly more accurate, bacterial identification are also reported on.

Due to the damage inflicted on milk constituents, especially the casein in milk, by enzymes produced by psychrotrophic bacterial contaminants and -biofilms, it would be advantageous to be able to monitor the proteolytic activity in the milk. Quantitative activity tests that could be evaluated include the Azocasein method, the Trinitrobenzenesulfonic acid (TNBS) method and

the Calbiochem Protease assay kit. Proteolytic assays on agar plates can also be considered, but these are more of a qualitative nature.

Finally, it is essential to reduce or prevent contamination and biofilm formation in milk-associated equipment. Procedures in this regard include i) Pre-conditioning surfaces to prevent biofilm formation; ii) Sanitizing with detergents and disinfectants; iii) Identification and application of quorum sensing antagonists and iv) Application of enzymes for the removal of biofilms. Much work is needed in this field and suggestions are made in the conclusions and recommendations section of this document to address challenges in this regard.

1. Definitions and basic concepts relating to psychrotrophic bacteria and biofilm formation

Milk is synthesized in specialized cells of the mammary gland and is virtually sterile when secreted into the udder (Tolle, 1980). Beyond this stage of milk production, the numbers and types of microorganisms in the raw milk reflect microbial contamination that generally can occur from three main sources (Bramley and McKinnon, 1990); i) from within the udder, ii) from the exterior of the udder and iii) from the surface of milk handling and storage equipment. The health and hygiene of the cow, the environment in which the cow is housed and milked, and the procedures used in cleaning and sanitizing the milking and storage equipment are all key factors in influencing the level of microbial contamination of raw milk.

Equally important is the temperature and length of storage time of the milk, which allows microbial contaminants to multiply and increase in numbers. All these factors will influence the bacterial count or load as well as the types of bacteria present in bulk raw milk. Psychrotrophic bacteria are not part of the normal udder microflora, so the numbers present in raw milk are related to sanitary conditions during production and to the length and temperature of storage before pasteurization. In South Africa, there are no standards regulating the numbers of psychrotrophic bacteria in raw milk intended for UHT processing or heat processing. The European Union (EU) standard for high quality raw milk requires a psychrotrophic count of less than 5,000 cfu/mL (3.7 log cfu/ml) (Samaržija et al., 2012).

Psychrotrophic bacteria are those bacteria, which can grow at 7ºC or less regardless of their optimal growth temperature (Frank *et al.,* 1993; Hassen & Frank, 2011 as quoted by Hugo *et al.,*

2017a). Some psychrotrophic bacteria are capable of growth at sub-zero temperatures whereas others can grow at temperatures as high as 30ºC. Psychrotrophic bacteria commonly isolated from raw milk and dairy products belong to a variety of Gram negative and Gram positive genera and they are important because these organisms cause spoilage by altering the constituents of milk (Abdou, 2003) The presence of psychrotrophs in particular, have an economic impact on the global dairy industry, causing significant negative effects on milk yield and also limiting the shelf-life of dairy products (Samaržija, et al, 2013)

Heat stable enzymes that are not inactivated by Ultra High Temperature (UHT) heat processing and negatively affect processed product quality are produced by several Gram-negative bacteria that are common in raw milk (Robinson, 2002). *Pseudomonas* species are the most detrimental of the oxidative Gram-negative rods and typically have the most impact on quality (Frank et al., 1993). The most damaging *Pseudomonas* species is usually *Pseudomonas fluorescens* (Cousin, 1982), During growth at low temperature, many strains produce lipases and proteases that, when produced in the raw milk, can survive heat treatment and cause sensory and textural defects in the processed dairy products (Frank et al., 1993) such as fruity and rancid tastes and bitter off-flavours in cheese, butter and UHT milk. as well as defects relating to casein degradation such as coagulation, thickening, and reduced cheese yield. This may adversely influence the quality and shelf life of most dairy products (Griffiths *et al.,* 1981, Hassen & Frank, 2011 as quoted by Hugo et al (2017b).

According to de Oliveira *et al*., 2013 as quoted by Hugo *et al.,* (2017b), two main steps should be taken to minimize the contamination of raw milk. The first step of control is to make sure that milk is obtained under sanitary conditions, and to ensure adequately cleaned and disinfected equipment used for the collection, transport, and storage of refrigerated raw milk. The second step is to prevent fouling of milk contact surfaces with milk film, which can support the growth of bacteria as multi-species biofilms that represent a source of contamination for any subsequent batch of milk.

Biofilms are surface-associated bacterial communities embedded in a matrix of self-produced or extracellular polymeric substances (EPSs). These EPSs consist of nucleic acids, polysaccharides, lipids, and proteins resulting from the successful attachment and growth of micro-organisms on a

surface. Biofilms develop in three critical stages, including adherence, proliferation, and the dispersion. The functionality of biofilms are dependent on their ability to take part in the complex web of symbiotic interactions and other factors, including pH, nutrient availability and the presence of organic and inorganic compounds and temperature (de Oliveira *et al.*, 2013 as quoted by Hugo *et al.*, 2017b).

Biofilms present problems in the dairy production and processing chain because more often than not, when contamination of dairy products takes place, the source of the problem is usually biofilm-related (Simões *et al.*, 2010 as quoted by Zachara and Myburgh, 2014). On top of that, biofilms may act as a source of recurrent contaminations, which cause spoilage and are a threat to the public's health. Owing to their resistance to heat treatments and to various cleaning agents, biofilms formed on dairy processing lines are difficult to remove. Moreover, re-contamination of milk processing lines can occur during cleaning-in-place procedures (CIP), because of what is known as a 're-adhesion phenomenon' (Malek *et al.*, 2012 as quoted by Zachara and Myburgh, 2014).

Various steps in the formation of a biofilm on a food contact surface is illustrated in Fig 1 (Simões et al., 2010 as quoted by Zachara and Myburgh, 2014) These steps include the preconditioning of the food contact surface by organic or inorganic molecules that may include milk proteins, or calcium phosphate precipitates etc. In a next step bacterial cells are deposited on the surface and, may adhere to the surface (described in Fig 1 as adsorption). If the cells are not able to adhere due to milk flow in pipelines or regular sanitation practices, they "desorp" into the surrounding fluid/matrix. Once the cells can adhere to the surface and can produce extracellular polymeric substances (ESPs), they start forming a biofilm (see also Fig 2).

In nature biofilms can be composed of a single species, but more commonly they are comprised of a consortium of species (Skandamis and Nychas, 2012 as quoted by de Oliveira *et al.*, 2015). The latter authors state that many *Pseudomonas* species utilise biofilm formation during plant colonisation to enhance persistence, resulting in the production of a variety of biofilm matrix molecules (Mann and Wozniak, 2012 as quoted by de Oliveira *et al.*, 2015). An intriguing feature of milk-spoiling *Pseudomonas* recovered from biofilms, according to de Oliveira *et al.*, (2015), is their ability to alter phenotypes via the process of phase variation (Marchand *et al.*, 2012). Through

this process, high-frequency phenotypic switching is mediated by mutation, reorganisation, or modification of the genome (Van Den Broek *et al.*, 2005 as quoted by Marchand *et al.*, 2012), contributing to the survival of the biofilm population during environmental stresses such as temperature fluctuations and frequent exposure to sanitizers during the cleaning of dairy processing and storage equipment (Marchand *et al.*, 2012).

As shown in Fig 1, bacteria in biofilms communicate with each other using chemical signalling molecules when specific cell densities are reached via a process termed quorum sensing (Fuqua *et al.*,1994; Liu *et al.*, 2007 as quoted by de Oliveira *et al.*, 2015). As a consequence of this process, gene expression can either be activated or repressed, and the behaviour of populations of single cells are synchronised in a manner similar to multi-cellular organisms (Bai and Rai, 2011; Smith *et al.*, 2004 as quoted by de Oliveira *et al.*, 2015).

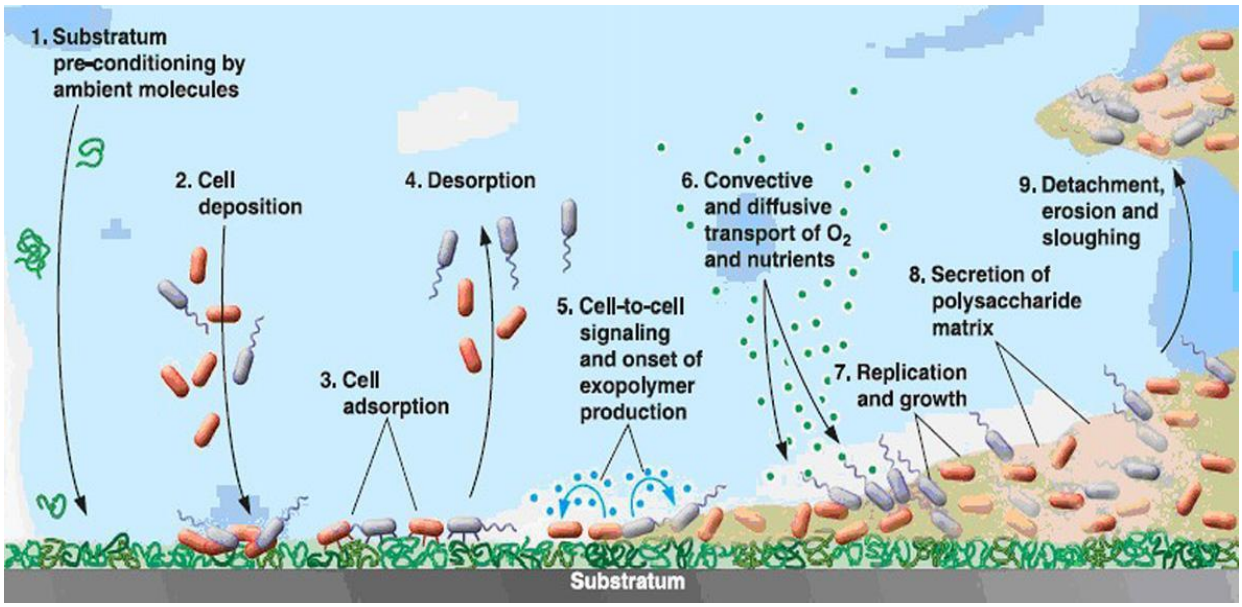

Figure 1. Mechanism of biofilm formation (Simões et al., 2010 as quoted by Zachara and Myburgh, 2014)

The cells in the biofilm feed by convective and diffusive transport of oxygen and nutrients (Fig 1) and in this way continue to replicate and grow, causing the biofilm to thicken due to secretion of the polysaccharide matrix and other polymers by the cells. The ability of the pseudomonads to resist cleaning is linked to the fact that many species are effective biofilm producers (Bai and Rai, 2011; Simões *et al.*, 2008 quoted by de Oliveira *et al.*, 2015). The complex and multi-layered structures of biofilms allow the bacterial communities to live in a sessile and protected

environment. While extracellular enzymes can be produced by the consortium of cells in the biofilm, cell-containing surface layers of the biofilms may slough off and enter the milk being pumped through pipelines and in this way contribute to the contamination and bacterial load of the milk.

2. Psychrotrophic bacteria in South African raw milk supplies

2.1 The emergence of psychrotrophic bacteria in South African raw milk supplies

In the mid 1960's milk was still delivered to dairies in cans. Even though Bloemfontein municipal regulations at that time required that milk immediately after production be cooled to a temperature not exceeding 7.2°C, the actual temperature of milk upon arrival at the city dairies (Jooste & Lategan 1967) was directly influenced by the prevailing atmospheric temperature especially in the summer months. Only 3.5% of the milk (on a mass basis) delivered to processing dairies between September and January of that year (1964 - 1965) had a temperature of less than 7°C. Altogether 63.6% of the milk supplies monitored arrived at temperatures of between 13 °C and 22 °C - temperatures allowing rapid growth of bacteria in the milk. Of the samples taken during winter, 40% exceeded a Total Bacterial Count of 200 000 colony forming units per ml (Legal limit specified by the Bloemfontein Municipal Milk and Dairy Regulations, 1961). Of the samples taken during summer, 84.8% exceeded the specified standard. This illustrated the dramatic effect that prevailing atmospheric temperatures had on the bacteriological quality of the milk upon delivery to the dairies.

The influence on the numbers of psychrotrophic or cold tolerant bacteria was less dramatic, possibly due at that stage to competition by mesophilic, possibly lactic acid producing, species. During the winter sampling period the mean counts were 74 000 cfu/ml compared to a mean summer count of 88 000 cfu/ml. It is important at this stage to take note of the fact that the mean psychrotrophic counts made up only 14.2 % of the so called total aerobic (mesophilic) count of the milk in the raw can-transported milk.

In the decade following this study, transport of milk in cans was replaced by bulk transport of milk in insulated tankers with refrigerated bulk tanks on the farm (Cousin 1982). This change was accompanied by dramatic changes in the microbial ecology of the milk. In a study done in

Bloemfontein in 1986 (Fischer et al 1987), the mean psychrotrophic count of the milk upon arrival at the processing plant instead of being lower, than the previously tested milk in cans, was up to 113 000 cfu/ml (in the winter) and 228 000 cfu/ml in summer. In the Pretoria milk supply similar counts were observed (Swart et al 1989) with winter samples having mean psychrotrophic counts of ± 70 000 cfu/ml and summer samples > 200 000 cfu/ml. These counts graphically illustrate the fact that the newer technology of bulk cooled, and bulk transportation of the refrigerated milk had introduced new problems. Not only had the actual numbers of the psychrotrophs increased, but also the percentage in relation to the total bacterial count. Psychrotrophic counts at this stage accounted for between 63.3 and 69.7% of the bacterial content of the milk (Swart *et al.*, 1989). This dramatic change in psychrotrophic bacterial content of bulk cooled milk versus milk transported in cans was, at that stage, ascribed to the selective pressure on the bacterial population exerted by the extended cold chain from farm to factory and growth of psychrotolerant bacteria in the milk. This theory however was possibly disproved to a large extent in later research and it was more recently postulated that the results rather had a bearing on contamination and growth in the pipelines and equipment on the farm and the emergence of psychrotrophic biofilms.

To more accurately rate the impact of the psychrotrophic population on the quality of the products, it was decided to examine the bacterial types themselves since the different bacterial genera have different physiological characteristics that can affect the quality of milk and milk products. (Fischer et al 1987). The Gram-negative bacterial population during the summer months was

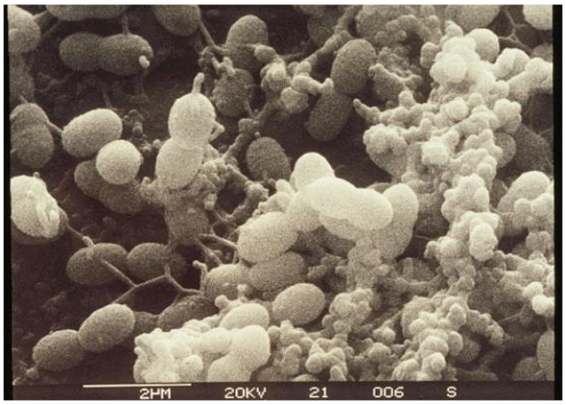

Fig 2. Electron micrograph of a psychrotrophic biofilm on a steel surface (Koutzayioutis *et al*, 1993)

dominated by *Acinetobacter* (34%), a relatively inert genus, followed by the more enzymatically active *Flavobacterium* (10.4%) and *Pseudomonas* (6.6%). This position changed dramatically

during the winter months with *Pseudomonas* (30.8%) assuming the dominant position followed by *Acinetobacter* (13.0%) and *Flavobacterium* (10.9%).

A puzzling fact was that this strong seasonal tendency existed even though the milk was bulk-cooled and transported. It was discovered in a later study (Koutzayioutis *et al.*, 1993) that psychrotrophic organisms (see Fig. 2) could adhere to steel milk contact surfaces such as pipelines. The conclusion consequently was that the main source of milk contamination by these organisms were the milk pipelines themselves. These pipelines are not cooled, and poorly sanitized pipelines inhabited by layers (biofilms) of the contaminating organisms are directly exposed to environmental temperatures, so explaining the seasonal tendency. It should be kept in mind that the psychrotrophic group of organisms are psychrotolerant (able to grow at refrigeration temperatures), but not necessarily psychrophilic (cold loving) and may have optimum growth temperatures in the order of 20 to 25ºC.

2.2 Counts and types of psychrotrophs in South African milk transported in bulk tankers

In a later study of refrigerated milk transported in bulk in road tankers (Mabunda and Jooste, 2007) additional evidence was found to strengthen the above theory that the milk is contaminated by biofilms growing in the pipelines and equipment and that high counts in the refrigerated milk are not necessarily as a result of the growth and multiplication of the psychrotrophs during transport and storage, as will be demonstrated below. Samples of bulk transported raw milk were obtained at a Clayville dairy factory, and four routes namely Escourt, Ixopo, Nestle, and Alexandria were targeted. The collection of the raw milk samples took place during June to November 2006. Samples were transported in bulk road tankers from the mentioned routes to Clayvillle and on route they were cooled at depots to keep the temperature in the tankers as low as possible. Using sterile bottles, the samples were then collected from the tankers immediately after arrival and then transported to the laboratory at TUT on ice and immediately analysed for microbiological load on arrival. One sample from each route was collected on each week of sampling, making a total of four samples per week. A total of 16 samples of bulk milk were analysed and subjected to psychrotrophic bacterial counts (Frank *et al*., 1993). After incubation, 6–10 representative colonies were picked from each countable agar plate. Bacterial isolates were then characterised and identified. Screening tests were performed to determine the type, morphology, and

biochemical properties of isolates before subjecting the isolates to identification in API 20NE galleries.

Fig. 3 shows that 55 % (11 out of 20) of the tanker milk samples analysed, had psychrotrophic counts exceeding the EU standard of Log_{10} 3.7 cfu/mL (5000 cfu/mL). The average count in these samples was Log_{10} 5.1613 cfu/mL (150 000 cfu/mL) with counts varying from 9600 to 800 000 cfu/mL. The average count of the 45 % of samples with counts less than 5000 cfu/mL was 2000 cfu/mL with counts varying from 230 to 4800 cfu/mL which showed that a psychrotrophic count standard of 5000 cfu/mL in bulk-transported refrigerated milk on arrival at the processing plant is not only feasible, but possible.

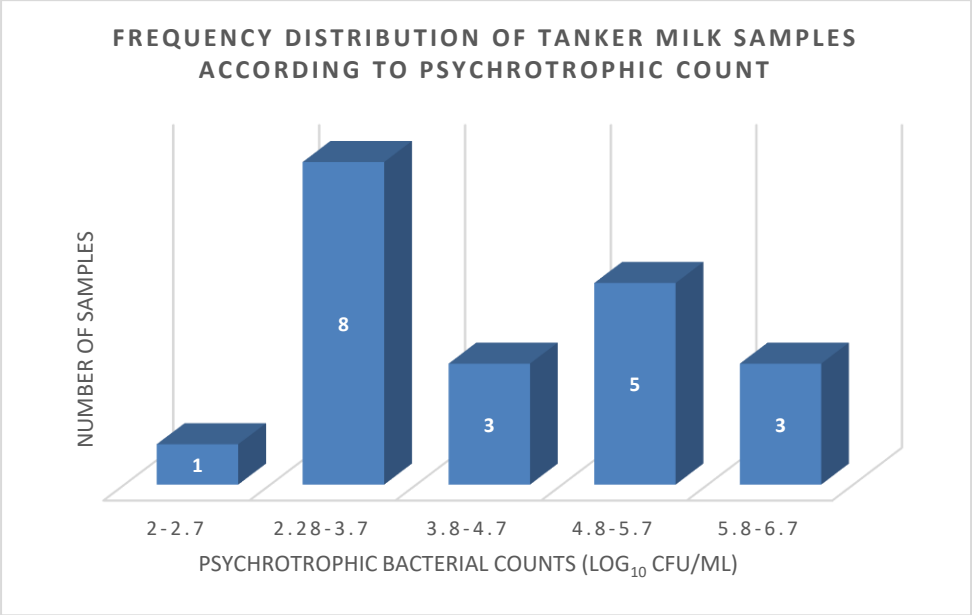

Figure 3: Distribution of psychrotrophic bacterial counts in log10 cfu/mL (7ºC for 10 days) in raw tanker milk samples upon arrival at the processing dairy plant.

To determine whether the high bacterial counts in the raw tanker milk were due to contamination or growth, the effect of route, transportation time and temperature on arrival on bacterial counts were considered. Fig 4 shows the mean psychrotropic counts of the samples from each of the four transport routes. Samples from the Estcourt route had the highest mean count of 73 000 cfu/mL This was followed by samples from the Nestle route with a mean count of 70 000 cfu/mL, followed by Ixopo samples (47 000 cfu/mL) and samples from the Alexandria route with the lowest mean count (29 000 cfu/mL) Statistically there was no significant difference between the counts, but all the mean counts were nevertheless much higher than the EU standard of 5000 cfu/mL.

No significant statistical relationship was found between transportation time and the microbial load in the raw tanker milk samples. The average time of transport was 14,2 h for Estcourt samples, 13.7 for Ixopo samples, 14 h for Nestle samples and 19,5 h for Alexandria samples. These findings show that high counts could not have been due to growth in the milk during transportation, but should rather be ascribed to contamination, since Alexandria samples that had the longest transport time had the lowest average counts. The transportation times for the other three routes were, more or less, the same.

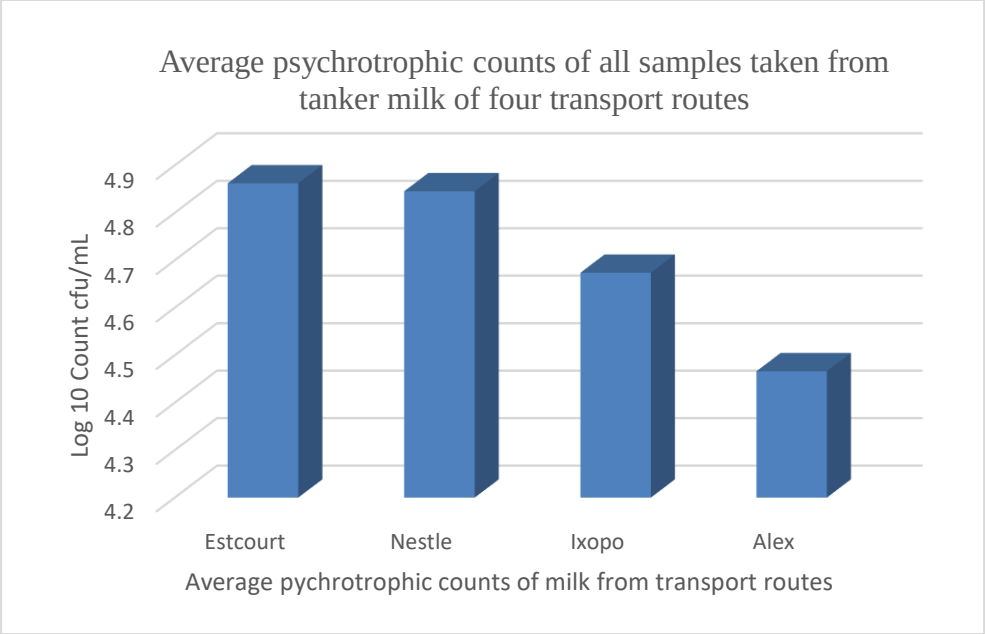

Figure 4: Psychrotrophic bacterial counts of all samples of raw tanker milk from different collection routes.

In Fig 5 the mean counts of samples from the four transport routes exceeding a count of 5000 cfu/mL are illustrated. A single Nestle route had the highest mean count of $\log_{10}$ 5.6628 cfu/mL (460 000 cfu/mL), followed by Ixopo with $\log_{10}$ 5.5085 (320 000 cfu/mL), Estcourt with $\log_{10}$ 5.0488 (110 000 cfu/mL) and Alexandria with the lowest mean count of $\log_{10}$ 4.9009 (80 000 cfu/mL). All these counts were much higher that the EU standard of 5000 cfu/mL.

No significant relationship between the temperature on arrival and microbial load of the raw tanker milk samples could be found either. The bacterial counts in the raw tanker milk samples consequently could not be ascribed to the temperature on arrival at the processing dairy plant and

consequent bacterial growth. The average temperature on arrival was 2.9 ºC for Estcourt samples, 3.3 ºC for Ixopo samples, 5.5 ºC for Nestle samples and 4.4 ºC for Alexandria samples. Once again, the assumption can be made that contamination of equipment and pipelines and growth in probable biofilms, but not in the milk during transportation, was the cause of the high count

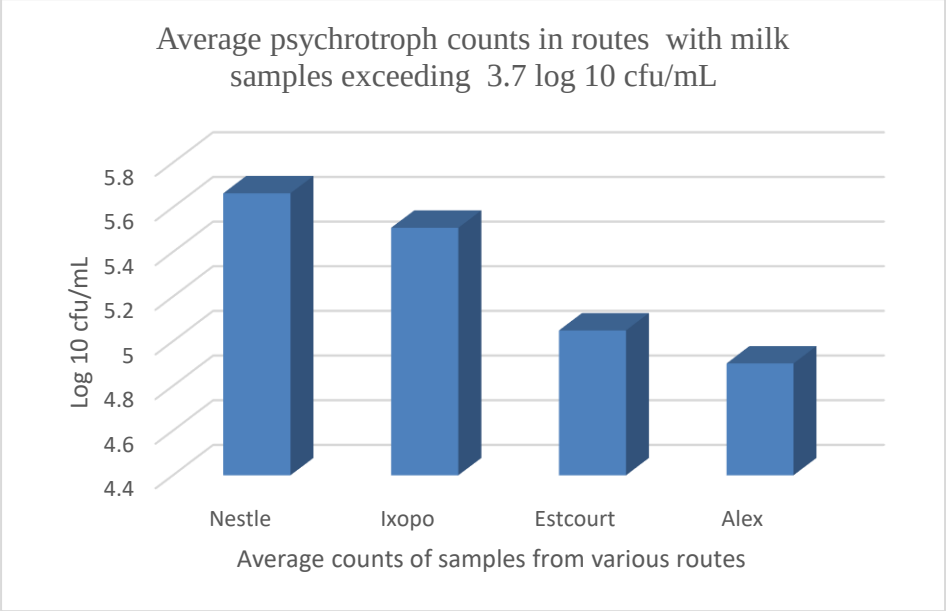

Figure 4: Psychrotrophic bacterial counts of raw tanker milk samples from different collection routes exceeding a count of 5000 cfu/mL

Altogether 34 Gram negative, non-fermentative bacterial types (regarded to be most important from a milk spoilage point of view) were isolated from the milk samples The organisms were identified as the following genera and species: 65% of the isolates were *Pseudomonas fluorescens*; 15 % were *Pasteurella* spp; 9% were *Pseudomonas putida*; 8% were *Sphingomonas paucimobilis* and 3% were *Chryseobacterium indologenes*. As referred to previously, *Pseudomonas fluorescens* is regarded as the most important psychrotroph in raw milk from a spoilage point of view. If *Pseudomonas putida* (9%) and *Sphingomonas paucimobilis* (8%), that both reside in the pseudomonad group, are added to the prevalence of *Ps fluorescens*, the pseudomonads would have constituted 82 % of the psychrotrophic isolates from the milk samples

2.3 Psychrotrophic counts and bacterial types in relation to the flocculation of raw milk by the Alizarol test on arrival at the processing dairy

Flocculation of raw milk on arrival at processing dairies due to a positive alizarol (% ?) platform test (The Dairy Mail, 2013), leads to rejection of such milk destined for UHT milk treatment. This has in the recent past caused problems in the South African dairy industry and continues to do so (MilkSA, 2016 as quoted by Hugo *et al.*, 2017b). In a recent study by Hugo *et al.*, (2017b), a 250 ml raw milk sample was collected aseptically, during the month of July, 2016 once a week for four weeks, from 10 different raw milk producers in the Bloemfontein area, Free State, South Africa. A total of 40 raw milk samples were obtained over the four week period. The samples were kept on ice and laboratory analysis followed within 24 h. After analysis, all milk samples were kept at 7^{o}C until flocculation occurred, as indicated by a positive alizarol test.

In evaluating the effect of psychrotrophic bacteria on the time to flocculation, a three-pronged approach was followed. In the first place an accelerated psychrotrophic plate count of the freshly sampled milk was conducted. Psychrotrophic bacteria commonly isolated from raw milk are important because these organisms cause spoilage by altering the constituents of milk (Abdou, 2003) The second approach was to employ a plate count of proteolytic psychrotrophs, the rationale being that flocculation of milk is allegedly associated with protease production by psychrotrophic bacteria; The third approach was to subject the samples to a plate count on *Pseudomonas* selective agar. The rationale here was due to the long-accepted belief that *Pseudomonas* species are the most detrimental of the oxidative Gram-negative rods and typically have the most impact on milk quality (Frank *et al.*, 1993). The techniques used to evaluate these psychrotrophic groupings will be discussed in greater detail in a following section.

The findings in the study of Hugo *et al.*, (2017b) showed that all three bacterial count groups, referred to above, had a significant bearing on the time of the raw milk to flocculation when the samples were incubated at 7^{o}C until they flocculated when subjected to the alizarol test. On the basis of the *Pseudomonas* plate count of milk samples from the ten farms, the farms could be grouped into four separate groups that differed significantly from each other on the basis of the average *Pseudomonas* count of samples taken over the four week period.. Group 1 included farms 3, 8 and 10 with the lowest count; Group 2 included farms 4 ,6 and 9 with significantly higher

counts; followed by Group 3 (farms1, 2 and 7) and Group 4 (farm 5) with the highest *Pseudomonas* count. Table 3 shows clearly how the other parameters relate to the mean *Pseudomonas* count of each group.

The count standard of the European Union (less than 5,000 cfu/ml (3.7 log cfu/ml) is also recommended by Hugo *et al.,* (2017b) and seems reasonable when taking the results in Table 3 into consideration. No count standards are specified by the EU for proteolytic psychrotophic bacteria, but Hugo *et al.,* (2017b) recommended a standard of <1000 cfu/mL for the raw milk which once again would serve to ensure a satisfactory time to flocculation. In the case of the *Pseudomonas* count, these organisms have a short generation time at refrigeration temperatures (< 4 h). Contamination with just a single cell can lead to their numbers increasing to greater than 10^6 cfu/ml in milk after eight days of storage at 4^{o}C (Samaržija *et al.,* 2012 as quoted by Hugo *et al.,* 2017b).

Table 3. Relationship of the mean proteolytic psychrotrophic count, the psychrotrophic plate count and the mean time to flocculation of milk samples incubated at 7^{o}C as compared to the *Pseudomonas* count (expressed as cfu/mL)

Group	*Pseudomonas* count (cfu/mL)	Proteolytic psychrotrophic count (cfu/mL)	Psychrotrophic plate count. (cfu/mL)	Time to flocculation of milk samples incubated at 7^{o}C (days)
1	0	20	40	9.05
2	2	58	1700	7.6
3	40	3000	12 000	7.6
4	200	10 000	250 000	5.0

Group 1=farms 3,8 and 10; Group 2 =farms 4,6 and 9; Group 3 = farms 1,2 and 7; Group 4 = farm 5

Hugo *et al*., (2017b) suggest that raw milk arriving at the dairy processing facility for UHT processing should have a *Pseudomonas* count of < 2.0 log $_{10}$ cfu/ml (<100 cfu/ml). Consequently, when compared to the results in Table 3, the lower the *Pseudomonas* count is on the farm, the longer the shelf-life of the refrigerated milk would be before it flocculates with the alizarol test. This is in agreement with dairy industry experience in Australia (DRDC Quarterly report 1993) where they regarded it as a "rule of thumb" that raw milk containing one or less psychrotrophs per mL in the freshly produced milk (which could be equivalent to one *Pseudomonas* cell since

Pseudomonas spp. are usually the prevalent psychrotrophs present) would have a shelf life at 4°C of 10 days. These count standards should, however, be confirmed by a larger study which would enable regression coefficients to be calculated that could confirm these estimations.

Finally, in the study of Hugo *et al.*,(2017b), similar to previous other studies (Samaržija, Zamberlin & Pogačić, 2012 as quoted by Hugo *et al.*,2017b), Gram-negative psychrotrophic bacteria were found to be the prevalent bacteria at the time that flocculation of milk occurred (after incubation of the milk samples at 7°C), which indicated that these bacteria and their proteolytic enzymes were associated with milk flocculation. *Pseudomonas* spp. and particularly *Ps. fluorescens,* were found by Hugo *et al.,* (2017b), to be the prevalent organisms at the time of flocculation. These findings agree with previous findings that *Pseudomonas fluorescens* is regarded as the most important psychrotroph in raw milk from a spoilage point of view. Mabunda and Jooste (2007) identified 65% of their isolates as *Pseudomonas fluorescens*. Other organisms identified by them included *Pseudomonas putida* (9%) and *Sphingomonas paucimobilis* (8%) that both reside in the pseudomonad group. If these organisms are added to the prevalence of *Ps fluorescens*, the pseudomonads would have constituted 82 % of the psychrotrophic isolates from the milk samples examined by Mabunda and Jooste (2007). This lends impetus to the importance of implementing the *Pseudomonas* count to monitor the quality of raw milk as recommended by Hugo *et al.,* (2017b). If it is accepted that the high bacterial counts in the milk samples were more probably caused by contamination of, and growth in biofilms in pipelines and milk contact surfaces and not so much by growth of the bacteria in the milk during storage and transport, it would seem reasonable that the EU standard of $\log_{10}$ 3.6990 cfu/m (5000 cfu/mL) for psychrotrophic bacteria in raw milk, should be regarded as the upper limit in raw milk for processing.. In the study of Mabunda and Jooste (2007) 45 % of samples of bulk milk transported in refrigerated tankers had counts of less than 5000 cfu/mL, which shows that this count of milk upon arrival at the processing dairy is attainable. Hugo *et al.,* (2017b) suggested that a reasonable standard when using the accelerated psychrotrophic count on standard plate count agar incubated at 18°C for 48 h, is <5,000 cfu/ml. For the proteolytic psychrotrophic count on SMCA, the recommended count is < 1,000 cfu/ml and when using the proteolytic psychrotrophic count on SMA medium the recommended count is < 250 cfu/ml. When using the *Pseudomonas* count on *Pseudomonas* agar, the recommended count is < 100 cfu/ml.

3. Problems caused by the occurrence of biofilms from the dairy farm to the processing plant

3.1 The nature of biofilms interferes with proper cleaning and sanitation

A biofilm is defined as a sessile microbial community characterized by adhesion to a solid surface and by production of a matrix that surrounds the bacterial cells and includes extracellular polysaccharides (EPSs), proteins and DNA (Wingender et al., 2001; Whitchurch et al., 2002; Costerton et al., 2003; Bjarnsholt et al., 2009 as quoted by Marchand et al 2012). According to Tsuneda *et al.,* (2003) as quoted by Simões *et al.,* (2010), proteins and polysaccharides account for 75–89% of the biofilm EPS composition, indicating that they are the major components. Biofilm development is a result of successful attachment and subsequent growth of microorganisms on a surface. The formation of biofilms results in surfaces of equipment used in food and beverage (such as milk) processing and handling commonly becoming contaminated by microorganisms, even following cleaning and disinfection procedures (Gibson *et al*.,1999; Marouani-Gadri *et al.*, 2010 as quoted by Marchand *et al.,* 2012).

It is well established that bacteria can switch between different habitation modes, namely from single cells (the planktonic or free-floating single cell state) and biofilms. In addition, it has been established that for each planktonic bacterium detected, there might be close to 1000 organisms present in biofilms (Momba *et al.,* 2000 as quoted by Marchand *et al.,* 2012). Bacteria within biofilms are protected from sanitizers due to multispecies cooperation and the presence of EPSs, by which their survival and subsequent contamination of processed milk products is promoted (Marchand *et al.,* 2012). The EPSs also serve many other functions such as providing an adhesive foundation, structural integrity, bacterial protection and promotion of intercellular communication (Loiselle *et al.,* 2003; Zhang *et al.*, 2005; de Carvalho, 2007; Ploux *et al*., 2007; Leroy *et al*., 2008 as quoted by Molobela *et al.,* 2010).

3.2 Biofilms are regulated by environmental and physiological triggers

Under suitable conditions, a biofilm in a milk processing environment develops initially through accumulation of organic matter on a metal surface, which is then colonized by bacteria. Transition from planktonic mode to biofilm mode is regulated by a variety of environmental and physiological triggers, such as quorum sensing, nutrient availability, and cellular stress. A biofilm community may comprise single and/or multiple species of bacteria and form a single layer or 3-

dimensional structures. Biofilms are large, complex, and organized bacterial ecosystems in which water channels are dispersed providing passages for nutrient, metabolite, and waste product exchange (Sauer *et al.,* 2007 as quoted by Marchand *et al.,* 2012). This organizational structure contributes to the difficulty in proper cleansing and sanitizing of pipelines and equipment.

3.3 The location of biofilms in the equipment contributes to corrosion and problems in sanitizing

Teixeira *et al.,* (2005, as quoted by Marchand *et al.,* 2012) illustrated that the short rubber milking tube (of the cluster in automatic milking machines) is one of the points more prone to biofilm formation.(Marchand *et al.,* 2012). Other possible hazards include biofilm accumulation and microbial colonization in milk pipelines, storage tanks, and milk silos (Shaheen *et al.,* 2010 as quoted by Marchand *et al.,* 2012). While a biofilm can spread at its own rate by ordinary cell division, it will also periodically release **"**pioneer" cells to colonize downstream sections of piping (Marchand *et al.,* 2012). Once established, biofilms accelerate corrosion and material deterioration. Dead ends, corners, cracks, crevices, gaskets, valves, and joints are all possible points for biofilm formation (Storgards *et al.,* 1999a; Storgards *et al.,* 1999b as quoted by Marchand *et al.,* 2012)

3.4 Biofilms are a source of heat-stable extracellular lipases, proteases, and lecithinases

Bacterial spoilage causes significant losses for the dairy industry. Milk contamination with psychrotrophic microorganisms is of concern to the dairy industry as dairy products
are stored and distributed at temperatures permissive for the growth of these organisms. *Pseudomonas* spp. can grow to high numbers and can form biofilms during refrigerated storage. Many of them produce heat-stable extracellular lipases, proteases, and lecithinases that contribute to milk spoilage (Shah 1994; Sorhaug and Stepaniak 1997; Marchand *et al.,* 2009b; as quoted by Marchand *et al.,* 2012). Furthermore, many of these enzymes remain active even following thermal processing steps that destroy their producing organisms (Garcia *et al.,* 1989; Sorhaug and Stepaniak 1997; Marchand *et al.,*2009b as quoted by Marchand *et al.,* 2012). Lipases hydrolyse tributyrin and other milk fat glycerides to yield free fatty acids, which cause milk to taste rancid, bitter, unclean, and soapy. Lecithinases degrade milk fat globule membrane phospholipids and increase the susceptibility of milk fat to the action of lipases (Cousin 1982; Shah 2000 as quoted by Marchand *et al.,* 2012). Peptidases/proteinases attack and destabilise the casein micelle causing problems that might result in the rejection of the milk when subjected to a platform test such as

the alizarol test and may contribute to gelation problems in heat treated long life milk. The hydrolytic products of milk fats and proteins always decrease the organoleptic quality of fluid milk products.

Since biofilms are frequently exposed to sanitizers during cleaning of dairy processing equipment, phenotypic switching may occur on a regular basis and even influence the enzyme production by pseudomonads adding an additional spoiling factor to the subsequently processed milk batch. This might certainly be the case if such *Pseudomonas* biofilms are present in the cooling equipment on farms or in holding silos in the dairy factory. Since the enzymes might be released from the biofilms into the milk, without bacterial detachment, the contamination might go unnoticed until problems arise with the shelf-life of the heat-treated dairy products. (Marchand *et al.,* 2012).

3.5 Resistance to antimicrobials

Bacteria in biofilms have intrinsic mechanisms that protect them from even the most aggressive environmental conditions, including the exposure to antimicrobials (Gilbert *et al.,* 2002; Cloete 2003; Davies 2003 as quoted by Marchand *et al.,* 2012). Dynes *et al.,* (2009) as quoted by Marchand *et al.,* (2012**)** investigated the effect of sub-inhibitory concentrations of four antimicrobial agents. Their results indicated that each antimicrobial agent elicited a unique response: *P. fluorescens* cells and biofilms changed their morphology and architecture, as well as the distribution and abundance of biomacromolecules, in particular the exopolymer matrix. In this regard, Norwood and Gilmour (2000) as quoted by Marchand *et al.,* 2012 investigated the effect of sodium hypochlorite on multispecies biofilms Their study confirmed that multispecies biofilms increased protective properties over monospecies biofilms. The authors attributed these observations to the shielding effect of increased numbers (or aggregation) of microorganisms but also to the production of greater amounts of EPS. Sommer *et al.,* (1999 as quoted by Marchand *et al.,* (2012**),** also found an increase of *Pseudomonas* biofilm resistance to chlorine with increasing age of the biofilm. Certainly, in pseudomonads, the capacity to alter EPS composition may be part of its intrinsic resistance to antimicrobials (Dynes *et al.,* 2009 as quoted by Marchand *et al.,* 2012).

The cells found in the deepest areas of the biofilm exhibit a lower growth rate due to the negative gradients of oxygen and nutrients (Brown, Allison, & Gilbert, 1988 as quoted by Gonzalez-Rivas

et al., 2018). These factors generate an almost inactive state (hibernation), which in turn produces increased resistance to biocides (Evans, Allison, Brown, & Gilbert, 1991; Van Houdt & Michiels,2010 as quoted by Gonzalez-Rivas et al., 2018).

4. Monitoring for psychrotrophic bacteria and biofilms in the dairy industry

4.1 Rapid techniques for the estimation of psychrotrophic bacteria in milk

4.1.1 The Psychro-Fast test

The Psychro-Fast test has been used as a rapid, qualitative test for the evaluation of the presence of psychrotrophs in milk in Australia (DRDC Quarterly report 1993). The results from the project of Hugo *et al.*, (2017b) illustrated that the rapid qualitative Psychro-Fast test, which gives results within 30 h after incubation at 30^{o}C, can be used to not only indicate the presence of psychrotrophic bacteria in raw milk, but the pink colour intensity can be used to indicate the degree of psychrotrophic bacterial contamination in raw milk. The findings showing the relationship of colour intensity with bacterial count in the milk is shown in Table 4

Table 4 Relationship between Psychro-fast test colour intensity and psychrotrophic count (Hugo *et al.*, (2017b)

Scale	1	2	3
Description	White - Light Pink	Pink	Dark pink/purple
Psychrotrophic count range (cfu/ml)	1-100	1,000 - 35,000	15,000 - 500,000

4.1.2 Plate counts for the rapid enumeration of psychrotrophic bacteria and proteolytic psychrotrophs in milk.

The standard psychrotrophic count method requires an incubation time and temperature combination of 10 days at 7^{o}C on Standard Plate Count Agar (SPCA), a standard medium corresponding to the American Public Health Association formulation for milk, water, food, and dairy products (Oxoid CM0463). The problem with this method is the long incubation time, which yields data that may only be of historical value (Fischer *et al.*, 1986 as quoted by Hugo *et al.*, 2017b). Statistical analysis by Hugo *et al.*,(2017b) indicated that when psychrotrophic plate counts are used, the accelerated incubation format (18^{o}C for 48 h; APC) is an acceptable replacement for the standard, time-consuming, incubation format (7^{o}C for 10 days). In the study of Mabunda and Jooste (2007) the APC was well correlated with the standard psychrotrophic

count (r = 0.7310) in a pour plate version of the APC. In the earlier study of Fischer *et al.,* (1986) the surface plated Accelerated Psychrotrophic Count (APC) with incubation temperature and time of 18^{o}C/45h had a correlation coefficient of r = 0.911 (n = 69) with the Standard Psychrotrophic Count (7^{o}C/10 days). No significant differences in the proportions of bacterial types with the two count formats were found to exist at a 1% level in the study of Fischer *et al.,* (1986).

For detection of proteolytic psychrotrophic counts, SPCA casein agar (SPCA-cas) or skim milk agar (SMA) could be used. The SPCA-cas however gave more accurate results. When the standard incubation format (7^{o}C for 10 days) was compared to the accelerated incubation format (18^{o}C for 48 h), no significant differences (p = 0.290) between the different incubation formats for either psychrotrophic or proteolytic psychrotrophic counts of the raw milk samples was observed (Hugo *et al.,* 2017b).

4.1.3 The *Pseudomonas* plate count

The *Pseudomonas* count was performed by Hugo *et al*., (2017b) on *Pseudomonas* agar base (Oxoid CM0559) with C-F-C supplement (Oxoid SR0102) using the pour plate method. Incubation was at 18^{o}C for 48. Colonies with blue-green or brown pigmentation, or that demonstrated fluorescence were counted and considered to be Pseudomonas spp. (Oxoid, 2007). The relationship between the *Pseudomonas* count, the rapid psychrotrophic- and the proteolytic psychrotrophic counts as determined by Hugo *et al.,* (2017b) are shown in Table 3 above.

4.1.4 Molecular techniques for enumeration of psychrotrophic bacteria by flow cytometry.

The fluorescence *in situ* hybridization (FISH) technique is described by Gunasekera *et al.,* (2003) as a method for the identification of psychrotrophic pseudomonads in 4.3.4.1. It utilizes fluorescently labelled DNA oligonucleotide probes to detect specific sequences of ribosomal RNA (rRNA) (Wallner *et al.,* 1993; Amann *et al.,* 1995 as quoted by Gunasekera *et al.,* 2003). rRNA provides a unique target for nucleic acid probes, as rRNA target molecules are generally present in high numbers in viable cells, and probes with various degrees of specificity (genus-, species- or even subspecies-level) may be designed (Wallner *et al.,* 1993 as quoted by Gunasekera *et al.,* (2003). The potential exists to detect and enumerate FISH probed bacteria in real time using flow cytometry (Wallner *et al.,* 1993, 1996 as quoted by Gunasekera *et al.,* (2003). Gunasekera *et al.,* (2003) conclude that the development of rapid assays for detection and enumeration of

Pseudomonas cells in milk are important steps towards quantitative population analysis of bacteria in milk and the rapid quantification of *Pseudomonas* in raw and pasteurised milk. The work reported by them suggests that FISH can play an important role in biological safety and quality issues.

4.2 Determination (detection) of biofilms on milk contact surfaces

The different methods employed for sampling and enumeration of bacteria in biofilms in a dairy plant are swabbing, rinsing, agar flooding, and agar contact methods. Grooves, crevices, dead-ends, and corrosion patches are areas where biofilms readily occur, but are hard to access, thus hampering sampling of such areas (Kumar and Anand 1998; Wirtanen 1995 as quoted by Marchand et al., 2012). Sensory evaluation of the dairy farm or -plant involves visually inspecting surfaces under good lighting, smelling for offensive odours and feeling for greasy or encrusted surfaces. This method is used as a process control technique to immediately rectify obvious shortfalls in sanitation. Microbiological assessments, that follow on such an examination, are typically used to ensure compliance with microbial standards and to optimize sanitation procedures (Holah, 1995 as quoted by Gonzalez-Rivas et al., 2018).

Microbiological assessment includes the standard surface sampling methods such as the swab, contact plates, and microorganism recovery by extraction rinses (Kasuga et al., 2011; Valentine et al., 2008 as quoted by Gonzalez-Rivas et al., 2018). The first method, using swabs or sponges, consists of rubbing the surface with a sterile sponge or a cotton swab and then resuspending it in a suitable recovery medium for microbiological testing (Lelieveld, Holah, & Napper, 2013 as quoted by Gonzalez-Rivas et al., 2018). Disadvantages of these methods is that not all the microorganisms on the surface can be recovered effectively because biofilms for example are not always easy to remove from the surface. Bacterial cells can also be retained in the swab or the sponge itself, decreasing the total count and producing erroneous results. The contact plate system is based on using a sterile agar plate that is pressed onto the surface to be evaluated and counting the bacterial colonies that develop after an incubation period. It is easy to apply, but only on flat, smooth surfaces with low counts because dilutions cannot be made. As referred to above, the microbiological status of the equipment and pipelines can also be tested by extraction rinses that can be examined by traditional microbiological tests such as counts after diluting the rinse buffer

and plating into suitable agar media. Traditional microbiological culture methods are however time consuming (Branda, Vik, Friedman, & Kolter, 2005 as quoted by Gonzalez-Rivas et al., 2018), and for the food industry it is very important to reduce the time required to confirm microbial contamination to know the hygienic state of the environment and be able to take fast corrective measures.

For the above reason, a product like BioFinder (INNOGIENE, Tania Garcia-Warner, tania@innogiene.co.za) is a good solution. The reagent can be sprayed onto surfaces being examined. The product is designed to react with catalase, an enzyme present in almost every living cell and which is universally found in biofilms. When biofilms are present the reagent undergoes a highly visible colour change and an effervescence of many small bubbles occurs as the reagent reacts with the catalase enzyme in the biofilms. BioFinder can also be used to check critical inspection points just before disinfection and to validate correct hygiene procedures at production sites (Ripolles-Avila, R´ıos Castillo, & Rodr´ıguez-Jerez, 2018 as quoted by Gonzalez-Rivas et al., 2018). The technique is of course only useful on exposed surfaces and while it is available in New Zealand and Australia, it is not certain whether this product is available in South Africa.

In a report to MilkSA (Du Plessis, 2016) discussions held with quality managers, factory managers and heads of laboratory at South African UHT processing plants, revealed that biofilms were usually detected using fluorescent techniques. More information will shed light on what these techniques entail. Advanced physical techniques that are available for examining biofilms on equipment surfaces include the application of Confocal laser scanning microscopy (CLSM) combined with a variety of fluorescent stains (Lawrence and Neu 1999; Manz *et al.*, 1999 as quoted by Marchand *et al.*, 2012). Viability and distribution of cells within the biofilm may be analysed as well. When using epifluorescence microscopy or CLSM, the choice of suitable fluorescent stains is critical to increase the contrast between organisms and the exopolymers in the biofilms. Nucleic acid stains such as 4,6-diamino-2-phenylindole (DAPI) or acridine orange have been used to stain the DNA of cells regardless of their viability. Other dyes sensitive to viable cells such as propidium iodine (PI) or 5-cyano-2,3-ditolyl tetrazolium chloride may be used to illustrate differences between viable and dead cells (Trachoo 2003; Donlan and Costerton 2002 as quoted by Marchand *et al.*, 2012).

Except for rinsing techniques, it is virtually impossible to examine pipelines or a closed system for microbiological contamination. Advanced techniques have however been developed for examining the intact equipment for the presence of biofilm. These methods report biofilm growth online, in real-time and non-destructively. One example is a method that uses duplicate turbidity measurement devices installed in the equipment. The one device is constantly cleaned. The difference of signals is proportional to the biomass developing on the non-cleaned window (Klahre and Flemming 2000 as quoted by Marchand et al., 2012). Another technique is the fibre active device (FOS), which is based on a light (optical) fibre integrated in the test surface, measuring the scattered light of material deposited on the tip (Tamachkiarow and Flemming 2003 as quoted by Marchand et al., 2012). A third in situ method that was developed, is a fouling cell assembly in 316L-grade stainless steel that may be placed in dairy pipelines and silos. Such assemblies enable monitoring of biofilm development without removal of the processing equipment out of the plant and can be used to generate objective data on the effectiveness of cleaning procedures (Fornalik 2008 as quoted by Marchand et al., 2012). A fourth mechatronic surface sensor that can detect biofilms in the early stages of development was developed by Pereira, Mendes, & Melo, (2009 as quoted by Simões et al., 2010). This sensor was also able to detect the presence of cleaning products on a surface, to determine when it was biologically and chemically cleaned and to measure the rate of cleaning.

4.3 Identification of psychrotrophic bacteria

4.3.1 Phenotypic identification

Munsch-Alatossava and Alatossava), (2006, as quoted by de Oliveira *et al.*, 2015) employed two commercial phenotypic identification systems (API-20NE and BIOLOG GN2) and reported difficulties in obtaining confident identification of many of the isolates. This was particularly the case for fluorescent pigment-producing pseudomonads, where the biochemical results were doubtful. Nevertheless, identification with the BIOLOG Gen lll identification system was successfully performed by Hugo *et al.*, (2017b) on pure isolates, following the Biolog test procedure (BIOLOG, 2013). Colonies were streaked on Biolog Universal agar (BIOLOG 70101) and incubated at 25^{o}C for 24 h. Colonies were inoculated onto Microplates and the procedure was carried out according to the manufacturer's (BIOLOG Inc.) instructions. In this study, the *Pseudomonas* isolates from SPCA (Standard Plate Count Agar) were represented by seven species

(% isolates out of 38 isolates) namely *Ps. fluorescens* (29%), *Ps. plecoglossicida* (24%), *Ps. fragi* (21%), *Ps. agarici* (13%), *Ps. syringae* (5%), *Ps. lundensis* (3%) and *Ps. marginalis* (3%).

According to de Oliveira et al (2017), an extensive body of published data exists on the prevalence of psychrotrophic bacterial types in milk. Nevertheless, they are of the opinion, based on the results of recent studies, that numerous gaps exist in our understanding of the biology of the psychrotrophic bacteria of importance for the dairy industry. The continued development of molecular tools for bacterial identification and their application to the analysis of microbial population structures and ecology in milk and dairy products has revealed the presence of psychrotrophic bacteria undetected by the use of traditional culture-based approaches (Almeida and Araujo, 2013; Marchand *et al.*, 2009b; Raats *et al.*, 2011 as quoted by de Oliveira *et al.*, (2017) and more work in this regard is encouraged.

Most studies on milk-associated psychrotrophs have focused on individual isolates grown as planktonic cultures (readily culturable). However, there is an increasing recognition that such approaches overlook potential interactions and cross-communication between different species of psychrotrophic bacteria, many of which are non-culturable, that are present within the biofilms that develop in milk storage and processing environments and that may exert an influence on milk quality and safety (Cleto *et al.*, 2012; Marchand *et al.*, 2012; as quoted by de Oliveira *et al.*, 2017).

4.3.2 Matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) for rapid identification and characterization of bacterial isolates.

This system has been used for the identification of bacterial types such as *E. coli* and *Listeria* in the laboratories of the Department of Consumer and Food Sciences at the University of Pretoria. Possible application of this system to identify psychrotrophic bacteria from milk should be discussed with Prof Elna Buys, HoD of the Department.

4.3.3 Dairy Standard Agency.

The analytical laboratory at the Dairy Standard Agency also has the capability of identifying bacterial isolates from milk using the BIOMERIEUX VITEK 2 system and use of their facilities in this regard can be discussed with the CEO, Mr Jompie Burger.

4.3.4 Molecular methods for the rapid identification of psychrotrophic bacteria

Molecular analyses of microbes offer some advantages over phenotypic methods, including speed and the ability to provide precise identification of microorganisms from the genus to the strain level, depending on the system used (de Oliveira *et al.,* 2015).

4.3.4.1 Fluorescent oligonucleotide probe hybridization

Fluorescent oligonucleotide probe hybridization is becoming an important tool in food microbiology to identify specific micro-organisms in mixed communities without the need for isolation in pure cultures. The fluorescence *in situ* hybridization (FISH) technique, referred to in section 4.1.4 above, utilizes fluorescently-labelled DNA oligonucleotide probes to detect specific sequences of ribosomal RNA (rRNA) (Wallner *et al.,* 1993; Amann *et al.,* 1995 as quoted by Gunasekera *et al.,* 2003). The rRNA provides a unique target for nucleic acid probes, as rRNA target molecules are generally present in high numbers in viable cells, and probes with various degrees of specificity (genus-, species- or even subspecies-level) may be designed (Wallner *et al.,* 1993). The *Pseudomonas*-specific FISH probe was tested on *Pseudomonas* spp. isolated from milk and various other bacteria commonly encountered in milk. The specificity of the probe was also validated using the polymerase chain reaction (PCR) method using the *Pseudomonas*-specific sequence as target for a PCR primer.

4.3.4.2 PCR amplification of the 16S DNA region, amplification of the 16S rRNA fragment and *rpoB* sequencing

Pseudomonas spp. were initially identified by PCR amplification of the 16S DNA region. The PCR reaction conditions were as described in Scatamburlo *et al.,* (2015) as quoted by Meng *et al.,* (2017). Amplicons (618 bp) were considered indicative of *Pseudomonas* spp. Isolates identified as *Pseudomonas* spp. (n143) were further analysed by amplification of the 16S rRNA fragment and *rpoB* sequences. Sequence data of the isolated *Pseudomonas* species were analysed using the Basic Local Alignments Search Tool (BLAST) program available from the National Center for Biotechnology Information (NCBI) (Meng et al 2017). Genus-specific PCR of 16S DNA fragments (618 bp) was used for preliminary characterization of their assignment at the *Pseudomonas* genus level. Of the 143 isolates, 58.7% belonged to the *Ps fluorescens* group, 27.3% to the *Ps chlororaphis* group,1.4% to the *Ps putida* group and 12.6% to an unknown group (consisting of a variety of pseudomonad species).

4.3.4.3 Sequencing of 16S rDNA in combination with restriction fragment length polymorphism (RFLP) analyses.

Munsch-Alatossava and Alatossava, (2006**;** as quoted by de Oliveira *et al.,* 2015) recommended the use of genotypic identification systems in future studies. A comparative evaluation of phenotypic and genotypic methods for the identification of 102 food-associated psychrotrophic *Pseudomonas* spp. clearly demonstrated that molecular methods (sequencing of 16S rDNA in combination with restriction fragment length polymorphism (RFLP) analyses) provided superior results (Franzettiand Scarpellini, 2007 as quoted by de Oliveira *et al.*, 2015).

High-throughput sequencing of 16S rDNA and real time quantitative PCR (qPCR) analysis were used in combination with flow cytometry to examine the microbial content of raw and pasteurised cow milk (Quigley *et al.*, 2013 as quoted by de Oliveira *et al.,* 2015). Quigley *et al.,* (2011; as quoted by de Oliveira *et al.,* 2015) recently reviewed several culture-dependent and -independent methods applicable to milk and cheese. Among the culture-independent methods, denaturing gradient gel electrophoresis (DGGE) based on the separation of complex mixtures of PCR amplicons of the same size, but with different nucleotide sequences, has emerged as the most commonly used fingerprinting technique applied to the study of populations of psychrotrophic bacteria associated with milk and dairy products (Ercolini, 2004; Raats *et al.,* 2011; Rasolofo *et al.*, 2010, as quoted by de Oliveira *et al.*, 2015)). An alternative molecular fingerprinting method called random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) was applied to 66 bacterial isolates from cold stored raw milk prior to nucleotide sequence analysis of the gene encoding the 16S ribosomal RNA (Ercolini *et al.*, 2009; as quoted by de Oliveira *et al.,* 2015).

4.4 Determination of proteolytic activity in milk

4.4.1 The Azocasein method (Becker *et al.*, 2015)

The proteolytic activity was assayed by the azocasein method according to Christen and Marshall (1984) and Deeth et al. (2002) with some modification by Becker *et al.,* (2015). Cell free extracts were used to determine the proteolytic activity and were prepared by removing 1 mL of milk from each sample and centrifuging for 15 min at 18 080 x g. The supernatant of each sample was used. For determination of proteolytic activity, 250 µL of the cell free extract was added to 1 ml 1%

azocasein (Sigma-Aldrich A2765; 10 $g \cdot L^{-1,}$ dissolved in phosphate buffer, pH 7.2). For the control sample, 250 µL of phosphate buffer was added in the place of the cell free milk extract. The mixtures were incubated for 1 h at 37 °C. After incubation, the reaction was terminated by adding 1 mL of 5% trichloroacetic acid (TCA). The mixture was centrifuged for 15 min at 18 080 x *g* and the absorbance of the supernatant was read at 345 nm (Christen and Marshall 1984; Deeth et al. 2002 as quoted by Becker *et al.,* 2015). One unit of proteolytic activity was defined as the amount of enzyme required to produce an absorbance increase at 345 nm of 0.01 within 1 hour under the assay conditions (Deeth et al., 2001 as quoted by Becker *et al.,* 2015).

4.4.2 The Trinitrobenzenesulfonic acid (TNBS) method (Meng *et al.,* 2017)

The proteolytic (bacterial peptidase) activity in milk was determined according to the protocol described by Caldera et al. (2016; as quoted by Meng *et al.,* 2017) to quantify the native proteolytic activity at the chosen incubation temperature. The trinitrobenzenesulfonic acid (TNBS) method was used to monitor the presence of free a-amino groups, indicators of protein hydrolysis (Polychroniadou, 1988; Marchand et al., 2009a as quoted by Meng *et al.,* 2017). The TNBS reagent (Sigma–Aldrich, Taufkirchen, Germany) was reacted with the released a-amino groups at pH 9.2 in the dark for 100 min. The intensity of the yellow-orange colour of the reaction products was measured by absorption values at 420 nm (VarioskanTM Flash Multimode Reader, Thermo Fisher Scientific, Waltham, MA, United States).

Bacterial growth in the milk was considered peptidase active if the measured absorption exceeded 2 mmol glycine equivalents per mL A standard curve was generated using glycine (Sigma–Aldrich). In the standard curve, relating glycine concentration to absorbance in the TNBS assay, the R^2 value was greater than 0.99. Bacterial growth in the milk was considered proteolytically active when the measured proteolytic activity exceeded 2 mmol of glycine equivalents per mL. Proteolytic activity was then confirmed by TNBS quantitative analysis and expressed as glycine equivalents per mL In their study Meng et al (2017) conclude by saying that it is necessary to acquire more information on *Pseudomonas* spp. with proteolytic activity and to develop sensitive and efficient tools to monitor for the presence of peptidases in raw milk.

4.4.3 Calbiochem Protease assay kit. Merck (Sigma/ Aldrich)

The commercial kit is allegedly suitable for the quantitative assay of a wide variety of proteases in biological samples, utilizing FTC-casein as a substrate. Enzyme activities that have been detected with this assay kit include chymotrypsin, elastase, plasminogen, PRONASE® Protease, subtilisin, thermolysin, and trypsin. Each kit provides sufficient reagents for 200 assays. The Protease Assay Kit can detect ~10 ng/ml of protease when a 24 h incubation is used. The sensitivity can be enhanced by up to 3-fold by using a fluorometer (excitation 490 nm and emission 525 nm) rather than a spectrophotometer to detect the proteolytic end products.

4.4.4 Identification of the *aprX* Gene (Meng et al 2017)

The presence of the *aprX* gene was detected in all the bacterial isolates that displayed extracellular peptidase activity.

4.4.5 Proteolytic assays on agar plates

4.4.5.1 On milk- and caseinate agar (Hugo *et al.*, (2017b)

The hydrolysis of casein on milk agar is an indication of proteolytic activity by psychrotrophic enzymes. Milk agar consists of agar with the addition of 10% skim-milk. Clear zones which are visible after incubation of the milk agar plates are evidence of casein hydrolysis. However, false positives may occur in which case the plates can be flooded with 1% hydrochloric acid which is a protein precipitant (Harrigan, 1998 as quoted by Hugo *et al.*, 2017b). According to Martley, Jayashankar and Lawrence (1970; as quoted by Hugo *et al.*, 2017b) standard methods agar with added caseinate (1% w/v), citrate (0.015 M) and Ca_2+ (0.02 M) is an improved medium with greater sensitivity compared to milk agar media for the detection of proteolytic organisms. The sensitivity of standard methods caseinate agar (SMCA) is due to its ability to detect the first stage of casein breakdown as shown by the formation of a white zone of precipitation. The precipitation is the deposition of the insoluble para-k-casein which is readily detected in the transparent medium. This medium can be used for the simultaneous determination of proteolytic and total bacterial counts (see 4.1.2 above)

4.4.5.2 Plate assays for proteolytic pseudomonads (Meng *et al.,* 2017)

Pseudomonas single colonies were streaked onto *Pseudomonas* agar (Oxoid Ltd.) supplemented with penicillin (100,000 IU/L, Dr. Ehrenstorfer GmbH, Augsburg, Germany), pimaricin (0.01 g/L, Dr. Ehrenstorfer GmbH), and UHT milk [10%, vol/vol, Modern Farming (Group) Co., Ltd., Hebei, China] (henceforth referred to as "milk agar") (Scatamburlo *et al.,* 2015; as quoted by Meng *et al.,* 2017). The plates were incubated at 25^{o}C for 5 days. Proteolytic halos in the inoculated areas were indicative of proteolytic activity (Scatamburlo *et al.,* 2015 as quoted by Meng *et al.,* 2017).

5. Reducing contamination, due to biofilms, throughout the dairy manufacturing chain: potential solutions

5.1 Pre-conditioning surfaces to prevent biofilm formation

Cloete and Jacobs (2001; as quoted by Simões *et al.,* 2010) reported that surface pre-conditioning with surfactants has potential to prevent bacterial adhesion. Non-ionic and anionic surfactants were evaluated in preventing the adhesion of *P. aeruginosa* to stainless steel and glass surfaces. The surfactants gave more than 90% inhibition of adhesion. More recently, other studies (Meylheuc, Renault, & Bellon-Fontaine, 2006; Pereira *et al.,* 2006; Splendiani, Livingston, & Nicolella, 2006 as quoted by Simões *et al.,* (2010) reinforced the idea of surface pre-conditioning and the efficiency of surfactants in the control of biofilm formation.

5.2 Sanitizing with detergents and disinfectants

To be able to clean and sanitize properly and effectively i.e. Cleaning-in-place (CIP) and Cleaning-out-of-Place (COP) – the importance of plant-design is crucial. A proper plant-design allows for effective CIP and COP and always includes the 4 parameters (which can be measured and quantified) according to which a Cleaning action/-step must take place i.e. Temperature, Flowspeed/Velocity (m/s); Concentration and Time. In order for any cleaning agent to be functionally and successfully applied, the design of the factory should be of such a nature that a proper cleaning action can be achieved and confirmed. Any mechanical cleaning action must be quantified; if not, the design is faulty and will not allow proper CIP/COP (Du Plessis, 2020, Personal communication)

The main strategy to prevent biofilm formation is to clean and disinfect regularly before bacteria attach firmly to surfaces (Midelet & Carpentier, 2004; Simões *et al.,* 2006 as quoted by

Simões *et al.,* 2010). Mechanical action is recognized as being highly effective in eliminating biofilms (Srinivasan, Stewart, Griebe, Chen, & Xu, 1995 as quoted by Simões *et al.,* 2010). An effective cleaning procedure must break up or dissolve the EPS matrix associated with the biofilms so that disinfectants can gain access to the viable cells (Simões *et al.*,2006 as quoted by Simões *et al.,* 2010). The cleaning process can remove 90% or more of microorganisms associated with the surface but cannot be relied upon to kill them all. Bacteria can redeposit at other locations and given time, water and nutrients can form a biofilm. Therefore, disinfection or sanitization is a necessary additive (Gram *et al.,* 2007 as quoted by Simões *et al.,* 2010). Sanitization entails the use of antimicrobial products to kill microorganisms. The aim of sanitization is to reduce the surface population of viable cells left after cleaning and to prevent microbial growth on surfaces before the production restarts. Sanitizers are more effective in the absence of organic material (fat, carbohydrates, and protein-based materials). Interfering organic substances, pH, temperature, water hardness, chemical inhibitors, concentration and contact time generally affect the efficacy of the sanitizer (Bremer, Monk, & Butler, 2002; Cloete, Jacobs, & Brozel, 1998; Kuda, Yano, & Kuda, 2008 as quoted by Simões *et al.,* 2010). The sanitizers must be effective, safe and easy to use, and easily rinsed off from surfaces, leaving no toxic residues that could affect the health properties and sensory quality of the final products.

The selection of sanitizers to be used in a dairy processing plant depends on the material of the processing equipment used and on the adhering microorganisms. The chemicals currently used in sanitization processes include the following: acidic compounds, aldehyde-based biocides, caustic or alkaline products; chlorine, hydrogen peroxide, iodine, isothiazolinones, ozone, peracetic acid, phenolics, biguanidines and surfactants (Bremer *et al.,* 2006; Dosti *et al.,* 2005; Rossmore, 1995; Simo˜es *et al.,* 2006; Wirtanen *et al.,* 2000 as quoted by Simões *et al.,* 2010).

These sanitisers are used to suspend and dissolve food residues by decreasing surface tension, emulsifying fats, and denaturing or dissolving proteins and destroying microorganisms (Forsythe & Hayes, 1998; Maukonen *et al.,* 2003 and Mosteller & Bishop, 1993 as quoted by Simões *et al.,* 2010). These chemicals can be used alone or in combination. A significant reduction in the number of attached *Pseudomonas fluorescens* cells was achieved on surfaces such as gasket materials made of rubber and Teflon when exposed to a hypochlorite sanitizer. Wirtanen and others (1995; as quoted by Marchand *et al.,* 2012) showed that a hydrogen peroxide-based

disinfectant was the most effective disinfectant against *Pseudomonas* biofilms when the microbiological activity was measured using conventional culture methods. The effect of hydrogen peroxide is based on the production of free radicals, which affect the biofilm matrix.

Biofilms are difficult to remove from milk processing environments due to the production of EPS materials and the difficulties associated with cleaning complex processing equipment and processing environments. Since stringent cleaning protocols are available, cleaning procedures should be accurately applied, and ideally, the cleaning efficiency should be evaluated. However, there is far too little knowledge on persisting contamination sources and existing innovative cleaning and disinfection techniques.

5.3 Quorum sensing antagonists

Cell–cell signalling has been demonstrated to play a role in cell attachment and detachment from biofilms (Daniels *et al.,* 2004 and Donlan, 2002 as quoted by Simões *et al.,* 2010). Bacteria, as they associate in biofilms, are considered to be far from solitary microorganisms, and in fact are colonial by nature and exploit elaborate systems of intercellular interactions and communications to facilitate their adaptation to changing environments (Davies *et al.*, 1998; Fuqua & Greenberg, 2002; Sauer & Camper, 2001 as quoted by Simões *et al*., 2010). The successful adaptation of bacteria to changing natural conditions is dependent on their ability to sense and respond to the external environment and modulate gene expression accordingly (Daniels et al., 2004 as quoted by Simões *et al*., 2010).

So-called "quorum sensing" is based on the process of autoinduction (Eberhard *et al.,* 1981 as quoted by Simões *et al.,* 2010). The process of quorum sensing provides a mechanism for self-organization and regulation of microbial cells (Parsek & Greenberg, 2005 as quoted by Simões *et al.,* 2010). It involves an environmental sensing system that allows bacteria to monitor and respond to their own population densities. The bacteria produce a diffusible organic signal, originally called an auto-inducer (Ai) molecule, which accumulates in the surrounding environment during growth (Fuqua & Greenberg, 2002 as quoted by Simões *et al.,* 2010). High cell densities result in high concentrations of signal and induce the expression of certain genes and/or physiological changes in neighbouring cells (Fuqua, Winans, & Greenberg, 1996; Parsek & Greenberg, 2005 as quoted by Simões *et al.,* 2010). A response to chemical signals in the

process of cell communication is a concentration dependent process, where a critical threshold concentration of the signal molecule must be reached before physiological response is elicited (Decho, 1999; Fuqua & Greenberg,2002 as quoted by Simões *et al.,* 2010).

Quorum sensing systems are known to be involved in a range of important microbial activities. These include extracellular enzyme biosynthesis, biofilm development, antibiotic biosynthesis, biosurfactant production, EPS synthesis and extracellular virulence factors in Gram-negative bacteria (Beck von Bodman & Farrand,1995; Daniels *et al.,* 2004; Davies *et al.,* 1998; Fux, Costerton,Stewart, & Stoodley, 2005; Passador, Cook, Gambello, Rust, & Iglewski, 1993; Pearson, Passador, Iglewski, & Greenberg, 1995 as quoted by Simões *et al.,* 2010).

The discovery that many bacteria use quorum sensing to form biofilms and use it to play a role in so many microbial activities, makes it an attractive target for control of biofilms (Dunstall, Rowe, Wisdom, & Kilpatrick, 2005; Rasmussen *et al.,* 2005 as quoted by Simões *et al.,* 2010). It is conceivable that quorum sensing inhibition may represent a natural, widespread, antimicrobial strategy with significant impact on biofilm formation (Dong, et al., 2005 as quoted by Simões *et al.,* 2010). A good understanding of the cell–cell signalling phenomenon of bacteria can be used to control the biofilm formation process by the identification of products that can act as quorum sensing antagonists (Simões *et al.,* 2009; Smith, Fratamico, & Novak, 2005 as quoted by Simões *et al.,* 2010). This property can lead to the development of new and efficient natural products for biofilm control. A recent study showed that 3-Indolylacetonitrile, a strong, stable indole derivative that acts as a signalling molecule inhibits the biofilm formation of *P. aeruginosa* and *E. coli* O157:H7 (Lee, Cho, & Lee, 2011 as quoted by Gonzalez-Rivas et al., 2018). Interest in studying signal molecules capable of inhibiting biofilm formation more thoroughly has risen since research is showing that many of them could be used as antibiotic agents in the future (Brackman & Coenye, 2015 as quoted by Gonzalez-Rivas et al., 2018).

5.4 Application of enzymes in the removal of biofilms

In a study by Lequette *et al.,* (2010) as quoted by Marchand *et al.,* 2017, the cleansing efficiency of polysaccharidases and proteolytic enzymes against biofilms of bacterial species found in food industry processing lines was analysed. Two serine proteases and an α-amylase appeared to be the most efficient enzymes. Proteolytic enzymes promoted biofilm removal of a larger range of

bacterial species than polysaccharidases, while more specifically, the serine proteases were more efficient in removing *Bacillus* biofilms and the polysaccharidases were better at removing *P. fluorescens* biofilms (Lequette *et al.,* 2010 as quoted by Marchand *et al.,* 2017). Solubilization of enzymes with a buffer containing surfactants and dispersing and chelating agents enhanced the efficiency of polysaccharidases and proteases in removing biofilms of *Bacillus* and *P. fluorescens*, respectively (Lequette *et al.,* 2010 as quoted by Marchand *et al.,* 2017).

Considering these results, a combination of enzymes, targeting several components of EPS, together with surfactants, and dispersing and chelating agents could be a good alternative to chemical cleaning agents. However, the use of enzymes in biofilm control is still limited due to the low prices of the chemicals used today compared with the costs of the enzymes. Another disadvantage was the finding that the performance of the enzyme action was significantly reduced in the presence of milk containing proteolytic enzymes. The specificity in the enzymes mode of action also makes it a complex technique, increasing the difficulty of identifying enzymes that are effective against all the different types of biofilms (Simões *et al.,* 2010).

Nevertheless, it is interesting and informative to take note of work done by Molobela, Cloete and Beukes (2010). The objective of their study was to determine the effect of commercial proteases and amylases on biofilms formed by *Pseudomonas fluorescens*. Biofilms were grown in diluted medium containing glass wool used as the attachment surface. The activity of proteases (Savinase, Everlase and Polarzyme) and amylases (Amyloglucosidase and Bacterial Amylase Novo) was tested on both biofilms and on extracted EPS. The EPS composition was found to consist predominantly of proteins. After testing the enzymes, biofilm integrity was evaluated by scanning electron microscopy. This yielded impressive photomicrographs of the reduction in biofilm attachment on the glass fibres by the enzymes. Everlase and Savinase were found to be the most effective enzymatic treatments for the removal of biofilms and degradation of the EPS.

6. Conclusions and recommendations

Research on South African milk, and much global research in this regard, has stressed the fact that psychrotrophic contamination of refrigerated milk is a challenge that must be reckoned with. In addition, psychrotrophic bacterial species such as *Pseudomonas* are active in producing biofilms on milk contact surfaces. These organisms occur as planktonic (single) cells or in the

form of biofilms that can negatively affect the quality of both unpasteurised and pasteurised milk and dairy products made from contaminated milk.

The transition of delivery of milk to processing dairies from cans in the mid 1960's to bulk transport of refrigerated milk in insulated tankers two decades later had a dramatic effect on the emergence of psychrotrophic bacteria in South African milk supplies that has caused unresolved challenges until the present time. In the first place the percentage of psychrotrophs in the total bacterial count increased dramatically. What was even more surprising was the fact that a seasonal effect became evident especially regarding the prevalence of bacterial types despite refrigeration on the farm and transport in insulated milk tankers. This pointed to the presence of bacterial contaminants, and the formation of biofilms, in pipelines and parts of the equipment that were exposed to environmental temperatures. Such a theory would explain the transition of bacterial types from relatively innocuous, and more mesophilic, species such as *Acinetobacter* dominating in Bloemfontein milk during the summer months, to domination in the winter months by psychrotrophic species such as *Pseudomonas,* with a higher spoilage potential.

In a more recent study of South African refrigerated milk transported in bulk road tankers in 2006, no significant statistical relationship was found between transportation time or temperature and the microbial load in the raw tanker milk samples. This provided additional evidence to strengthen the theory that the milk is contaminated by biofilms growing in the pipelines and equipment and milk contact surfaces exposed to environmental temperatures; and that high counts in the refrigerated milk are not necessarily the result of growth and multiplication of psychrotrophs in the milk during transport and storage. While only 45% of the milk samples tested were within the EU standard of $\log_{10}$ 3.7 cfu/mL (5000 cfu/mL) on arrival at the processing dairy, it showed that a psychrotrophic count standard of 5000 cfu/mL in refrigerated bulk transported milk is not only feasible, but possible.

A recent study by Prof Celia Hugo at Free State University in 2017, supported by MilkSA, was aimed at determining the effect of psychrotrophic counts and bacterial types in milk on the flocculation of raw milk after refrigerated storage. The rapid, qualitative Psychro-Fast test, which gives results within 30 h after incubation at 30ºC, was used to not only indicate the presence of psychrotrophic bacteria in raw milk, but the intensity of the pink colour could also be used to

indicate the degree of psychrotrophic bacterial contamination in raw milk. The accelerated psychrotrophic count, the proteolytic psychrotrophic count and the *Pseudomonas* count had a significant bearing on the time of the raw milk to flocculation after incubation at 7^{o}C. With the *Pseudomonas* plate count of milk samples from the ten farms as reference, it clearly showed how the other parameters correlate with the mean *Pseudomonas* count. As the *Pseudomonas* count decreased, the other two counts decreased accordingly and the time that the milk could be stored at 7^{o}C before flocculation increased. *Pseudomonas* spp. and particularly *Ps. fluorescens*, were found by Hugo *et al.*, (2017b) to be the prevalent organisms at the time of flocculation This lends impetus to the importance of implementing the *Pseudomonas* count as measure of the hygienic quality of the raw milk. Hugo *et al.*, (2017b) suggested that the EU standard of 5000 cfu/mL for psychrotrophic bacteria in raw milk, should be regarded as the upper limit in raw milk for processing. In the study of Mabunda and Jooste (2007) 45 % of samples of bulk milk transported in refrigerated tankers had counts of less than 5000 cfu/mL, which shows that this count of milk upon arrival at the processing dairy is attainable. Hugo *et al.*, (2017b) suggested that a reasonable standard when using the accelerated psychrotrophic count on standard plate count agar incubated at 18^{o}C for 48 h, is <5,000 cfu/ml. For the proteolytic psychrotrophic count on SMCA, the recommended count is < 1,000 cfu/ml and when using the proteolytic psychrotrophic count on Skimmilk agar medium (SMA) the recommended count is < 250 cfu/ml. When using the *Pseudomonas* count on *Pseudomonas* agar, the recommended count is < 100 cfu/ml. These standards should be shown to be attainable and acceptable to the industry in follow-up studies and in collaboration with the large processing companies in South Africa.

Problems caused by biofilms on milk contact surfaces include the following: i) Bacteria within biofilms are protected from sanitizers due to multispecies cooperation and the presence of extracellular polymeric substances, by which their survival and subsequent contamination of processed milk products is promoted; ii) Biofilms are large, complex, and organized bacterial ecosystems that are regulated by a variety of environmental and physiological triggers, such as quorum sensing, nutrient availability, and cellular stress; iii) Once established on milk contact surfaces, biofilms accelerate corrosion and material deterioration of the equipment; iv) Many biofilms produce heat-stable extracellular lipases, proteases, and lecithinases that contribute to milk spoilage; v) Bacteria in biofilms have intrinsic mechanisms that protect them from even the most aggressive environmental conditions, including the exposure to sanitisers. These

characteristics of biofilms should be kept in mind in maintaining the necessary levels of hygiene in equipment used for milk production, transport, and processing.

Effective monitoring for psychrotrophic bacteria and biofilms in the dairy industry remains an ongoing challenge. Accelerated tests for psychrotrophic bacteria in milk include the Psychro-fast test; plate counts for the rapid enumeration of psychrotrophic bacteria, proteolytic psychrotrophs in milk and the *Pseudomonas* plate count. It is important that these tests be included in the testing regimes of the Dairy Standard Agency and in the laboratories of processing companies with such facilities. While more sophisticated testing techniques are more expensive, tests could be outsourced to laboratories with such facilities if necessary One such a system to detect and enumerate bacteria in real time is flow cytometry in conjunction with the fluorescence *in situ* hybridization (FISH) technique.

The detection of biofilms on milk contact surfaces is the responsibility of each processing dairy. This should include the producer farms delivering milk to such processing plants. Visually inspecting surfaces under good lighting, smelling for offensive odours and feeling for greasy or encrusted surfaces is recommended to detect and rectify obvious shortfalls in sanitation. Microbiological assessments, that follow on such an examination, are typically used to ensure compliance with microbial standards and to optimize sanitation procedures. The different methods employed for sampling and enumeration of bacteria in biofilms on the farm and in the dairy plant include swabbing, rinsing and agar contact methods. Traditional microbiological culture methods are however time consuming and not always consistent. For immediate results, a spray-on technique such as Biofinder (INNOGIENE, Tania Garcia-Warner, tania@innogiene.co.za) could be useful.

In situ techniques have been developed for monitoring the intact equipment for the presence of biofilms. These methods report biofilm growth online, in real-time and non-destructively. One example is a method that uses duplicate turbidity measurement devices installed in the equipment. Another technique is a optical fibre device, which is based on a light (optical) fibre integrated in the test surface, measuring the scattered light of material deposited on the tip. A third *in situ* method, is a fouling cell assembly in 316L-grade stainless steel that may be placed in dairy pipelines and silos. The cell assembly, if possible, can then be examined by microscopic

techniques such as confocal laser scanning microscopy (CLSM) combined with a variety of fluorescent stains, if such facilities are available. A fourth mechatronic surface sensor that is able to detect biofilms in the early stages of development is also able to detect the presence of cleaning products on a surface, to determine when it was biologically and chemically cleaned and to measure the rate of cleaning.

Identification of bacteria involved, form an integral part of monitoring for psychrotrophic contamination and biofilms. Techniques based on phenotypic traits of the bacteria include systems such as the commercially available API-20NE galleries, the BIOLOG Gen lll system available at Free State University (Prof Celia Hugo) and the MALDI-TOF-MS identification system available in the laboratories of Pretoria University (Prof Elna Buys). Facilities for the identification of bacteria, using the BIOMERIEUX VITEK 2 system, are also available in the laboratories of the Dairy Standard Agency (Mr Jompie Burger).

Several DNA based molecular techniques for rapid, and possibly more accurate, bacterial identification are also available and more work in this regard is encouraged. The fluorescence *in situ* hybridization (FISH) technique, utilizes fluorescently labelled DNA oligonucleotide probes to detect specific sequences of ribosomal RNA (rRNA). The rRNA in bacterial cells provides a unique target for nucleic acid probes and probes for identification of bacterial isolates with various degrees of specificity (genus-, species- or even subspecies-level) may be designed. In another study *Pseudomonas* spp. were initially identified by PCR amplification of the 16S DNA region. Isolates identified as *Pseudomonas* spp. were further analysed by amplification of the 16S rRNA fragment and *rpoB* sequences. A comparative evaluation of phenotypic and genotypic methods for the identification of 102 food-associated psychrotrophic *Pseudomonas* spp. clearly demonstrated that molecular methods (sequencing of 16S rDNA in combination with restriction fragment length polymorphism (RFLP) analyses) provided superior results. Among the culture-independent methods, denaturing gradient gel electrophoresis (DGGE) based on the separation of complex mixtures of PCR amplicons of the same size, but with different nucleotide sequences, has emerged as the most commonly used fingerprinting technique applied to the study of populations of psychrotrophic bacteria associated with milk and dairy products.

Due to the damage inflicted, by enzymes produced by psychrotrophic bacterial contaminants and -biofilms on milk constituents, especially the casein in milk, it would be advantageous to be able to monitor the proteolytic activity in the milk. Such information will also enable prediction of the integrity of the milk proteins if such tests can be correlated with bacterial counts in the milk and the time to flocculation by the alizarol test after incubation at refrigeration temperatures. Quantitative activity tests that should be evaluated include the Azocasein method, the Trinitrobenzenesulfonic acid (TNBS) method and the Calbiochem Protease assay kit. Further tests should also be looked for in the literature. The evaluation of such tests and the setting of acceptable and applicable standards for bacterial peptidase/protease induced proteolytic activity in the raw milk should be worthwhile as a MilkSA supported research project. It would also be useful if existing tests for the determination of the role of indigenous plasmin activity in the milk could be evaluated. Bacterial protease activity should be distinguished effectively from plasmin activity.

Finally, it is essential to reduce or prevent contamination and biofilm formation in milk-associated equipment. Procedures in this regard include i) Pre-conditioning surfaces to prevent biofilm formation; ii) Sanitizing with detergents and disinfectants; iii) Identification and application of quorum sensing antagonists and iv) Application of enzymes for the removal of biofilms. Pre-conditioning of surfaces is still in an experimental stage, but information in this regard should be reckoned with. The identification and application of quorum sensing antagonists could be useful in combatting the functioning and integrity of the biological ecosystem in biofilms. An in-depth literature study on this topic and the necessary research expertise at a master's or PhD academic level will however be required to contribute in this regard. The application of enzymes for the removal of biofilms shows much promise, but the cost of employing such measures in comparison with chemical sanitisers may prove to be a factor.

The most important and effective procedure in combatting bacterial contamination and removal or prevention of biofilms, however, lies in the domain of chemical detergents and sanitisers. This matter has been touched upon in this report in terms of the various types of detergents and sanitisers that are recommended in the literature. This matter however should be taken further in the form of a research project funded by Milk SA in close collaboration with milk processors that are willing to participate. Cleaning chemicals and procedures should be identified and accurately applied, and the cleaning efficiency should be evaluated. Since the reactive, and possibly even

the pro-active, treatment of equipment to combat biofilms is a costly exercise it is sometimes, for this reason, avoided by factory staff, as related by Du Plessis (2016) in a report to MilkSA. Close cooperation between a researcher(s), identified factory personnel and possibly a person with economic expertise, could yield valuable outcomes for the dairy industry in South Africa.

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ADDENDUM A

Procedures and possible projects emanating from the Psychrotrophic bacteria / Biofilm report of P J Jooste. (Read in conjunction with the Conclusions and Recommendations section)

- Possible follow-up studies or procedures to confirm the recommended bacterial count standards as stated in 2.3 of the report for the accelerated psychrotroph tests in milk as acceptable and attainable in collaboration with industry.This would entail inclusion of the accelerated psychrotroph tests (4.1.1 to 4.1.3 of the report) in the testing regime of DSA and larger processing plants.

- Outsourcing or creation of a facility for performing flow cytometry in conjunction with the fluorescence *in situ* hydridization technique for detecting or enumerating psychrotrophs in real time.

- Are procedures for detecting biofilms on the farm and in the processing plant in place? If so what are the procedures and who are they performed by?

- Are there companies that have installed *in situ* techniques, as referred to in the report, in the factory or on the farm for monitoring intact equipment for biofilms? Such methods are able to report on biofilm growth on-line, in real time and non-destructively.

- Facilities should be identified that can perform molecular (DNA based) techniques for the rapid identification of psychrotrophs and a project could be run to apply such techniques.

- A project (in my opinion) is sorely needed to evaluate tests for the proteolytical activity of bacterial proteases in milk and to propose acceptable and attainable standards in this regard. This should include a survey of the baseline proteolytic activity in milk after production and these samples should then be incubated at 7^{o}C until the samples flocculate with the alizarol test. The proteolytic activity should be measured at regular intervals and also when the milk flocculates. Accelerated proteolytic psychrotroph / *Pseudomonas* counts should be run concurrently to determine the statistical correlation between proteolytic activity, bacterial counts and time to flocculation.

- Although a procedure for determining the activity of indigenous plasmin activity has not been included in the report, such tests do exist and a study in this regard can be run simultaneously with the previous project on bacterial protease activity. It will be useful to determine the effect of indigenous plasmin activity on casein stability in milk in comparison with that of bacterial proteases.

- Projects are needed to prevent or combat biofilm contamination on the farm and the processing dairy.
    - A study on quorum sensor antagonists could be done at a Masters or PhD level.
    - A study should be done on the application and economic feasibility of applying enzymes to remove biofilms
    - An in depth study should be done to determine what sanitation reagents and sanitation procedures are applied in Souh African factories to eliminate biofilms on milk contact surfaces. Because of the economic implications of such procedures it should be a

collaborative study between a researcher(s), milk processors and reagent suppliers who are willing to collaborate and an economist to determine the costs involved.

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