

**EFFECT OF EXTENDED SHELF LIFE MILK PROCESSING ON THE
BACTERIAL COMPOSITION ASSOCIATED WITH THE NOZZLES
OF FILLING MACHINES**

By

Sandile Khoza

Submitted in partial fulfilment of the requirements for the degree

Master of Science

Food Science

In the

Department of Food Science

Faculty of Natural and Agricultural Sciences

University of Pretoria

Pretoria

December 2015

DECLARATION

I declare that the dissertation herewith submitted for the degree MSc Food Science at the University of Pretoria has not previously been submitted by me for a degree at any other university or institution of higher learning.

Sandile Khoza

ACKNOWLEDGEMENTS

I would like to thank God, the Almighty, for having made everything possible by giving me strength and courage to do my Master's degree.

My deepest gratitude to Prof EM Buys, my supervisor, for her unselfishness, encouragement and guidance and patience she demonstrated during my study.

I sincerely want to thank the following people and institutions for helping me to successfully complete this degree:

My fellow students in the Food Microbiology Lab and the staff in the Food Science department for the support and kindness of everyone was a true inspiration.

The University of Pretoria for financial assistance.

To all my friends, my parents and family members for their love and support.

Desmond Mugadza, Mathew Aijuka, Victor Ntuli and Lanre Fayemi for their invaluable advice, encouragement and especially the help with Maldi-Tof.

Special thanks to Alan Hall (Laboratory for Microscopy and Microanalysis, University of Pretoria) who provided assistance for scanning electron microscopy.

DEDICATION

This work is dedicated to my parents, Gladys (my late mother) and Richard, and to my siblings, Simphiwe, Thandeka, Thabile, Mapule and Shirley.

ABSTRACT

EFFECT OF EXTENDED SHELF LIFE MILK PROCESSING ON THE BACTERIAL COMPOSITION ASSOCIATED WITH THE NOZZLES OF FILLING MACHINES

By Sandile Khoza

Supervisor: Prof. Elna. M. Buys

Department: Food Science

Degree: MSc: Food Science

One of the possible reasons for post-contamination of extended shelf life (ESL) milk could be attachment and formation of biofilms on stainless steel pipe surfaces. In South Africa, ESL milk processors are still facing challenges extending the shelf life beyond 14 days. It is hypothesized that post-contamination along the milk processing line is responsible of reducing the shelf life of ESL milk. This assumption was investigated by assessing the microorganisms associated with the nozzles of aseptic filling machines post CIP process and this study was designed and divided into two phases. Phase one involved isolation and characterisation of the bacteria associated with the nozzles of aseptic filling machines after CIP process with the aim of determining the diversity of microorganisms attached to the nozzles of aseptic filling machines. Swab samples were collected from a plant processing ESL milk. Twenty swabs were taken from ten different nozzles of aseptic filling machines during 4 visits (n=80). The swab samples were plated on the day of sampling. A total bacterial count ranging from 1.75 - 1.95 log CFU/cm³ with an average of 1.81 log CFU/cm³ (n=80). MALDI-TOF revealed a high percentage of Gram-positive rods (69%), followed by Gram-positive cocci (20%) and then Gram-negative rods (7%). The Gram-positive rods belonging to genus *Bacillus* were identified as *Bacillus cereus*, followed by *B. pumilus*, *B. subtilis* and *Paenibacillus* spp. The Gram-positive cocci included *S. hominis*, *S. epidermidis*, *Micrococcus luteus* and *Anaerococcus* spp. The Gram-negative rods were identified as *Acinetobacter junii*. The prevalence of *Bacillus* spp. noted in the nozzles of ESL aseptic filling machines is attributed to their ability to resist heat treatment during CIP process and their ability to attach to stainless steel surfaces. There was a degree of similarity in terms of MALDI-TOF MS profiles for the strains of *B. cereus*,

Staphylococcus spp. and *Paenibacillus* spp. originating from the nozzles of aseptic filling machines and the packaged ESL milk product. Furthermore, these isolates show close relatedness. These bacteria are likely to originate from the nozzles, dispensed into the final ESL milk during filling process. Phase two aimed at determining cell surface hydrophobicity of the isolates originating from the nozzles of aseptic filling machines. Co-currently the study further determined the ability of *B. cereus*, *S. epidermidis*, *M. luteus* and *Paenibacillus* spp. to attach and form biofilms on stainless surfaces. The bacterial strains were isolated from the nozzles of aseptic filling machines. The degree of hydrophobicity of the spore formers ranged from 8-91% while non-spore formers ranged from 6-67%. Hydrophobicity of *S. hominis*, *S. epidermidis*, *Acinetobacter junii* and *Arthrobacter castelli* differed significantly ($p \leq 0.05$). Hydrophobicity of *B. pumilus*, *Paenibacillus* spp. and *B. cereus* ($p \leq 0.05$) differed significantly. Spore formers showed the highest hydrophobicity to solvents, this can be contributed to their hydrophobic nature and their ability to attach to stainless surfaces. A continuous flow reactor system was used to grow biofilm of the isolates in skim milk. The skim milk was inoculated with spore suspension of *B. cereus*, *Paenibacillus* spp. and bacterial suspension of *M. luteus* and *S. epidermidis*. The bacterial suspensions were run separately over a period of 20h at 37°C. Stainless steel strips were submitted to Scanning Electron Microscopy (SEM) after 22h. The results suggested that spores of *B. cereus* and *Paenibacillus* spp. can only attach whilst *M. luteus* and *S. epidermidis* can attach and form biofilms on stainless steel. The ability of these isolates to form biofilms on stainless steel strips could be the main cause of contamination of ESL milk. Strains of *Bacillus* spp. can form biofilm on stainless steel and limit the shelf life of milk and milk products. However, toxins produced by some of these strains of *B. cereus* might be contagious to humans. The results confirmed that one of the reasons of contamination of ESL milk could be the ability of *B. cereus* to attach to stainless surfaces and *M. luteus* to form biofilms. Over and above the fact that the spores of *B. cereus* can lead to spoilage of milk and milk products, a concern is that the toxins produced by some of the strains of *B. cereus* are detrimental to human health.

TABLE OF CONTENTS

DECLARATION	i
ACKNOWLEDGEMENTS.....	ii
DEDICATION.....	iii
ABSTRACT.....	iv
LIST OF FIGURES	8
LIST OF TABLES.....	10
CHAPTER 1: PROBLEM STATEMENT.....	Error! Bookmark not defined.
CHAPTER 2: LITERATURE REVIEW	Error! Bookmark not defined.
2.1 EXTENDED SHELF LIFE (ESL) MILK PROCESSING OPERATIONS.....	Error! Bookmark not defined.
2.2 PROCESSING STEPS OF ESL MILK	Error! Bookmark not defined.
2.2.1 PASTEURISATION.....	Error! Bookmark not defined.
2.2.2 BACTOFUGATION.....	Error! Bookmark not defined.
2.2.3 MICROFILTRATION	Error! Bookmark not defined.
2.2.4 ASEPTIC PACKAGING	Error! Bookmark not defined.
2.3 BACTERIA ASSOCIATED WITH DAIRY MANUFACTURING.	Error! Bookmark not defined.
2.3.1 <i>BACILLUS CEREUS</i>	Error! Bookmark not defined.
2.3.2 <i>ENTEROCOCCUS</i> SPP.	Error! Bookmark not defined.
2.3.3 <i>MICROCOCCUS</i> SPP.	Error! Bookmark not defined.
2.3.4 <i>PSEUDOMONAS</i> SPP.	Error! Bookmark not defined.
2.3.5 <i>STAPHYLOCOCCUS</i> SPP.....	Error! Bookmark not defined.
2.4 BIOFILMS IN THE DAIRY INDUSTRY	Error! Bookmark not defined.
2.4.1 IMPACT OF BACTERIAL BIOFILMS IN DAIRY PROCESSING PLANTS	Error! Bookmark not defined.
2.4.2 IMPORTANCE OF <i>BACILLUS</i> SPECIES IN THE DAIRY INDUSTRY ...	Error! Bookmark not defined.
2.5 BACTERIAL BIOFILMS.....	Error! Bookmark not defined.
2.5.1 MECHANISM OF BIOFILM FORMATION	Error! Bookmark not defined.
2.7 MICROBIAL HYDROPHOBICITY	Error! Bookmark not defined.
2.8 OBJECTIVES AND HYPOTHESES.....	Error! Bookmark not defined.
2.8.1 HYPOTHESES	Error! Bookmark not defined.
2.8.2 OBJECTIVES	Error! Bookmark not defined.

CHAPTER 3: RESEARCH	Error! Bookmark not defined.
3.1 INTRODUCTION	Error! Bookmark not defined.
3.2 IDENTIFICATION AND CHARACTERISATION OF BACTERIA ASSOCIATED WITH ASEPTIC FILLING NOZZLES OF ESL MACHINES AFTER CIP	Error! Bookmark not defined.
3.2.1 INTRODUCTION	Error! Bookmark not defined.
3.2.2 MATERIALS AND METHODS	Error! Bookmark not defined.
3.2.3 RESULTS	Error! Bookmark not defined.
3.2.4 DISCUSSION	Error! Bookmark not defined.
3.3 ADHESION OF BACTERIAL STRAINS ISOLATED FROM ESL FILLING MACHINES TO STAINLESS STEEL	Error! Bookmark not defined.
3.3.1 INTRODUCTION	Error! Bookmark not defined.
3.3.2 MATERIALS AND METHODS	Error! Bookmark not defined.
3.3.3 RESULTS	Error! Bookmark not defined.
3.3.4 DISCUSSION	Error! Bookmark not defined.
3.3.5 CONCLUSION	Error! Bookmark not defined.
CHAPTER 4: GENERAL DISCUSSION	Error! Bookmark not defined.
4.1 CRITICAL REVIEW OF METHODOLOGY	Error! Bookmark not defined.
4.2 RESEARCH FINDINGS AND FUTURE WORK	Error! Bookmark not defined.
CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS	Error! Bookmark not defined.
5.1 CONCLUSIONS	Error! Bookmark not defined.
5.2 RECOMMENDATIONS	Error! Bookmark not defined.
CHAPTER 6: REFERENCES	Error! Bookmark not defined.

LIST OF FIGURES

Figure1: Process concept for the production of ESL milk and conventionally pasteurised milk. http://www.drgailbarnes.com/2013/01/extended-shelf-life-future-for-chilled.html [Available online, accessed 10 Feb 2015]	4
Figure 2: Contamination route of spores of <i>Bacillus cereus</i> in a production chain (Heyndrickx, 2011)	11
Figure 3: The different layers and structure of <i>Bacillus cereus</i> spores (Andersson <i>et al.</i> , 1995).....	11
Figure 4: Stages of bacterial biofilm development (Kokare <i>et al.</i> , 2009)	13
Figure 5: Phase 1 identification and characterisation of bacterial associated with aseptic ESL filling nozzles	20
Figure 6: Phase 2 determination of the isolates to attach to hydrocarbons and stainless steel strips	21
Figure 7: (A) Aseptic filling machine used in the production of ESL milk (B) Nozzles of plastic bottle rotary aseptic filling machine. www.sfds.eu/rules-of-conduct/7.html [Accessed 20 March 2015]	25
Figure 8: Schematic diagram of the Extended Shelf Life milk processing at the dairy plant assessed during this study	27
Figure 9: Total bacterial count for four weeks obtained from the nozzles in aseptic filling machines after CIP process	28
Figure 10: Composition of isolates from the nozzles of aseptic ESL filling machines after CIP procedures	29
Figure 11: Dendrogram of <i>Bacillus cereus</i> strains isolated from the nozzles of aseptic ESL filling machines and the packaged ESL milk products	31
Figure 12: Dendrogram of <i>Paenibacillus</i> spp. and <i>Paenibacillus amylolyticus</i> strains isolated the nozzles of aseptic ESL filling machines and packaged ESL milk product	32
Figure 13: Dendrogram of <i>Staphylococcus</i> spp. strains isolated the nozzles of aseptic ESL filling machines and packaged ESL milk products	33
Figure 14: Dendrogram of <i>Bacillus pumilus</i> strains isolated the nozzles of aseptic filling machines and the final ESL milk product	34
Figure 15: Phase contrast image of <i>Bacillus cereus</i> spores after 4 days at 37°C	39
Figure 16: Schott bottles with inoculated 40ml UHT skim-milk incubated at 37°C in a shaking water-bath at 65rpm for 20h	42

Figure 17: Laboratory biofilm reactor system used to evaluate the ability of the isolates to attach and form biofilm on the stainless steel strips	44
Figure 18: Hydrophobicity of the spore formers as measured by bacterial adhesion to hydrocarbons. Horizontal line: values above 50% indicates strong hydrophobic cell surface (Basson <i>et al.</i> , 2008)]	45
Figure 19: Hydrophobicity of the non-spore formers as measured by bacterial adhesion to hydrocarbons Horizontal line: values above 50% indicates strong hydrophobic cell surface (Basson <i>et al.</i> , 2008)	46
Figure 20: Sterile stainless steel strip surface (control)	46
Figure 21: (A) <i>Staphylococcus hominis</i> isolated from aseptic ESL filling nozzles attached on stainless steel under static reactor system after 20h of contact in skim milk at 37°C. (B) Channels and pores between the densely packed cells of <i>S. hominis</i> adhered to stainless steel and connected together forming a matrix at higher-magnifications (blue-arrows)	47
Figure 22: (A) Spores of <i>Paenibacillus</i> spp. isolated from aseptic ESL filling nozzles attached on stainless steel under static reactor system on stainless steel after 20h of contact in skim milk at 37°C. (B) Hair-like structures extending from the spore surfaces (blue-arrows)	48
Figure 23: (A) Spores of <i>Bacillus pumilus</i> isolated from aseptic ESL filling nozzles attached on stainless steel under static reactor system on stainless steel after 20h of contact in skim milk at 37°C. (B) Hair-like structures extending from the spore surfaces (blue-arrows)	49
Figure 24: Spores of <i>Bacillus cereus</i> isolated from aseptic ESL filling nozzles attached on stainless steel under static reactor system on stainless steel after 20h of contact in skim milk at 37°C. Hair-like structures extending from the spores of <i>B. cereus</i> adhered on stainless steel surface (blue-arrows)	50
Figure 25: <i>Acinetobacter junii</i> isolated from aseptic ESL filling nozzles attached on stainless steel under under static reactor system on stainless steel after 20h of contact in skim milk at 37°C	50
Figure 26: Spores of <i>Bacillus cereus</i> isolated from aseptic ESL filling nozzles attached on stainless steel under under continuous flow system on stainless steel after 20h of contact in skim milk at 37°C. (B) Hair-like structures extending from the spore surfaces (blue-arrows)	51
Figure 27: (A) <i>Staphylococcus epidermidis</i> isolated from aseptic ESL filling nozzles attached on stainless steel under under continuous flow system on stainless steel after 20h of contact in skim milk at 37°C. Clustered cells connected together forming a matrix (blue-arrow). (B-C) Cells of <i>S. epidermidis</i> adhered on stainless steel at higher-magnifications	52

Figure 28: (A) *Micrococcus luteus* isolated from aseptic ESL filling nozzles attached on stainless steel under continuous flow system on stainless steel after 20h of contact in skim milk at 37°C. Clustered cells covered by a layer (blue-arrow) and a broken layer covering the cells revealing the surface of stainless steel (red-arrow). (B) Cells of *M. luteus* adhered on stainless steel at higher magnification..... 53

LIST OF TABLES

Table 1: Predominant biofilm micro-flora in different food processes (Kokare, Chakraborty, Khopade, and Mahadik, 2009)	9
Table 2: Novel approaches for control of biofilm (Mogha <i>et al.</i> , 2014)	15
Table 3: Hydrophobicity levels of <i>Bacillus</i> spp., as determined by three methods (Simmonds <i>et al.</i> , 2003)	17
Table 4: Predominant bacteria in the nozzles of aseptic ESL filling machines for isolates (n=80) sampled after CIP over four weeks	30

CHAPTER 1: PROBLEM STATEMENT

Milk is thermally processed to reduce the microbial load for both safety and keeping quality of milk and milk products. Multiple milk heat treatment options are pasteurisation, extended shelf-life (ESL) and ultra-high temperatures (UHT) processing (Rysstad and Kostad, 2006). ESL refers to milk products that have been treated in a manner to reduce the microbial count beyond normal pasteurisation, packaged under aseptic conditions, and which have a defined prolonged shelf life under refrigerated conditions (Rysstad and Kostad, 2006; Schmidt, Kaufmann, Kulozik, Scherer and Wenning, 2012). Appropriate temperature selection and duration of heating are crucial for the required microbial quality of the milk product (Janstova and Lukasova, 2001). New manufacturing techniques have been introduced for the production of ESL milk in South Africa with a taste like fresh milk, but a prolonged shelf life of up to 5 weeks in cold chain distribution. The shelf life of traditionally pasteurised milk is between 7 and 10 days, provided the product is stored at or below 6°C (Buys, 2001). The shelf life of ESL milk is between 18 and 40 days (Rysstad and Kostad, 2006), provided the cold chain is maintained and the product is not re-contaminated post-pasteurisation by the surfaces of the processing equipment.

In the dairy industry, equipment surfaces are recognized to be a major source of contamination of processed milk with spoilage and pathogenic microorganisms. These contaminating microorganisms appear as adherent bacteria or as more complex structures called biofilms (Costerton, Lewandowski, Caldwell, Korber, and Lappinscott, 1995). Adhered bacteria can detach and contaminate the product as it passes the processing surfaces (Malek, Mousa-Boudjemaa, Khouani-Yousfi, Kalai and Kihel, 2012). Biofilms are of concern in the dairy manufacturing plants, as bacteria within biofilms are more difficult to eliminate than free living cells (Flint, 1998). Biofilms of *Bacillus* spp. are important contaminants in the dairy industry and are recognized as a serious problem (Elhariry, 2008). If post-pasteurisation contamination occurs in the final products, it may be due to the biofilm formation on and in milk processing equipment which may contaminate the pasteurised milk and milk products (Dhillon, 2012). For pasteurised milk, the filling machines have been shown as the main source of recontamination, with the filler nozzles, aerosols and the water at the bottom of the filling machine being of particular concern (Rysstad and Kostad, 2006). Eneroth, Ahrne and Molin (2000), shown that recontamination of pasteurised milk with Gram-negative psychrotrophic bacteria occurs during filling of consumers packages. Contamination of pasteurised milk with *B. cereus* may occur

during the filling process (Eneroth, Svensson, Molin, and Christiansson, 2000). Recontamination of ESL milk with *B. subtilis* and *Paenibacillus* spp. was also observed by Mugadza and Buys (2013). Rysstad and Kostad (2006) stated that the shelf life of pasteurised milk can be extended by introducing better hygiene transfer from processing to filling machines and filling process.

Up to now, there is limited information available on the factors limiting the shelf-life of ESL milk produced by bacto-fugation and subsequent pasteurisation and there's also limited information available on the relationship between storage temperature and the bacterial quality of ESL milk produced in South Africa. The shelf life of ESL milk might be limited by bacterial attachment and biofilm formation in dairy processing environment, particularly the nozzle of the filling machines because equipment surfaces used in the dairy industry and filling machines are recognized to be the main source of post-pasteurisation contamination of pasteurised milk. Therefore the nozzles of aseptic ESL filling machines might be a reservoir of bacteria that could lead to post-pasteurisation contamination of ESL milk products. Post-pasteurisation contamination encompasses the recontamination of the product anywhere downstream of the processing line and it can occur in the regeneration or cooling sections, in storage tanks and in the final packaging of the product, due to poor hygienic practices (Lewis and Deeth, 2009). Studies indicate that the source of the contamination lies not within the processing lines but in external sources entering near the final stages of milk and milk products. To our knowledge there is no information available about the microorganisms associated with nozzles of aseptic ESL filling machines used for the production of ESL milk. The purpose of this study was to isolate and characterise the bacteria associated with biofilm in the nozzles of ESL aseptic filling machines after CIP process. To determine the ability of the isolates to attach and form biofilm to stainless steel strips.

CHAPTER 2: LITERATURE REVIEW

2.1 EXTENDED SHELF LIFE (ESL) MILK PROCESSING OPERATIONS

ESL processing technology is a recent addition to the South African dairy industry and fills the gap between High-Temperature Short-Time (HTST) pasteurised milk which has a shelf life of about 7 days when kept refrigerated and ultra-high temperature (UHT) milk, which is able to be stored at ambient temperature for up to 9 months (Fitzgerald, 2012). ESL milk may also be used to describe the conventionally pasteurised milk products that are processed under stringent hygienic conditions designed to prevent post-pasteurisation contamination with the goal of extending the shelf life or keeping quality of ESL milk products (Robinson, 2005). The ESL processing technology reduces the bacterial load by a much greater degree than traditional pasteurisation method (Figure 1). Based on the processing concept of ESL milk (Figure 1), we can say that ESL milk products will have a better shelf life than conventionally pasteurised milk products provided there is no post-pasteurisation contamination of ESL milk products by the stainless steel pipes, as a result of bacterial biofilms formed in the fillers. Cromie (1991) reported that the factors that influence the shelf life of pasteurised milk include the quality of the raw material, resistant microorganisms to pasteurisation (particularly psychrotrophics), the presence and activity of post pasteurisation contaminants (Figure 1), the packaging system and storage temperature post pasteurisation which had the greatest impact on the stability of the product. Due to the hygienic design, the aseptic ESL filling machines provides a much greater degree of protection against recontamination from the filling environment, therefore ESL milk products are likely to have lower microbial count (Figure 1) during storage and distribution compared to pasteurised milk products.

2.2 PROCESSING STEPS OF ESL MILK

2.2.1 PASTEURISATION

Pasteurisation is a heat treatment aimed at reducing the number of any harmful microorganisms in milk and liquid milk products, if present, to a level at which they do not constitute a significant health hazard. In addition, it results in prolonging the keep ability of milk or the liquid milk product and in only minimal chemical, physical and organoleptic changes (Lewis and Deeth, 2009). Pasteurisation of raw milk can be achieved by combination of time and temperature, with the most widely applied comprising a 15sec heat at 72°C (Munsch-

Alatossava, Ghafar and Alatossava, 2013). The manufacturing process of ESL milk relies on pasteurisation temperatures of 72-73°C for 15sec at an early stage of manufacturing with the aim of destroying heat-sensitive spoilage and pathogenic bacteria in the raw milk and also relies in aseptic filling process. Foodstuffs, Cosmetics and Disinfectant Act, 1972 (Act No. 54 of 1972) states that, raw milk with a plate count of more than 200 000 cfu/ml may not be consumed by humans, and no person is allowed to sell pasteurised milk with a standard plate count exceeding 50 000 cfu/ml (Department of Health (DOH), 2002). Milk pasteurisation was introduced as a public health measure in order to destroy the most heat resistant, non-spore forming human pathogens (*Mycobacterium paratuberculosis* and *Coxiella burnetti*) likely to be present in raw milk (Dumalisile, 2004; Dhillon, 2012).

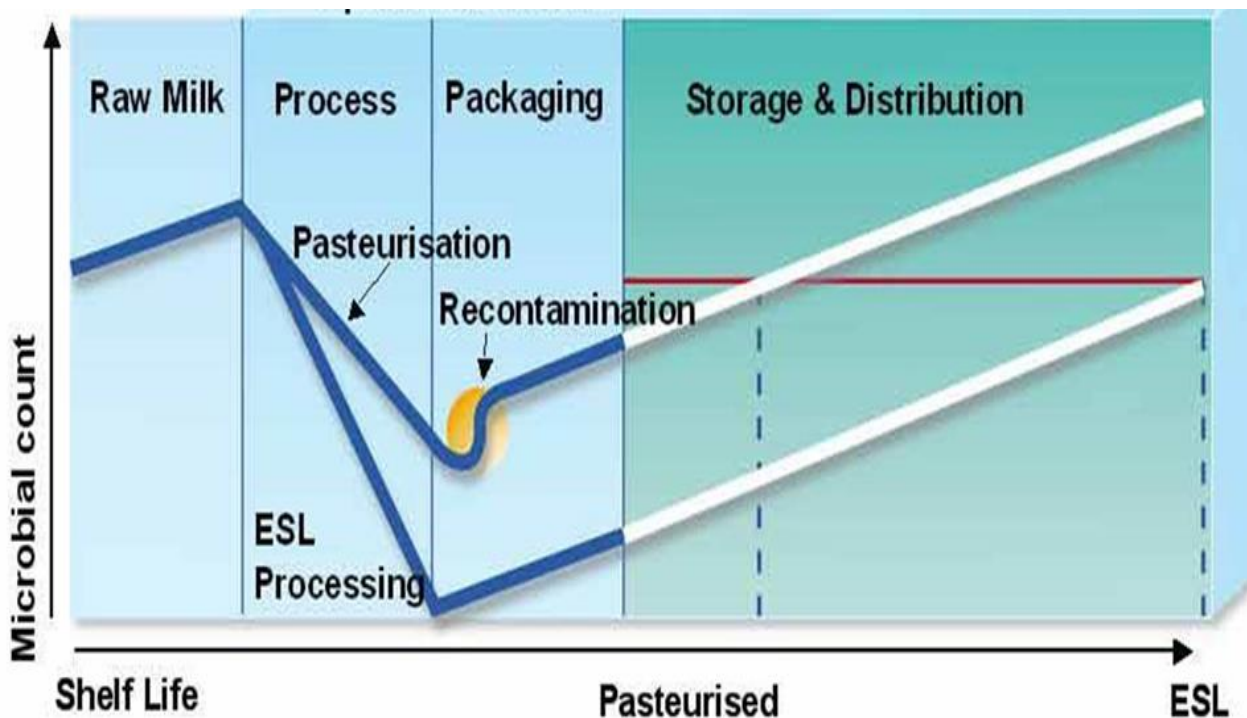


Figure 1: Process concept for the production of ESL milk and conventionally pasteurised milk <http://www.drgailbarnes.com/2013/01/extended-shelf-life-future-for-chilled.html> [Available online, accessed 10 Feb 2015]

2.2.2 BACTOFUGATION

Bactofugation is a complementary processing technology to conventional HTST pasteurisation of milk (Henyon, 1996). Bactofugation employs centrifugal force for removal of bacteria from milk, especially heat resistant spores, have a significantly higher density than milk and can be efficiently removed by application of centrifugal force (Henyon, 1996). Bactofugation has proved to be an efficient way of reducing the number of spores in milk (Faccia, Mastromatteo,

Conte and Del Nobile, 2013). According to Faccia *et al.* (2013), on the average milk treated with a bactofuge contains 90% fewer microorganisms than untreated milk.

2.2.3 MICROFILTRATION

The principle of this technique in ESL processing is to remove bacterial cells and spores from milk mechanically using ceramic membrane with pore diameter of 0.8-1.4mm for processing (Rysstad and Kokstad, 2006). As the pore size of the membrane used is much greater than in the cases of reverse osmosis and ultra-filtration the process known as microfiltration. First approaches of using microfiltration for the reduction of the microbial load have been undertaken for more than 25 years and by applying this method, raw milk is separated into skimmed milk and milk fat (Schmidt *et al.*, 2012). The skimmed milk is microfiltered through ceramic membranes and subsequently pasteurised (Schmidt *et al.*, 2012). However, there are constraints on the application of microfiltration to ESL processing since the pores allow some bacteria to pass through the membrane and thus the milk must be pasteurised to ensure the elimination of vegetative pathogens (Robinson, 2005; Rysstad and Kokstad, 2006).

2.2.4 ASEPTIC PACKAGING

There are two main aseptic packaging systems that are used commercially. First, the type that uses pre-formed containers, and second, the type that forms, fill and seals the containers in the aseptic packaging system (von Bockelmann and von Bockelmann, 1998). According to Ansari and Datta (2003), aseptic packaging involves the filling and sealing of microbiologically stable, which is commercially sterile product into sterilized containers under conditions that prevent microbial recontamination of the product, the containers, and their closures. In aseptic packaging, raw or unprocessed product is heated, sterilized by holding at high temperature for a predetermined amount of time, then cooled and delivered to a packaging unit for packaging, while packaging material and equipment surface may be sterilized by various methods such as heat, hydrogen peroxide, irradiation, infrared light etc. and combinations of methods (Carlson, 1998; Ansari and Datta; 2003). In contrast to UHT milk, ESL milk is not a commercially sterile milk product but it is packaged under aseptic conditions in a closed system.

2.3 BACTERIA ASSOCIATED WITH DAIRY MANUFACTURING

ESL milk might contain spore-forming bacteria which are resistant to the heat treatment. In a study by Mayr, Gutser, Busse and Seiler (2004), ESL milk produced at 127°C for 5s, Gram-

positive non-spore forming bacteria were found to be the most common spoilage organisms including species of *Rhodococcus*, *Anquinibacter*, *Arthrobacter*, *Microbacterium*, *Enterococcus*, *Staphylococcus* and *Micrococcus*. In the dairy industry most of the spore formers are believed to be re-contaminants and not spores which survived the heat process. Additionally, in the dairy environments the most commonly encountered bacteria belong to the genera *Bacillus*, *Pseudomonas*, *Enterococcus*, *Enterobacter*, *Staphylococcus*, *Micrococcus* and *Acinetobacter* (Wiedmann, Weilmeier, Dineen, Ralyea and Boor, 2000; Waak, Tham and Danielsson, 2002; Sharma, Anand and Prasad, 2003).

2.3.1 *BACILLUS CEREUS*

B. cereus is a gram-positive, spore-forming bacterium. It causes two types of food poisoning: diarrheal by enterotoxin production in the small intestine and emetic by toxin which is formed in food (Valik, Gorner and Laukova, 2003; Wijman, de Leeuw, Moezelaar, Zwietering and Abee, 2007). *B. cereus* spores are both highly resistant to numerous stresses such as heat and cold temperatures and are hydrophobic, which causes them to adhere easily to food processing equipment (Fallie, Jullien, Fontaine, Bellon-Fontaine, Slomianny and Benezech, 2002; Elhariry, 2008). *Bacillus* spores adhere to surfaces that are frequently used for food processing materials such as stainless steel, polystyrene and rubber (Oosthuizen, Steyn, Lindsay, Brozel and von Holy, 2001; Peng, Tsai and Chou, 2002; Harimawan, Zhong, Lim and Ting, 2013). Adhesion of these spores may initiate biofilm formation which can cause cross-contamination of milk and milk products and subsequently decrease their shelf-life (Harimawan *et al.*, 2013). *B. cereus* has been found to account for 12.4% of the constitutive bacteria associated with biofilm, in a commercial dairy plant (Sharma and Anand, 2002). Biofilm formation by *B. cereus* has also recently been investigated, since biofilms produced by this bacterium are considered a potential health hazard in the dairy industry (Pretorius, 2010). This might be the cause for concern in the dairy industry since *B. cereus* is known for establishing biofilms in milk processing equipment, and contamination of milk and milk products due to the spores being able to survive the pasteurisation process (Flint, 1998). Wijman *et al.*, 2007, observed biofilm formation by *B. cereus* on stainless steel coupons. Their results showed that *B. cereus* biofilms may develop within storage and piping systems when either partially filled or when liquid residues remain during production. In addition, increase in spore formation by *B. cereus* within biofilms can potentially cause recontamination and equipment failure during food production. Lindsay, Brozel, Mostert and von Holy (2000), isolated *Bacillus* species from alkaline wash solutions

used for CIP in South Africa dairy factories and suggested that *Bacillus* species to contaminate surfaces of dairy processing equipment and result in post pasteurisation spoilage of milk and milk products.

2.3.2 *ENTEROCOCCUS* SPP.

Enterococcus spp. are ubiquitous Gram-positive, catalase-negative cocci that often occur in large numbers in vegetables, plant materials and foods, especially those of animal origin such as dairy products (Franz, Holzapfel and Stiles, 1999; Citak, Yucel and Mendi, 2005). *Enterococci* presence in dairy products can have conflicting effects, of either a risk as a foreign or intrusive flora indicating poor hygiene during milk handling and processing (Gimenez-Pereira, 2005). *Enterococci* may present an emerging threat as human pathogens associated with nosocomial infections, particularly, the strains of *E. faecalis* as a predominant species and to lesser extent *E. faecium* have been reported to be involved in human pathogenesis (Dardir, Aba-Alkhail and Abdel-All, 2011). McAuley, Gobius, Britz and Craven (2012) reported that strains of *E. faecalis*, *E. faecium*, *E. durans* and *E. hirae* exhibit heat resistant characteristics. Teh, Flint and French (2010) demonstrated that cells of *E. faecalis* can attach and form biofilm on stainless steel surfaces. Biofilm formed in the dairy plants pose a threat to the smooth and continuous running of the plant (Dhillon, 2012).

2.3.3 *MICROCOCCUS* SPP.

Micrococcus spp. are Gram-positive, spherical, saprotrophic bacteria that belong to the family Micrococcaceae (Thomas and Prasad, 2012). They are found in soil, dust, water and air, and as part of the normal flora of the mammalian skin. These microorganisms are usually not motile and non-spore forming and their optimum growth temperature is 25-37°C (Sutton, 2004). *Micrococcus* spp. are commonly found in pasteurised milk and they usually find their way into the milk supply from improperly cleaned and sterilized utensils at the producing farm (Thomas and Prasad, 2012). *Micrococcus* spp. have been reported to show potential for biofilm formation on stainless steel (Dhillon and Kaur, 2014) and have been isolated by Malek *et al.*, (2012), from different segments of dairy processing lines. *Micrococci* are frequently isolated from the dairy processing lines along with *Pseudomonas* and *Staphylococcus* species (Sutton, 2004).

2.3.4 *PSEUDOMONAS* SPP.

Pseudomonas spp. are the predominant Gram-negative bacteria found in chilled milk, owing to their ability to grow within the bulk phase and on the surface of refrigerated milk containers (Seale, Bremer, Flint, Brooks and Palmer, 2015). They are found in food environment such as processing equipment and drains (Hood and Zottola, 1995). Their ability to produce extracellular polymers, which facilitate their attachment on the stainless steel surfaces, makes them superior biofilm former (Barnes, Lo, Adams and Chamberlain. 1999). *P. fluorescens* is important as a biofilm-forming bacterium capable of contaminating milk previously processed (Caixeta, Scarpa, Brugnera, Freire, Alves, Abreu and Piccoli, 2012). Dhillon (2012) demonstrated the ability of *P. fluorescens* to form biofilm on stainless steel surfaces. The most common shelf-life limiting organisms found in dairy processing plants are *P. fluorescens* and *P. putida* (Cleto, Matos, Kluskens and Vieira, 2012). These microorganisms are most commonly found in refrigerated milk and have the ability to grow under refrigeration as well as to produce a number of heat-stable spoilage enzymes (Dogan and Boor, 2003).

2.3.5 *STAPHYLOCOCCUS* SPP.

Staphylococcus spp. are Gram positive non-motile, non-spore forming, facultative anaerobic cocci occurring in pairs or irregular clusters and they are disseminated in the environment, with a number of species inhabiting specific ecological niches (Heikens, Fleer, Paauw, Florijn and Fluit, 2005). They are found on the skin and mucous membranes of warm-blooded animals and humans, which generally imply a commensal or symbiotic relationship with their host. *Staphylococci* are also isolated from a wide range of foodstuffs such as meat, cheese and milk, and from environmental sources such as soil, sand, air and water (Heikens *et al.*, 2005). *Staphylococcus* spp., including *S. epidermidis*, have been well recognized as bacteria which may attach, form biofilms and survive on the contact surfaces in the milk processing plants (Sharma and Anand, 2002). The adhered cells of *Staphylococci* are considerably more resistant to sanitizers and heat and are able to form biofilms in post-pasteurisation processing lines (Seale *et al.*, 2015).

2.4 BIOFILMS IN THE DAIRY INDUSTRY

2.4.1 IMPACT OF BACTERIAL BIOFILMS IN DAIRY PROCESSING PLANTS

Dairy processing equipment biofilm forming bacteria on stainless steel surfaces due to mass production of products, lengthy production cycles and vast surface areas for biofilm development (Lindsay and von Holy, 2006). The presence of established biofilms in milk processing lines can have a serious effect on processing efficiency in a dairy manufacturing plant. The adherence of denatured proteins to stainless steel surfaces can promote bacterial adhesion and growth (Seale *et al.*, 2015). Bacteria within biofilms are more difficult to eradicate and once established they become significant source of contamination of dairy products (Flint, 1998). It also provides a protective barrier for the bacteria contained within it by providing not only a physical barrier from stresses like fluid flow, but also a chemical barrier. Antimicrobials have a limited effect on bacteria in a biofilm for several reasons centered on its structure and the bacteria themselves (Leslie, 2011). Biofilms formed in food-processing environments are of special importance as they may consist of pathogenic bacteria (Table 1) and may be persistent source of contamination leading to the spoilage of final product. Bacterial contamination can adversely affect the quality, functionality and safety of products produced by the dairy industry (Mogha, Shah, Prajapati and Chaudhari, 2014). When contamination of dairy products occurs evidence suggests that biofilms on the surfaces of milk processing equipment are a major source (Flint, Brooks, Elzen and Bremer, 1997). In dairy manufacturing plants biofilms can be divided into two categories, biofilms that are unique to dairy manufacturing plants which have been termed "process" biofilms e.g. those which form on surfaces (e.g. heat exchanger) in direct contact with flowing product, and biofilms which form in the general milk processing environment. Biofilm develops on the sides of gaskets despite of cleaning-in-place procedures (Mogha *et al.*, 2014). Bacteria form biofilms on the surfaces of stainless steel equipment in food processing industries, releasing bacteria that compromise the safety and quality of the final product (Zottola, 1994).

Table 1: Predominant biofilm micro-flora in different food processes (Kokare, Chakraborty, Khopade, and Mahadik, 2009).

Food borne pathogen	Growing surface
<i>Listeria monocytogenes</i>	Dairy processing plant, conveyor belt
<i>Pseudomonas</i> spp.	Dairy, vegetable, meat surface
<i>Bacillus</i> spp.	Pipe-line, joint processing environment, hot fluid
<i>Salmonella</i> spp.	Poultry processing environment

2.4.2 IMPORTANCE OF *BACILLUS* SPECIES IN THE DAIRY INDUSTRY

Bacillus spp. is found throughout dairy processing plants (Oosthuizen, Steyn, Lindsay, Brozel and Holy, 2001). *Bacillus* spp. survives heat processing and accumulates on pipelines and joints in the processing environment (Jeong and Frank 1994). If hot fluid continuously flows over a surface for over 16h, *Bacillus* and other thermotolerant bacteria may form a biofilm (Chmielewski and Frank, 2003). The importance of *Bacillus* spp. in the dairy industry has been recognized since 1938, when the occurrence of bitty cream was first recorded attributed to *B. cereus* (Flint, 1998). Spore-formers including *B. cereus* have been associated with sweet curdling of pasteurised milk stored at refrigerated temperatures (Heyndrickx, 2011). *B. cereus* can be introduced into milk from a variety of sources during production, handling and processing (Figure 2). According to Heyndrickx (2011), during recent decades, there are several possible contamination routes (Figure 2). *B. cereus* in pasteurised milk have been described with either raw-milk or post-pasteurisation contamination of the pasteurised milk being the source. *B. cereus* is causing major problems for the dairy industry. If milk is not re-contaminated after pasteurisation, the keeping quality of milk is determined by the number of *B. cereus* cells/spores in the product (Heyndrickx, 2011). *Bacillus cereus* is of great significance among *Bacillus* spp. because it can grow quickly, therefore the species may grow at low temperatures and produce enzymes which result in sweet curdling (IDF, 1994). Spores of *Bacillus* spp. can remain in a heat exchanger, adhere to the surface, germinate and grow. If these spores detach, the equipment can be a continuous source of contamination (Te Griffet and Beumer, 1998). The spores of *B. cereus* are very adhesive to different surfaces and this is a major reason for its presence and the difficulty of control. The strong adhesion of *B. cereus* spores is mainly due to three characteristics: the high relative hydrophobicity, the low spore surface charge and the spore morphology (Figure 3). The spores are surrounded by appendages (Figure 3) and these promote adhesion (Andersson, Ronner and Granum, 1995).

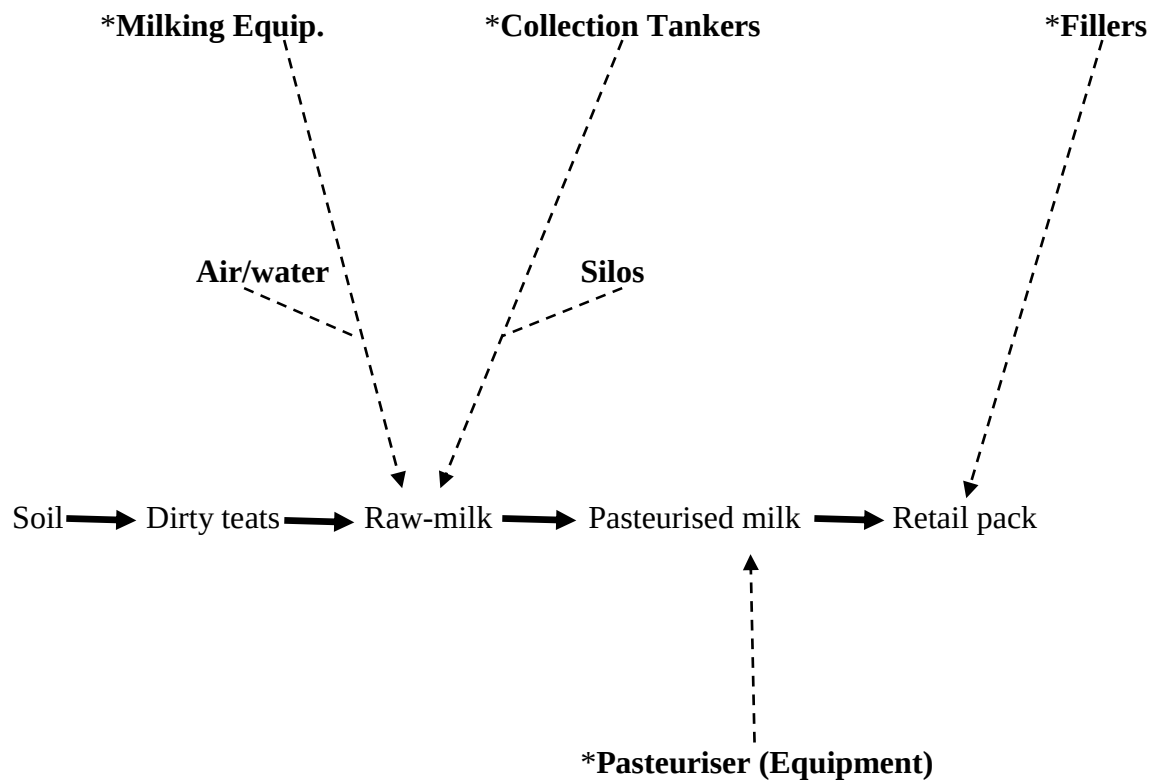


Figure 2: Contamination route of spores of *Bacillus cereus* in a production chain (Heyndrickx, 2011).

*Possible sources or routes of contamination is indicated by dotted arrow

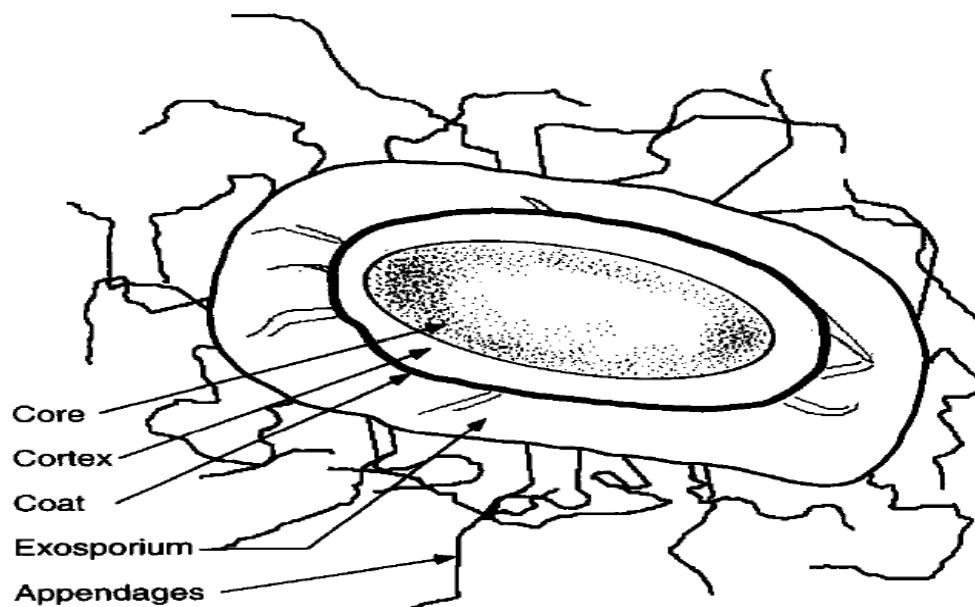


Figure 3: The different layers and structure of *Bacillus cereus* spores (Andersson *et al.*, 1995).

2.5 BACTERIAL BIOFILMS

Biofilms are not a phenomenon of recent time, have been investigated for more than 20 years in the food processing industry (Vlkova, Babak, Seydlova, Pavlik and Schegelova, 2008). They can be defined as a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to stainless steel surfaces (Costerton, Stewart and Greensberg, 1999). Elder, Stapleton, Evans and Dart (1995), defined a biofilm as a functional consortium of microorganisms organized within an extensive exopolymer matrix adhering to a surface. From these definitions development of biofilms requires three important components: microbial cells, extracellular polymeric substances (EPS) and surface, therefore if one of these components is removed from the system, a biofilm doesn't develop. Mixed species biofilms are generally more commonly found in nature as they are more stable than single cells (Dhillon, 2012), because biofilms formed by mixed species of microorganisms protect one another during the application of cleaning chemical agents.

2.5.1 MECHANISM OF BIOFILM FORMATION

Bacterial attachment and the formation of biofilms appear to take place in different stages (Figure 4) involving physicochemical and biological factors (Marchand, De Block, De Jonghe, Coorevits, Heyndrickx and Herman, 2012). The biological factors include cell-to-cell signalling between the biofilm bacteria, growth rates of the bacteria, extent of EPS production, motility of the biofilm bacteria as well as possible competition or cooperation between the bacteria (Stoodley, Sauer, Davies and Costerton, 2002). As the bacteria grow, the biofilm matures, thus, producing EPS and developing water channels (Dhillon, 2012).

Formation of conditioning layer

In dairy, organic and inorganic molecules like proteins from milk gets adsorbed to the surface forming a conditioning film (Mogha *et al.*, 2014). The conditioning of the solid surface also plays a significant role in the rate of microbial attachment (Myszka and Czacyk, 2011). These conditioning layers form when organic molecules are attracted to the substrate by a variety of interactions, of which charge plays a major role (Chmielewski and Frank, 2003). The conditioning layer alters the physicochemical properties of the surface including the hydrophobicity, surface energy and electrostatic charges and also results in increased nutrient

concentration on the substrate surface, all influencing microbial attachment (Kumar and Anand, 1998 and Dhillon, 2012).

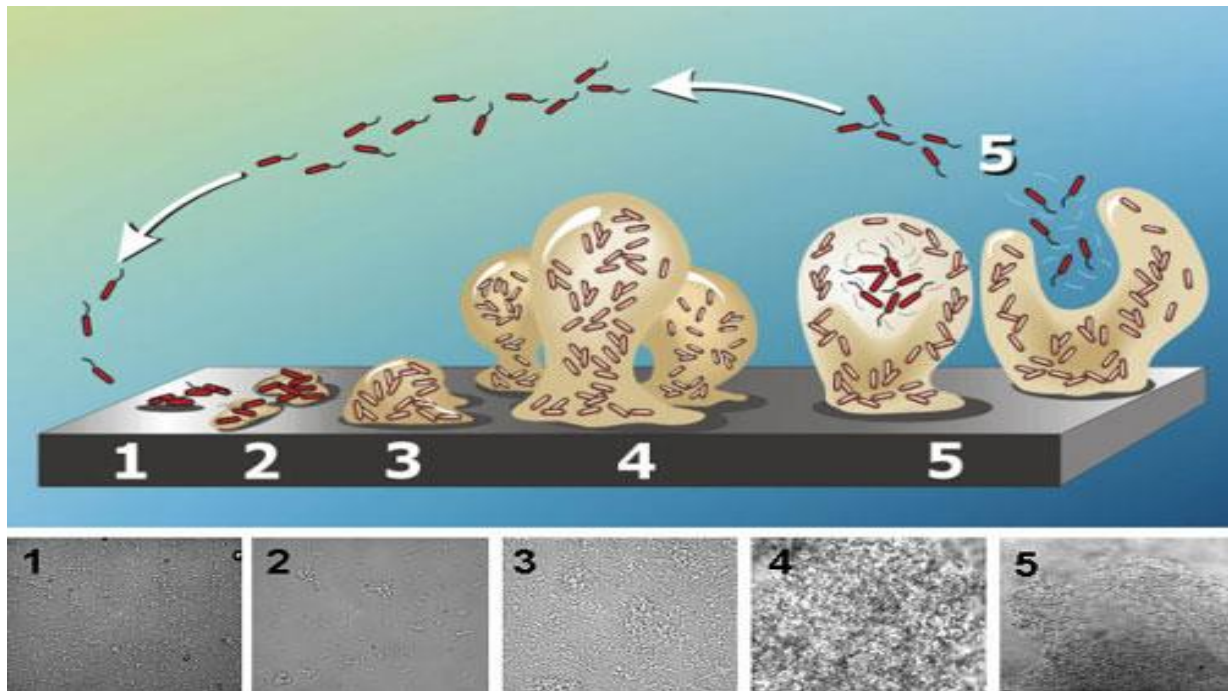


Figure 4: Stages of bacterial biofilm development (Kokare *et al.*, 2009).

Bacterial adhesion

Adhesion of bacterial cells to the conditioning layer is the second step in the formation of biofilms (Dhillon, 2012). Formation of bacterial biofilm begins with the attachment of single cells to the solid surface. This process is time dependent and can be divided into two phases: the reversible and irreversible phase (Myszka and Czaczyk, 2011). The reversible adhesion is initiated when the bacteria comes into contact with the surface via van der Waal's, electrostatic forces and hydrophobic forces (Kumar and Anand, 1998). The bacterial cells are not tightly attached and can be easily removed by applying moderate shear force (Dhillon, 2012). The interactions in the irreversible phase involves short range forces such as dipole-dipole forces, hydrogen, covalent and ionic forces (Kumar and Anand, 1998). In irreversible attachment of cells, the repulsive forces prevent the bacterial cells in making direct contact with the surface, still the bacterial attachment is mediated by fimbriae, pili, flagella, and bacterial extracellular polymeric substances (ESP) that act to form a bridge between bacteria and the conditioning film (Kokare *et al.*, 2009). In this process removal of cells requires much stronger forces such as scrubbing or scrapping (Mogha *et al.*, 2014). Once irreversibly attached, the entire biofilm

develops through the growth of the bacteria attached to the surface and through addition of more bacteria (Dhillon, 2012).

Bacterial growth

The irreversibly attached cells grow and divide by using nutrients present in the conditioning film and the surrounding fluid environment (Poulsen, 1999). This leads to the formation of microcolonies, which enlarge and coalesce to form a layer of cells covering the surface (Myszka and Czacyk, 2011). Cell-cell signalling and production of EPS help in maintaining the microcolony (Dhillon, 2012).

Biofilm formation

According to Dhillon (2012), continuous attachment of cells over a period of time and their growth results in the formation of biofilm and suitable growth conditions lead to the maturation of biofilms over time. During this step, the anchored cells also synthesis additional EPS that assists in the attachment process of the cells to the surface and protect the cells aggregate from fluctuations of the environment (Kumar and Anand, 1998).

Detachment and dispersion of single cells

As the biofilm matures, the attached bacteria, in order to survive and colonise new niches, detach and disperse from the biofilm (Myszka and Czacyk, 2011). Detachment and dispersion of cells from biofilms may either be initiated by the bacteria themselves or mediated by the external forces such fluid shear, abrasion and cleaning (Bremer, Flint, Brooks and Palmer, 2015). At least three distinct modes of biofilm dispersal have been identified: erosion, sloughing and seeding (Kaplan, 2010; Bremer *et al.*, 2015). Erosion is the continuous release of single cells or small clusters of cells from a biofilm at low levels, owing to either cell replication or an external disturbance to the biofilm. Sloughing is the sudden detachment of large portions of the biofilm, usually during the later stages of its growth, perhaps as conditions with it change or it becomes unstable due to its size. Seeding dispersal is the rapid release of a large number of single cells or small clusters of cells and is always initiated by the bacteria (Kaplan, 2010). The detached bacteria may be transported to newer locations and again restart the biofilm formation process (Myszka and Czacyk, 2011).

2.6 TREATMENT FOR CONTROLLING BIOFILMS

Table 2: Novel approaches for control of biofilm (Mogha *et al.*, 2014)

Treatments
Mechanical treatment with chemical agent
1. Air injected CIP system
2. Cleaning of surface with short intervals
3. Control of operating time between cleaning and sanitation
4. Electropolishing the surface of AISI 316 SS
5. Inclusion of germinant in CIP regime
6. Natural disinfectant scallop shell powder slurries
7. Peracetic acid
8. Surface pre-conditioning with surfactants
Biological treatment with green chemicals
1. Bacteriophage was engineered for expressing biofilm degrading enzyme
2. Protease an enzyme based detergent used as biocleaners
3. Biopreservatives such as nisin, lauricidin, reuterin and pediocin

Cleaning procedures used in dairy manufacturing plants are limited mainly to the use of cheap chemicals (caustic, acid and chlorine) and most sanitizing regimes have remained unchanged since the early 1900's (Mogha *et al.*, 2014). The chemicals currently used in disinfection processes belong to the following types: acidic compounds, aldehyde-based biocides, caustic products; chlorine, hydrogen peroxide, iodine, isothiazolinones, ozone, peracetic acid, phenolics, biguanidines, surfactants (Dosti, Guzel-Seydim and Greene, 2005; Bremer *et al.*, 2006; Simoes, Simoes, Machado, Pereira and Vieira, 2006). Biofilms are more resistant to cleaning chemical agents than planktonic cells of the same species (Dhillon, 2012). Within a processing environment, the renowned difficulty in removing biofilms is caused by a wide variety of factors associated with plant design and operation, as well as the inherent properties of biofilms and the cells within them (Bremer *et al.*, 2015). Five factors are involved in the development of biofilms in dairy processing plants, namely: the species of microorganisms involved; the type of product being manufactured; the operational conditions; the surface material and its condition; and the cleaning and sanitation regimes employed (Bremer *et al.*, 2015). Therefore it can be said that the current cleaning methods need to be reassessed to ensure the control of biofilms in the dairy industry. Table 2 enlists the novel approaches for control of biofilms and there is no known effective cleaning method that is able to control the formation of biofilms. Besides chemical methods, biological control can be used to eliminate biofilms

(Simoese, Simoese and Vieira, 2010). Lu and Collins (2007) engineered a bacteriophage to express a biofilm degrading enzyme that had the ability to attack the bacterial cells in the biofilm and the biofilm matrix, substantially reducing the biofilm (more than 99.9% of removal). The technology has not yet been successfully developed and relatively little information is available on the action of bacteriophages on biofilms. Augustin, Ali-Vehmas and Atroshi (2004) demonstrated the potential application of enzymatic cleaning products against biofilms formed by microorganisms commonly found in dairy products. Formulations containing several different enzymes seem to be fundamental for a successful biofilm control strategy. Moreover, the use of enzymes in biofilm control is still limited due to the low prices of the chemicals (Augustin *et al.*, 2004; Mogha *et al.*, 2014).

2.7 MICROBIAL HYDROPHOBICITY

In biological systems, hydrophobic interactions are usually the strongest of all long range non-covalent forces and adhesion to surfaces is often mediated by these types of interactions (Briandet, Herry and Bellon-Fontaine, 2001). Microbial hydrophobicity is defined by the energy of attraction between a-polar or slightly polar cells immersed in an aqueous phase (van Oss and Giese, 1995). Cell surface hydrophobicity is influenced by structures and components found on the bacterial cell surface, such as pili, fimbriae polysaccharides and flagella, which can vary between bacterial strains (Reid, Bialowska-Hobrzanska, Van der Mei and Busscher, 1999) and change throughout the bacterial life cycle (Xu, Zou and Lee, 2010). Three methods have been employed for evaluation of cell hydrophobicity includes: contact angle measurement, hydrophobic interaction chromatography and bacterial adhesion to hydrocarbons (Simmonds, Mossel, Intaraphan and Deeth; 2003). Spores of *B. cereus*, *C. perfringens* and *B. subtilis* have hydrophobic characteristics, but their affinities for liquid hydrocarbons are different (Simmonds *et al.*, 2003). Hydrophobicity levels of *Bacillus* spores (Table 3) using the three methods reported by Simmonds *et al.*, (2003).

Table 3: Hydrophobicity levels of *Bacillus* spores determined using the three methods reported by Simmonds *et al.*, (2003).

Organisms	MATH %	HIC %	CAM 0
<i>B. cereus</i>	69-86	99	20-58
<i>B. licheniformis</i>	28-45	26-28	14-53
<i>B. stearothermophilus</i>	14-75	74-79	34-99

***MATH – Microbial Adhesion to Hydrocarbons, HIC – Hydrophobic Interaction Chromatography, CAM – Contact Angel Measurement

Bacterial adhesion to hydrocarbon (BATH)

Cell surface hydrophobicity is often assessed by using BATH assay (Pembrey, Marshall and Schneider, 1999). A cell suspension is mixed with hydrocarbons for a predetermined period to allow optimal interaction of the bacteria with the hydrocarbon phase (Pembrey *et al.*, 1999). Many investigators have modified the original BATH test and have found that seemingly small variations in experimental conditions as the diameter of the test tubes, the pH of the suspension medium and the volume of hydrocarbons used, can significantly alter the results (Bunt, Jones and Tucker, 1993). According to Doyle (2000), the best method for determining bacterial hydrophobicity is by contact angle measurements. These other methods are based on the adhesion of cells to either liquids or solid materials and dependent on factors such as temperature, time, pH, ionic strength and relative concentration of interacting species and all of these factors conspire to influence the adhesive event (Oliveira, Azeredo, Teixeira and Fonseca, 2001).

Contact angle measurement (CAM)

The assay consists of depositing a drop of water on a film of cells and determining the angle between the film surface and the tangent to the drop at the solid-liquid-air meeting point (Ronner, Husmark and Henriksson, 1990). In principle, the contact angle of an air bubble on a solid surface in aqueous solution is determined by the mechanical equilibrium under the action of three interfacial tensions, i.e., solid-air surface ‘tension’ ($\gamma_{s/a}$), solid–liquid interfacial tension ($\gamma_{s/l}$), and liquid-air surface tension ($\gamma_{l/a}$) (Chau, Bruckard, Koh and Nguyen, 2009). The contact angle determined by balancing the surface tension forces is known as Young’s contact angle, θ_Y , and the relationship describing the balance of surface forces is known as Young’s equation as given:

$$\gamma_{s/a} = \gamma_{s/l} + \gamma_{l/a} \cos \theta_Y$$

Hydrophobic interaction chromatography (HIC)

According to Walter, Syldatk and Hausmaan (2010), HIC is a chromatographic procedure based on hydrophobic interaction between the nonpolar groups on a hydrophobic chromatographic resin and the nonpolar regions of a particle. A bacterial suspension is drained into a gel bed of hydrophobized sepharose. Hydrophobic microbes are retained by the gel and the degree of adsorption of the cells to the gel can be measured by turbidity of the eluate or by bacteria count (Sen, 2010; Palmer, Seale and Flint, 2015). For desorption of the adherent

microbes, the ionic strength of the buffer is decreased. HIC is very convenient because screening and isolation of potential strains can be combined in one step (Sen, 2010). Pruthi and Cameotra (1997), reported that HIC is a reliable screening method for biofilm formation and the technique is also valid for comparative analysis of the hydrophobic properties of microorganisms.

2.8 OBJECTIVES AND HYPOTHESES

2.8.1 HYPOTHESES

1. A variety of microorganisms will be identified from the nozzles of ESL filling machines after CIP process. Additionally, *Bacillus* spp. will dominate the identified microorganisms. Previous studies reported a numerous occurrence of bacteria from different point of processing lines including filling machines post CIP treatment (Mattila, Manninen and Kylasiurola, 1990; Svensson, Eneroth, Brendehaug and Christiansson, 1999; Sharma and Anand, 2002; Rysstad and Kokstad, 2006; Malek *et al.*, 2012). *B. cereus* was found among the bacteria of pasteurised milk production line and it is also known for its resistance to heat and chemical disinfectants (Fallie *et al.*, 2001; Peng *et al.*, 2002).
2. Spore forming and non-spore forming bacteria will attach and form biofilm on stainless steel strips. Micrococci and Staphylococci are strong biofilm formers on a range of surfaces including stainless steel (Krolasik, Zakowska, Krepska and Klimek, 2010; Palmer *et al.*, 2015). (Marchand, De Block, De Jonghe, Coorevits, Heyndrickx and Herman, 2012) noted the adhesion of *B. cereus* spores on stainless steel surfaces, this bacteria is described as an excellent biofilm former due to the pronounced ability to its spores to adhere to stainless steel surfaces. Flint *et al.*, (2001) demonstrated that *B. stearothermophilus* can attach and form biofilms on clean stainless steel surfaces. Spores of *Bacillus* are hydrophobic, resulting in firm adhesion to surface frequently used for food processing materials such as stainless steel (Harimawan, Zhong, Lim and Ting, 2013).

2.8.2 OBJECTIVES

1. To identify and characterise the bacteria associated with the nozzles of ESL aseptic filling machine after CIP process.
2. To determine the ability of isolates isolated from the nozzles of ESL aseptic filling machine to attach and form biofilms on stainless steel strips.

CHAPTER 3: RESEARCH

3.1 INTRODUCTION

This study was divided into 2 phases. First phase aimed to identify and characterise the bacterial biofilms associated with the nozzles of aseptic ESL filling machines (Figure 5). The second phase aimed to study the ability of the isolates originating from the nozzles of aseptic ESL filling machines to form biofilms on stainless steel strips (Figure 6). Phase 2 involved examining the cell surface hydrophobicity of the isolates and attachment of the isolates to stainless steel strips with Scanning Electron Microscopy (SEM).

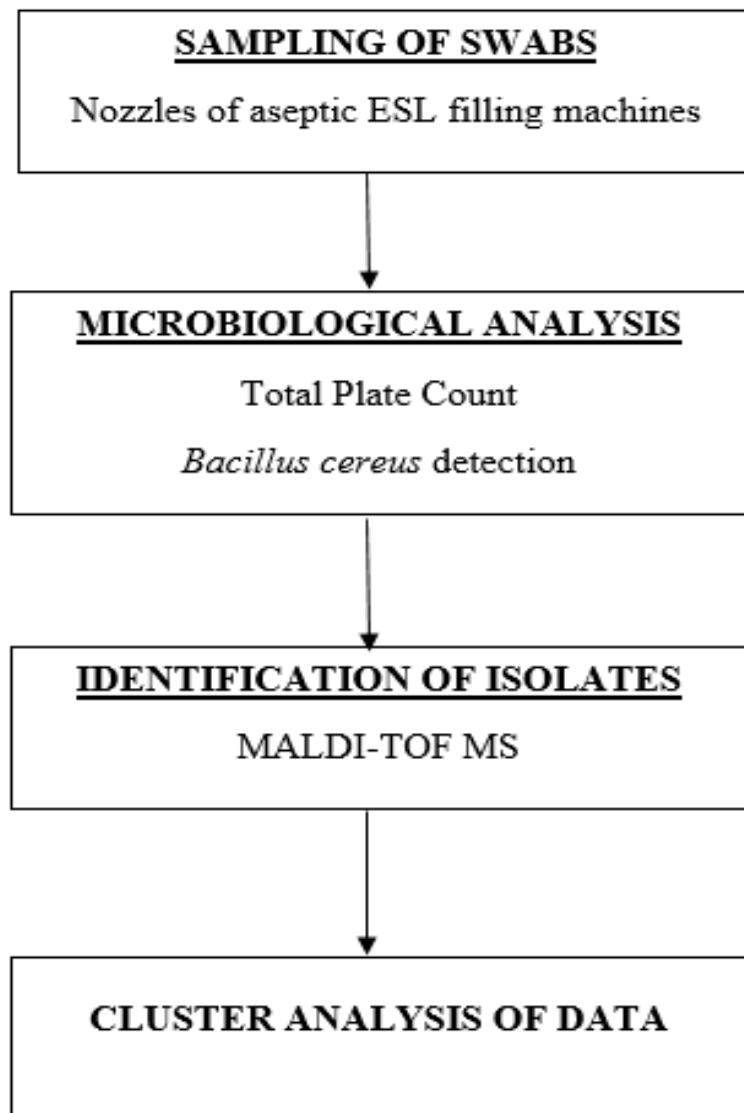


Figure 5: Phase 1 identification and characterisation of bacteria associated with the nozzles of aseptic ESL filling machines

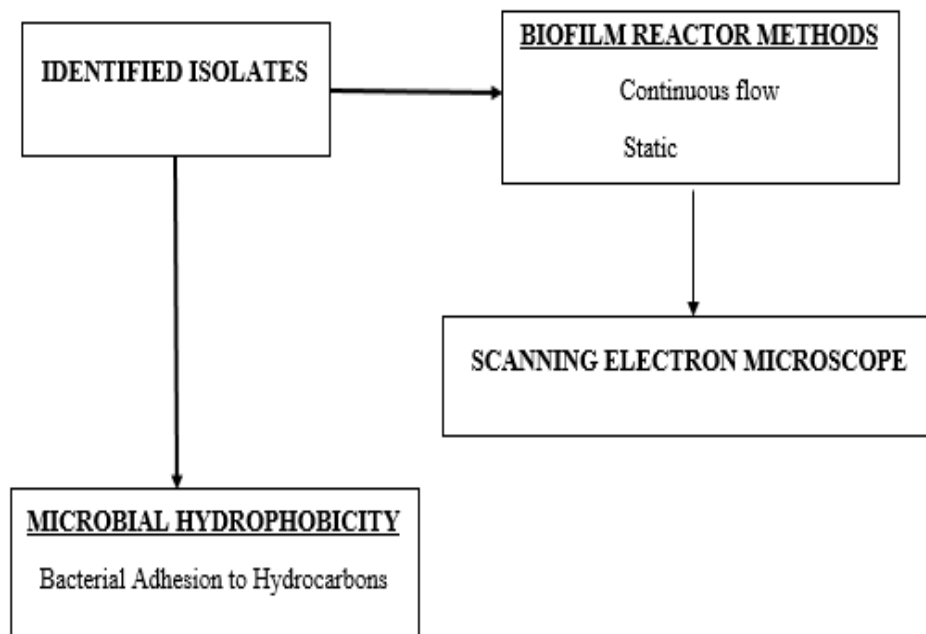


Figure 6: Phase 2 determination of the attachment of bacterial isolates to hydrocarbons and stainless steel strips

3.2 IDENTIFICATION AND CHARACTERISATION OF BACTERIA ASSOCIATED WITH ASEPTIC FILLING NOZZLES OF ESL MACHINES AFTER CIP

ABSTRACT

In South Africa extended shelf life (ESL) milk processors are still facing challenges extending the shelf life beyond 14 days. It is hypothesized that post-contamination along the milk processing line is responsible of reducing the shelf life of ESL milk. This assumption was investigated through assessing the microorganisms associated with the nozzles of aseptic filling machines post CIP process, this study was designed and divided into two phases. Phase one involved isolation and characterisation of the bacteria associated with the nozzles of aseptic filling machines after CIP process with the aim of determining the diversity of microorganisms attached to the nozzles of aseptic filling machines. Swab samples were collected from a dairy plant processing ESL milk. Twenty swabs were taken from ten different nozzles of aseptic filling machines during 4 visits (n=80). The swab samples were plated on the day of sampling. A total bacterial count ranging from 1.75 - 1.95 log CFU/cm³ with an average of 1.81 log CFU/cm³ (n=80). MALDI-TOF revealed a high percentage of Gram-positive rods (69%), followed by Gram-positive cocci (20%) and then Gram-negative rods (7%). The Gram-positive rods belonging to genus *Bacillus* were identified as *B. cereus*, followed by *B. pumilus*, *B. subtilis* and *Paenibacillus* spp. The Gram-positive cocci included *S. hominis*, *S. epidermidis*, *Micrococcus luteus* and *Anaerococcus* spp. The Gram-negative rods were identified as *Acinetobacter junii*. The prevalence of *Bacillus* spp. noted in the nozzles of ESL aseptic filling machines is attributed to their ability to resist heat treatment during CIP process and their ability to attach to stainless steel surfaces. There was a degree of similarity in terms of MALDI-TOF MS profiles for the strains of *B. cereus*, *Staphylococcus* spp. and *Paenibacillus* spp. originating from the nozzles of aseptic ESL filling machines and the packaged ESL milk product. These bacteria are likely to originate from the nozzles and dispensed into the final ESL milk during filling process.

3.2.1 INTRODUCTION

Milk processing lines are treated with cleaning-in-place (CIP) procedures (Austin and Bergeron, 1995). The basic sequence of the CIP procedure is: 1. a pre-rinse with cold water to remove gross residues; 2. the circulation of detergent to remove remaining minor residues; 3. an intermediate cold water rinse to flush out detergent; 4. the circulation of disinfectant to inactivate and kill any residual microorganisms; 5. a final cold water rinse to flush out detergent (Simoes *et al.*, 2010). However, even with acceptable CIP systems, bacteria may remain on equipment surfaces and may accumulate and form biofilms (Sharma and Anand, 2002). A proper cleaning and sanitation programme is critical to improve the keeping quality of pasteurised and extended shelf life (ESL) milk and protecting the consumer against foodborne disease (Austin and Bergeron, 1995). The formation of biofilms on dairy industry equipment can lead to serious hygiene problems and economic losses due to food spoilage and equipment impairment (Simoes *et al.*, 2010). In dairy processing operations, biofilms have been reported on bends in pipes, rubber seals, gaskets, conveyor belts, floors, plate-heat exchangers, the pasteurised milk section of the pasteuriser and the filler nozzles (Eneroth *et al.*, 2000; Sharma and Anand, 2002; Malek *et al.*, 2012).

Biofilms formed in food-processing environments are of special importance as they have the potential to act as a persistent source of microbial contamination that may lead to spoilage of final product or transmission of disease (van Houdt and Michiels, 2010). Biofilms are known to affect the quality and safety of dairy products and to significantly reduce their shelf-life. Due to their resistance to heat treatments and to antimicrobial agents, biofilms on dairy processing lines are also difficult to remove, even with acceptable cleaning and disinfection procedures (Brooks and Flint, 2008). A number of food-spoilage bacteria, including *Pseudomonas aeruginosa*, *P. fragi*, *Micrococcus* spp., *B. subtilis* and pathogenic bacteria such as *Listeria monocytogenes*, *Yersinia enterocolitica*, *Escherichia coli* O157:H7 and *Campylobacter jejuni* have been shown to be associated with biofilms in food processing environments (Sharma and Anand, 2002). *Bacillus* spp. found in milk are hydrophobic and are therefore drawn to the surfaces of the pipes in the processing equipment and form biofilms (Poulsen, 1999). Knowledge of biofilm ecology is necessary to elaborate efficient cleaning and disinfection procedures that would target dominant species and successfully eliminate biofilms from dairy processing lines (Malek *et al.*, 2012). This study was aimed at identification and characterisation of the bacteria associated with the nozzles of aseptic ESL filling machines after CIP process.

3.2.2 MATERIALS AND METHODS

SAMPLING OF NOZZLES OF THE ASEPTIC ESL FILLING MACHINES

Swab samples were collected from a dairy processing plant in the Gauteng, South Africa, processing ESL milk. The plant practices the following cleaning procedure: rinsing of the processing lines with cold water for 20-30 min followed by cleaning with caustic (1-1.5%) for 30-45 min and rinsing with cold water for 20-30 min. Followed by cleaning with super-acid (1-2%) and the last stage of the clean-in-place (CIP) process is rinsing with cold water for 10-20 min. The processing lines are sanitized using chemical disinfectors followed by rinsing with water. Twenty swabs were taken from ten different nozzles of aseptic filling machines during 4 visits (n=80) from one dairy plant processing ESL milk. Two swabs from each nozzle (swabbing the inlet and outlet) with 45mm diameter (Figures 7 and 8). After swabbing, two swab was transferred into separate 10ml buffered peptone water (Biolab, Midrand, South Africa). The swab samples were transported to the department of Food Science, University of Pretoria, Pretoria, South Africa and plated on the day of sampling.

ENUMERATION OF BACTERIA

The swabs were vortexed for 120s to release cells attached to the surface of the cotton-tip, serially diluted and plated in duplicate on nutrient agar (Biolab) plates. The plates were incubated at 30°C for 96h.

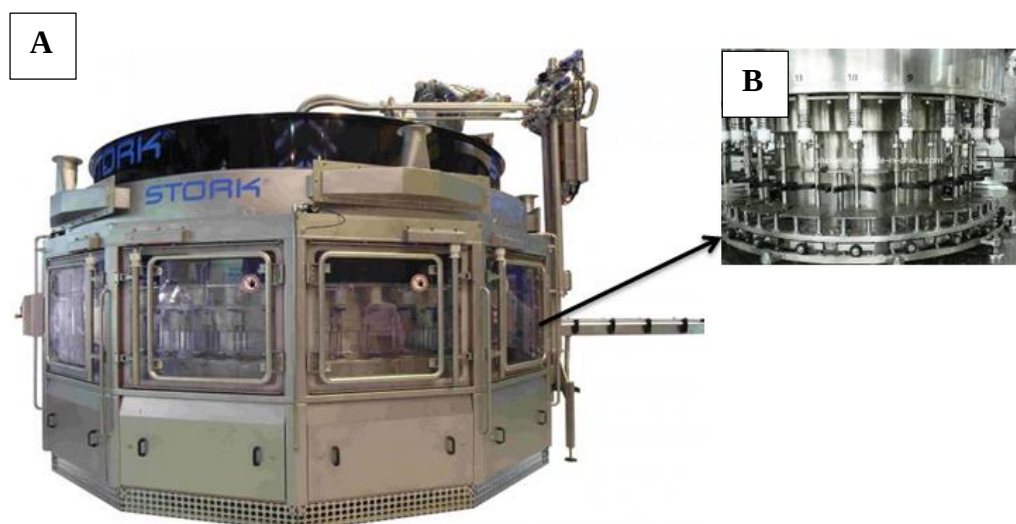


Figure 7: (A) Aseptic filling machine used in the production of ESL milk (B) Nozzles of plastic bottle rotary aseptic filling machine. www.sfds.eu/rules-of-conduct/7.html [Accessed 20 March 2015]

IDENTIFICATION OF BACTERIA FROM THE NOZZLES OF ASEPTIC ESL FILLING MACHINES

Three colonies were randomly selected from plates with highest dilution. Gram staining reactions and 3% potassium hydroxide (KOH) test was done of purified isolates. Isolates were transferred to 20% sucrose and stored at -20°C for further analysis. Identification of isolates was conducted with MALDI-TOF biotyper (Bruker Daltonics, Bremen, Germany).

IDENTIFICATION OF ISOLATES USING MALDI-TOF AND DENDROGRAM CREATION

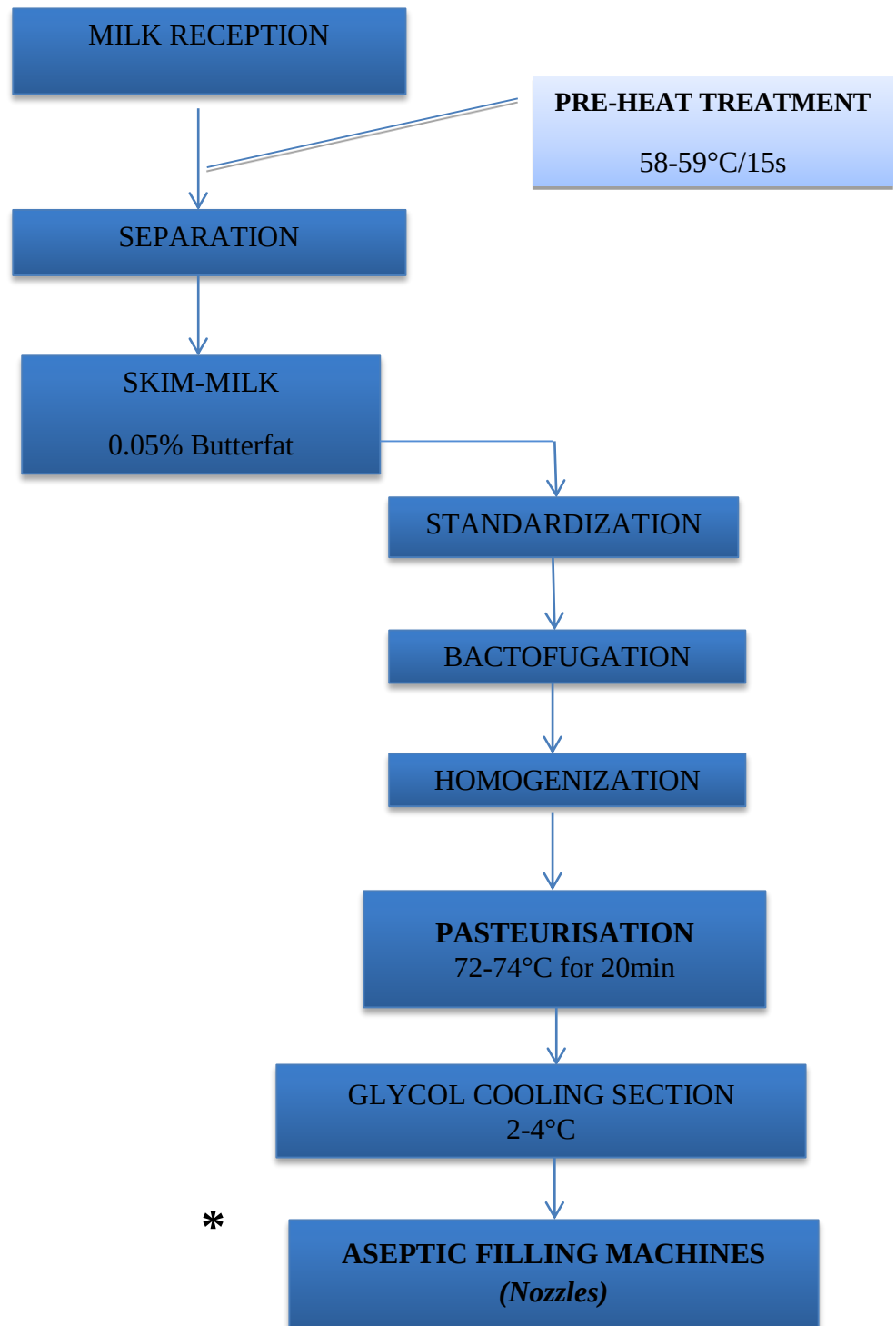
Isolates were sub-cultured twice on plate count agar (Biolab) for 24-48h. Purified colonies were placed on a ground-steel MALDI target plate (Sigma-Aldrich, USA) in duplicate and air-dried at room temperature. Samples were then overlaid with 1 μ l of HCCA solution (α -cyano-4-hydroxycinnamic acid, 20 μ g μ l⁻¹), crystallized by air-drying at room temperature and directly screened by MALDI-TOF MS. The MALDI-TOF mass spectra were acquired on an AutoFlex III Smartbeam instrument (Bruker Daltonics) using the instruments pre-programmed Flex Control 3.0 software (Bruker Daltonics) which stipulated the following parameter settings: linear positive mode, 60Hz laser frequency, 20 kV acceleration voltage, 170 ns extraction delay and a 2000-20 137 m/z range. For each sample spot (approximately 5mm in diameter on the ground steel MALDI target plate), an average of 600 shots was delivered at one point, and the final spectrum was an average accumulation of all spectra gathered from at least six different points on a sample spot. The protein molecular detection limit was set in the medium range between 2,000 and 20,000 Da. To validate the accuracy of mass spectral data generated by the MALDI-TOF MS instrument, each batch of samples contains the Bruker bacterial standard (BTS). Identification was performed using the MALDI software which compared the acquired spectra to reference spectra from library. Species identification was considered reliable, when the score calculated by the MALDI biotyper 2.0 exceeded 2, 000.

Creation of a MSP dendrogram to determine the appropriate relatedness of strains of the isolates was conducted using the Bruker Daltonics MALDI Biotyper 3.0 software. Comparative clustering of mass spectra was performed by the ClinProTools 2.1 software to statistically evaluate MALDI-TOF mass spectra. Spectra were normalized and recalibrated, using the respective functionalities of the software.

The data of the isolates originating from raw milk samples, pasteurised milk from holding tanks and the final ESL milk products were obtained from the research work done by Mugadza and Buys (2013). This included the creation of dendrograms showing the cluster analysis of the isolates from the nozzles of aseptic filling machines and the final ESL milk products. Isolates with >75% similarity were regarded as closely related strains.

STATISTICAL ANALYSIS

A single factor analyses of variance (ANOVA) was performed using Statistica software for Windows version 12 (Statsoft Inc, Tulsa, Oklahoma, USA, 2011) to determine if there was a significant difference in the bacterial counts and hydrophobicity in the nozzles of aseptic ESL filling machines at the 95% level of significance.



*Sampling point

Figure 8: Schematic diagram of the Extended Shelf Life milk processing at the dairy plant assessed during this study

3.2.3 RESULTS

TOTAL PLATE COUNT OF THE NOZZLES OF THE ASEPTIC FILLING MACHINES

A total bacterial count ranging from 1.75-1.95 log CFU/cm³ with an average of 1.81 log CFU/cm³ (n=80) was observed after CIP process from the nozzles in aseptic filling machines used in the production of ESL milk (Figure 9).

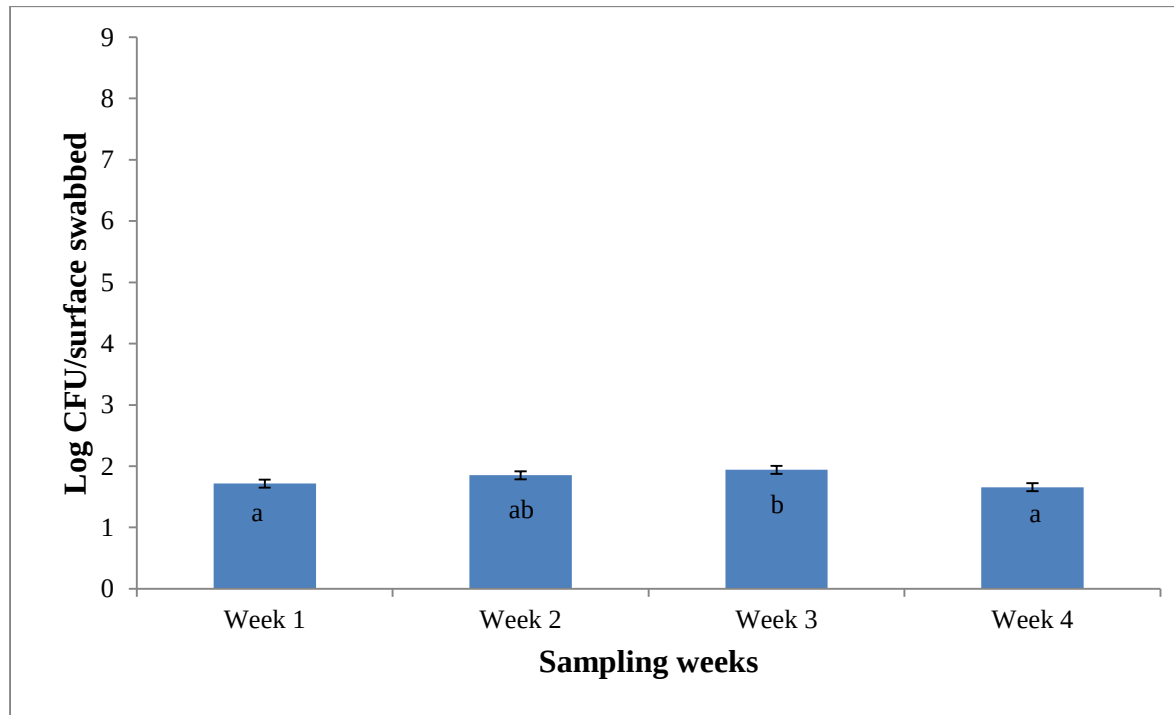


Figure 9: Total bacterial count for four weeks obtained from the nozzles in aseptic filling machines after CIP process. [Bars with different letters differ significantly ($p \leq 0.05$)] (n=80)

CHARACTERISATION AND PROFILING OF BACTERIAL STRAINS ISOLATED FROM THE NOZZLES OF ASEPTIC ESL FILLING MACHINES

A high percentage of Gram-positive rods (69%) was observed (Figure 10a), dominating the identified groups of bacteria, followed by Gram-positive cocci (20%) and then Gram-negative rods (7%). The Gram-positive rods (Figure 10b), belonging to genus *Bacillus* were identified as *B. cereus* (41%), followed by *B. pumilus* (9%), *B. subtilis* (7%) and *Paenibacillus* spp. (4%). The Gram-positive cocci included *S. hominis* (5%), *S. epidermidis* (5%), *Micrococcus luteus* (5%) and *Anaerococcus* spp. (5%). The Gram-negative rods were identified as *Acinetobacter junii* (7%).

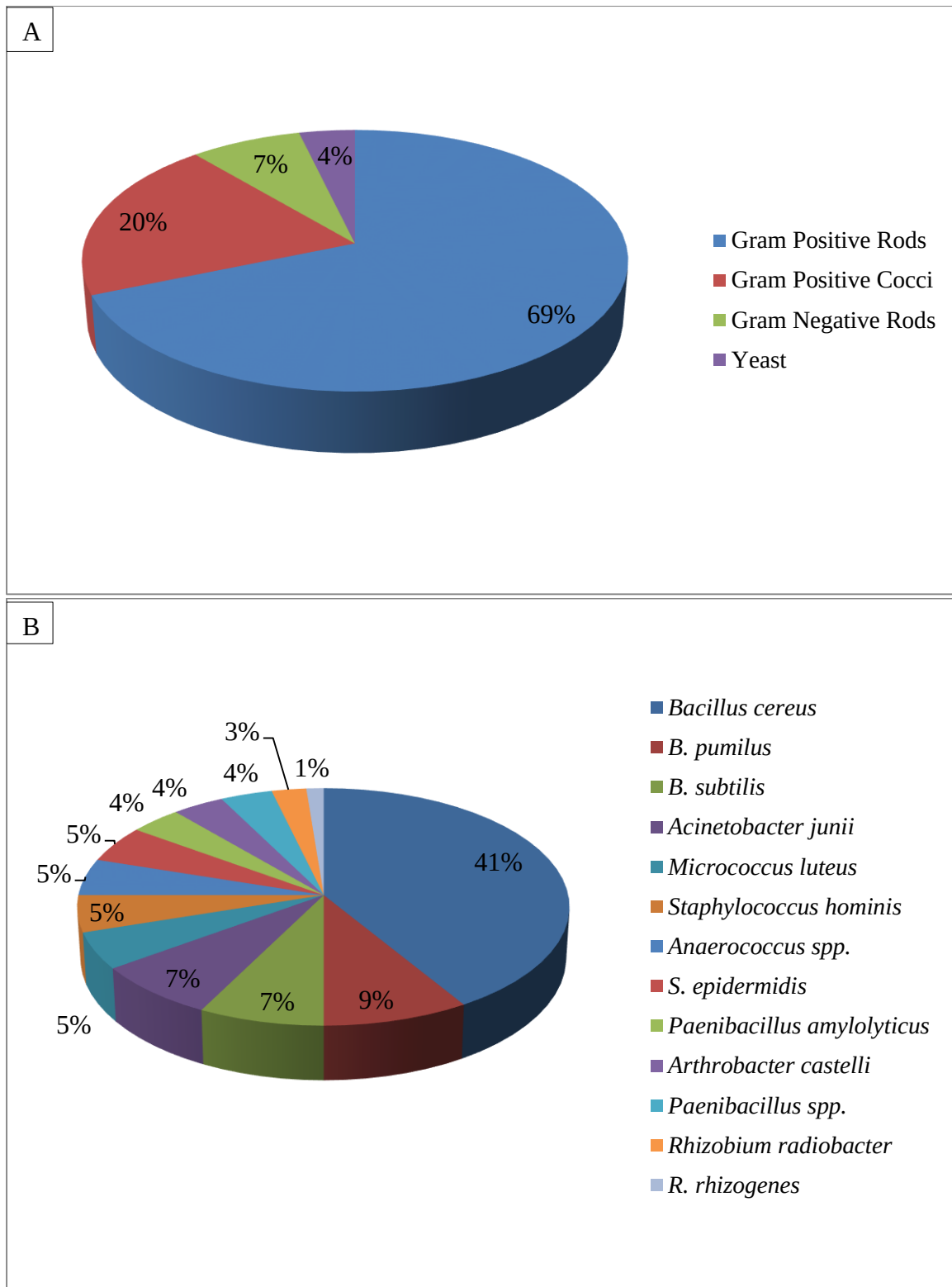


Figure 10: Composition of isolates from the nozzles of aseptic ESL filling machines after CIP procedures.

Species belonging to *Bacillus* spp. (41.3%) were recognised to dominate the bacterial composition associated with the nozzles of aseptic filling machines. *B. cereus*, *B. pumilus* and *A. junii* were present in all weeks of sampling (Table 4). *B. pumilus* and *S. hominis* were only isolated in weeks 1-3 of sampling. *B. subtilis* was present in weeks 2-4.

Table 4: Predominant bacteria in the nozzles of aseptic ESL filling machines for isolates (n=80) sampled after CIP over four weeks.

Identified isolates	PERCENTAGE OF ISOLATES (%)				Total number (%)
	Week 1	Week 2	Week 3	Week 4	
<i>Bacillus cereus</i>	37	45	40	43	33 (41,25)
<i>B. pumilus</i>	10,5	5	13	5	7 (8,75)
<i>B. subtilis</i>		15	9	5	6 (7,5)
<i>Acinetobacter junii</i>	10,5	10	5	5	6 (7,5)
<i>Micrococcus luteus</i>	16		5		4 (5)
<i>Staphylococcus hominis</i>		10	9		4 (5)
<i>Anaerococcus</i> spp.				21	4 (5)
<i>S. epidermidis</i>	5		5	11	4 (5)
<i>P. amylolyticus</i>	10,5			5	3 (3,75)
<i>Arthrobacter castelli</i>		15			3 (3,75)
<i>Paenibacillus</i> spp.			9	5	3 (3,75)
<i>Rhizobium radiobacter</i>	10,5				2 (2,5)
<i>R. rhizogenes</i>			5		1 (1,25)
Total number					80 (100)
Log CFU/surface swabbed	1.75±0.21	1.77±0.14	1.95±0.05	1.75±0.12	

CLUSTER ANALYSIS OF *B. CEREUS*, *PAENIBACILLUS* SPP., *B. PUMILUS* AND *STAPHYLOCOCCUS* SPP. ORIGINATING FROM VARIOUS STAGES OF ESL MILK PROCESSING

B. CEREUS

For the cluster analysis of *B. cereus* strains, two distinct clusters, cluster 1 and cluster 2 were observed. Within the two clusters, two different sub-clusters were observed. This yielded six groups. Groups 1-4 consisted of single strains. The bacterial strains in group 1 was isolated from the packaged ESL milk product and the strains in groups 2-4 were isolated from the nozzles of aseptic filling machines. Group 5 consisted of three strains that were closely related (75%), with all strains originating from the aseptic filling machines (Figure 11).

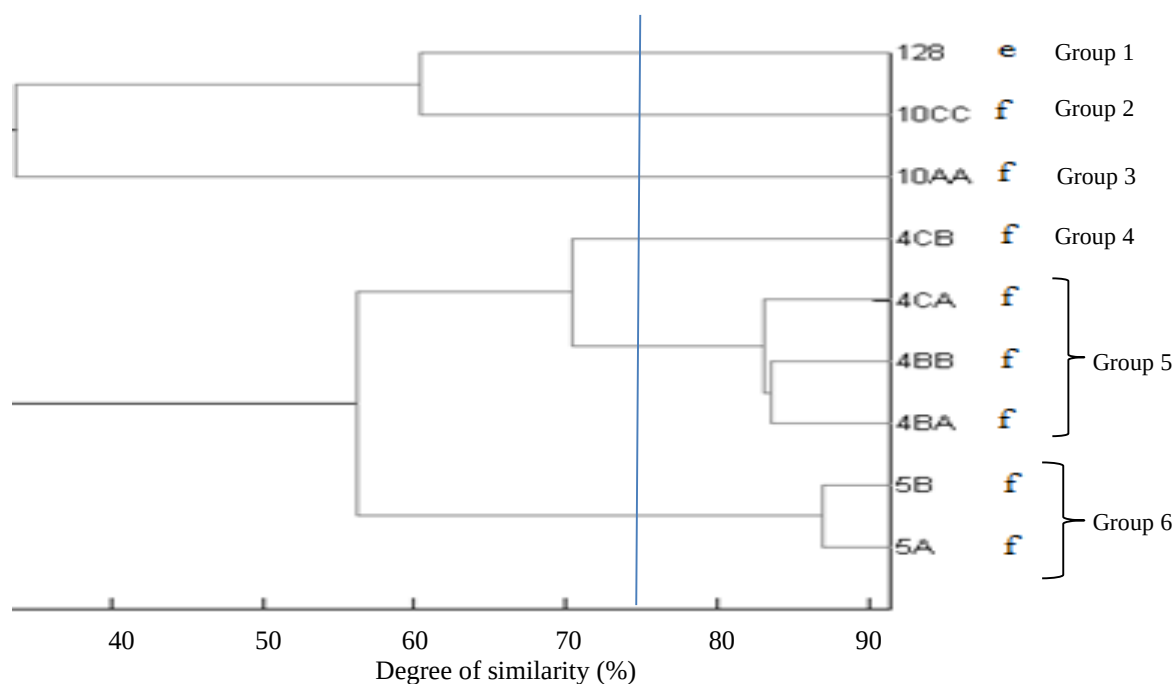


Figure 11: Dendrogram of *Bacillus cereus* strains isolated from the nozzles of aseptic ESL filling machines and the packaged ESL milk product

*Sources of isolates: f- Aseptic filling nozzles and e- Packaged ESL milk product

Vertical line: above 75% indicates similarity of the strains to genus and species levels

PAENIBACILLUS SPP.

For the cluster analysis of *Paenibacillus* spp. strains, two distinct clusters, cluster 1 and cluster 2 were observed. Within cluster 2, two different sub-clusters were observed. This yielded three groups (Figure 12). Group 1 consisted of a single strain originating from the nozzles of aseptic filling machines. Group 2 consisted of two strains originating from the nozzles and the pasteurised milk, these strains were closely related (>75%).

STAPHYLOCOCCUS SPP.

For the cluster analysis of *Staphylococcus* strains, two distinct clusters, cluster 1 and cluster 2 were observed. Within cluster 2, four different sub-clusters were observed. This yielded four different groups (Figure 13). Groups 1-3 consisted of single strains originating from the nozzles of the aseptic filling machine (group-1), the pasteurised milk (group 2) and the final ESL milk product (group 3). Strains in group 4 originating from the nozzles of aseptic filling machines were closely related (>75%).

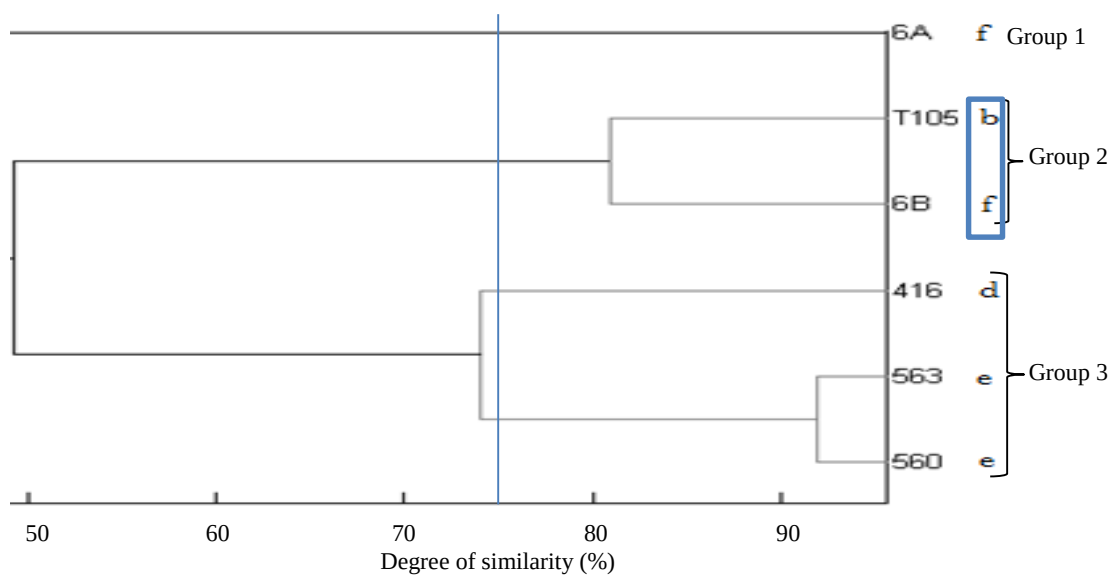


Figure 12: Dendrogram of *Paenibacillus* spp. and *Paenibacillus amylolyticus* strains isolated the nozzles of aseptic ESL filling machines and packaged ESL milk product

*Isolate sources: b – Pasteurised milk, d- Packaged ESL milk products, e – Stored ESL milk at 7°C and f – Nozzles of aseptic filling machines.

*Strains: *Paenibacillus* spp. (f) and *P. amylolyticus* (b, d & e)

Vertical line: above 75% indicates similarity of the strains to genus and species levels

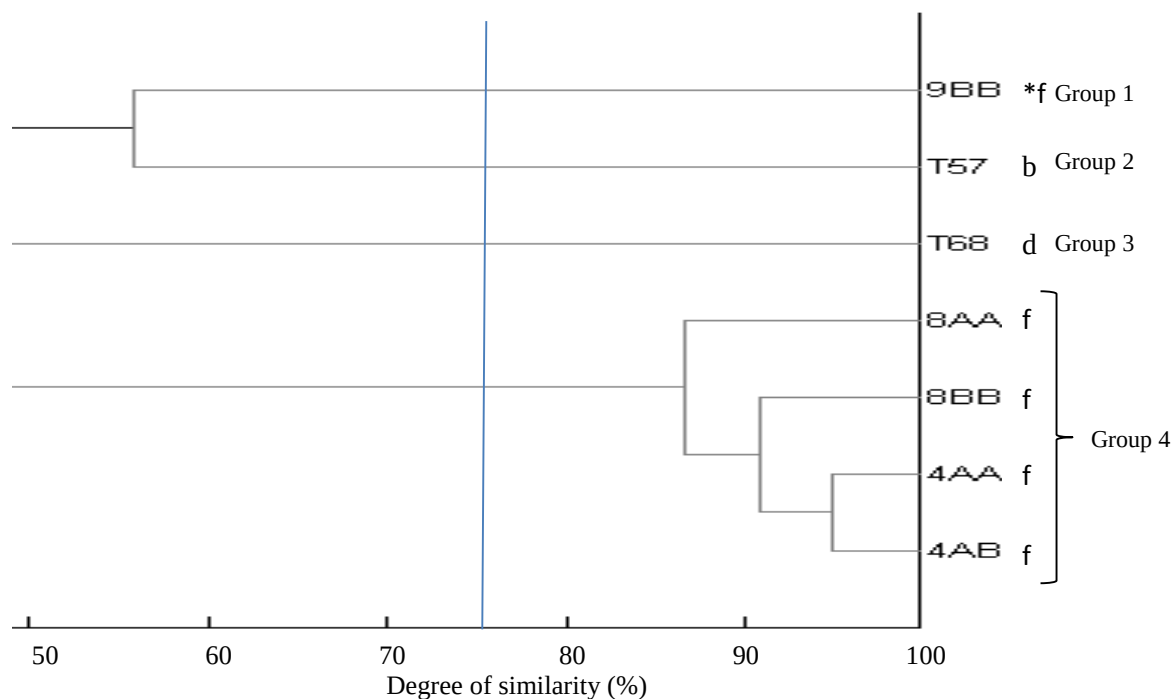


Figure 13: Dendrogram of *Staphylococcus* spp. strains isolated the nozzles of aseptic ESL filling machines and packaged ESL milk product

*Isolate sources: b – Pasteurised milk, d- Packaged ESL milk products and f –Nozzles of aseptic filling machines.

*Strains: *Staphylococcus epidermidis* (*f), *S. capitis* (b & d) and *S. hominis* (f)

Vertical line: above 75% indicates similarity of the strains to genus and species levels

B. PUMILUS

For the cluster analysis of *B. pumilus* strains, two distinct clusters, cluster 1 and cluster 2 were observed. Within cluster 1, nineteen groups were observed and within sub-cluster 2, fifteen sub-clusters were observed (Figure 14). This yielded five different groups 1-5. Groups three and four consisted of strains originating from raw milk, samples from the pasteurised milk holding tanks, the nozzles of aseptic filling machines and the packaged ESL milk product. Strains within groups three and four were closely related (>75%).

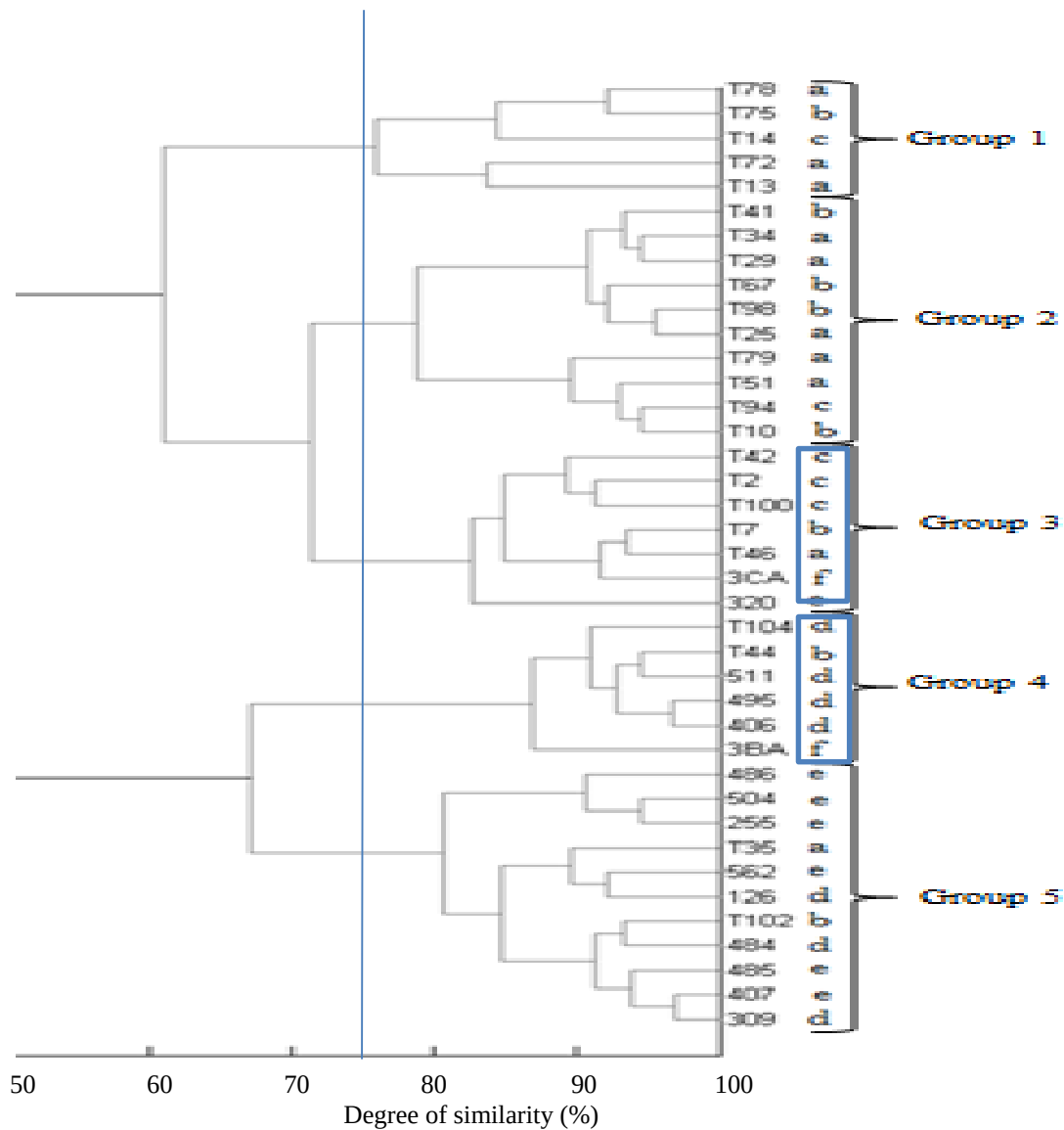


Figure 14: Dendrogram of *Bacillus pumilus* strains isolated the nozzles of aseptic filling machines and the final ESL milk product

*Isolate sources: a – Raw milk, b – Pasteurised milk, c – Packaged ESL milk and f –Nozzles of aseptic filling machines

Vertical line: above 75% indicates similarity of the strains to genus and species levels

3.2.4 DISCUSSION

The low levels of total plate count (TPC) noted in the nozzles of aseptic filling machines indicates that (CIP) process was effective. However, there are no CIP standards to be used as a benchmark to determine the efficiency of CIP processes practiced in the dairy industry. Other authors have indicated that the CIP process has been found to effectively reduce the levels of microbial counts in the nozzles of filling machines (Salustiano, Andrade, Soares, Lima, Bernardes, Luiz and Fernandes, 2009).

Despite the low levels of TPC noted in this study, the presence of the identified isolates in the nozzles of aseptic filling machines could indicate that the species isolated were resistant to the sanitizers and disinfectants used in the cleaning programme. Furthermore, the cleaning programme was not effective enough to eliminate some of the microbial cells in the nozzles of aseptic filling machines. It has also been noted by (Flint, 1998; Bremer, Fillery and McQuillan, 2006; Brooks and Flint, 2008), that routine CIP procedures used in the dairy industry are not always effective enough to remove all attached bacterial cells. The identified isolates in this study could indicate that there's a multispecies community of microbial cells attached onto the nozzles of the aseptic ESL filling machines.

Although all the bacteria isolated in this study have previously been isolated from the dairy processing lines including raw milk tanks, pasteurisers, pasteuriser-gaskets, pasteurised milk holding tanks, pasteurised milk fillers (Eneroth *et al.*, 2000; Vlkova *et al.*, 2008; Salustiano *et al.*, 2009; Malek *et al.*, 2012), this is the first report on the microbial quality of the nozzles of aseptic filling machines for ESL milk products. Also *Paenibacillus* spp. had not previously been reported to have been isolated from the nozzles of ESL aseptic filling machines.

Simmonds *et al.*, 2003 reported that when spores are attached to stainless steel surfaces, their heat resistant increases significantly. The prevalence of *Bacillus* spp. noted in the nozzles of ESL aseptic filling machines can be attributed to their ability to resist heat treatment during CIP process and their ability to attach to stainless steel surfaces. *Bacillus* spp. has been isolated from dairy processing lines including pasteurisers and pasteurised milk fillers after CIP process (Eneroth *et al.*, 2000; Sharma and Anand, 2002; Malek *et al.*, 2012). Lindsay *et al.*, (2000) reported that *B. cereus* traps planktonic cells to form biofilms. The presence of *Bacillus* spp. could have also enhanced the attachment of the other isolates noted in the nozzles of the aseptic

filling machines. Additionally, *Bacillus* spp. could have protected the other isolates from the chemical agents applied during the CIP process. Therefore, this explains the presence of the strains of *Staphylococcus*, *Micrococcus*, *Anaerococcus*, *Arthrobacter*, *Acinetobacter* and *Paenibacillus* spp. on the nozzles of aseptic ESL filling machines and these strains may be resistant to the chemical agents used for cleaning procedures. These strains may then be dispersed into the final ESL milk product during subsequent filling. As a consequence, the aseptic ESL filling nozzles act as a potential source of contamination of final ESL milk products.

There was degree of similarity between the strains of *B. cereus*, *Staphylococcus* spp. and *Paenibacillus* spp. obtained from the nozzles of aseptic filling machines and the packaged ESL milk products. This suggests that the nozzles of aseptic filling machines could be the reservoir for *Bacillus* spp. and other identified bacterial species including *Paenibacillus* spp. *S. hominis* and *S. epidermidis* were also present in the ESL milk products. It has been demonstrated that for pasteurised and extended shelf life (ESL) milk, the filling machines are the main source of recontamination (Eneroth *et al.*, 2000; Rysstad and Kostad, 2006; Dhillon, 2012). This is an indication that the cross contamination of the ESL milk product could take place during the filling process and may ultimately lead to microbial spoilage of ESL milk products.

3.2.5 CONCLUSION

In this study we have shown that there's a range of microorganisms, most probably in the form of biofilms, attached to the nozzles of aseptic filling machines. The identified microorganisms may have been resistant to cleaning and disinfection processes. Additionally, gram positive bacteria belonging *Bacillus* spp. were shown to be dominant bacteria of the aseptic ESL filling nozzles. These bacteria are likely to be dispensed into the final ESL milk during the filling process. Ultimately, they may multiply at refrigeration temperatures and during distribution chain. This may compromise food safety and public health of ESL milk products.

3.3 ADHESION OF BACTERIAL STRAINS ISOLATED FROM ESL FILLING MACHINES TO STAINLESS STEEL

ABSTRACT

Equipment surfaces are recognised to be a major source of contamination of processed milk with both spoilage and pathogenic microflora. One of the reasons for contamination of ESL milk could be attachment of persistent spores to such surfaces. The aim of this study was to evaluate the ability of *B. cereus*, *B. pumilus*, *Paenibacillus* spp., *M. luteus* and *S. epidermidis* to form biofilm on SS-306 stainless steel strips. The bacterial strains were isolated from the nozzles of aseptic filling machines. A continuous flow reactor system was used to grow biofilm of the isolates in skim milk. The skim milk was inoculated with spore suspension of *B. cereus*, *B. pumilus*, *Paenibacillus* spp. and bacterial suspension of *M. luteus* and *S. epidermidis*. The bacterial suspensions were run separately over a period of 22h at 37°C. Stainless steel strips were submitted to SEM Scanning Electron Microscopy (SEM) after 20h. The results suggested that spores of *B. cereus*, *B. pumilus* and *Paenibacillus* spp. can only attach whilst *M. luteus* and *S. epidermidis* can attach and form biofilms on stainless steel. The ability of these isolates to form biofilms on stainless steel strips could be the main cause of contamination of ESL milk. Strains of *Bacillus* spp. can form biofilm on stainless steel and limit the shelf life of milk and milk products. However, toxins produced by some of these strains of *B. cereus* might be contagious to humans. The results confirmed that one of the reasons of contamination of ESL milk could be the ability of *B. cereus* to attach to stainless surfaces and *M. luteus* to form biofilms. Over and above the fact that the spores of *B. cereus* can lead to spoilage of milk and milk products, a concern is that the toxins produced by some of the strains of *B. cereus* are detrimental to human health.

3.3.1 INTRODUCTION

Microbial hydrophobicity is the key factor in the adhesion of microbial cells to solid surfaces such as stainless steel (Simmonds *et al.*, 2003). Other factors such as surface properties of the substrate and the suspending medium may also play a role in attachment (Simmonds *et al.*, 2003). Surface hydrophobicity is generally associated with bacterial adhesiveness and varies from organism to organism, from strain to strain and is influenced by the growth medium, bacterial age and bacterial surface structure (Basson, Flemming and Chenia, 2008). The bacterial cell surface charges differ between bacterial strains and it depends on the surface chemistry of the functional groups such as carboxyl, phosphate and amino groups (Buszewski, Dziubakiewicz, Pomastowski, Hryniewicz, Ploszaj- Pyrek, **Kramer, Talik, and Albert, 2015**). The physicochemical properties of the microbial cell surface, including the presence of (glycol-) proteinaceous material at the cell surface results in higher hydrophobicity, whereas hydrophilic surfaces of microbial cells are associated with the presence of polysaccharides (Abdulla, Abed and Saeed, 2014). Busscher, van der Belt-Gritter and van der Mei (1995), measured the zeta potential values of hydrocarbons and have found that hydrocarbons are negatively charged. Dunne (2002) reported that electrostatic interactions tend to favour repulsion, because most bacteria and hydrocarbons are negatively charged. In the absence of electrostatic interactions, the difference observed in microbial adhesion to polar and non-polar solvents can be attributed to unfavourable acid-base interactions and unfavourable interactions are expressed by the decrease in microbial cell affinity to a polar solvent (Bellon-Fontaine, Rault and Van-Oss, 1996). Microbial cell affinity to hydrocarbons is considered to be the result of interplay of electrostatic, van der Waals and Lewis acid-based interactions, in the same way as microbial adhesion to stainless steel surfaces (Bellon-Fontaine *et al.*, 1996). Therefore the attachment of microbial cells to stainless steel surfaces may be connected with the cell surface hydrophobicity.

Elhariry (2008) reported that the hydrophobicity of *Bacillus* spores plays an important role in the hydrophobic interactions in the adhesion of bacteria to the surface of stainless steel. Stainless steel is commonly used in food manufacturing plants because of its heat transfer efficiency, corrosion resistance and strength (Brooks and Flint, 2008). In the dairy industry, stainless steel surfaces are recognized to be major source of contamination of processed milk with spoilage and pathogenic micro-flora (Malek *et al.*, 2012). Biofilms are of concern in dairy manufacturing plants, as bacteria within biofilms are more difficult to eliminate than free living

cells and once established can act as a source of contamination of product and other surfaces (Flint, 1998). The aim of this study was to determine the ability of the bacteria isolated from the nozzles of aseptic ESL filling machines to adhere to hydrocarbons and their potential to attach and form biofilms on stainless steel strips.

3.3.2 MATERIALS AND METHODS

PREPARATION OF ISOLATES FOR SURFACE CHARACTERISTICS

NON-SPORE FORMERS

Isolates (18h) of *S. hominis*, *S. epidermidis*, *A. junii*, *A. castelli* and *M. luteus* isolated from the ESL milk filling machines were plated on Tryptic Soy Agar (TSA) and incubated at 37°C for 48h and stored at 4°C for further analysis.

SPORE FORMERS

Isolates (18h) of *B. cereus*, *B. pumilus* and *Paenibacillus* spp. were plated on nutrient agar supplement with 12mg of manganese sulphate per liter to induce sporulation. The agar plates were incubated at 37°C for 4 days. VS3 Microscope phase contrast (Micromet Scientific, Johannesburg, South Africa) was used to monitor the presence of spores on the agar plates after spore staining method was conducted. More than 95% spore formation was observed on the slides using phase contrast microscopy (Figure 15). Spores were then harvested by flooding the agar plate surface with sterile distilled water and scraping with sterile metal spreader until opaque suspension was achieved. The spore suspension was transferred to 18mm test tubes and washed three times by centrifuging at 3000 x g for 15min. The spore suspensions were re-suspended into McCartney bottle with peptone saline and stored at 4°C for further analysis.

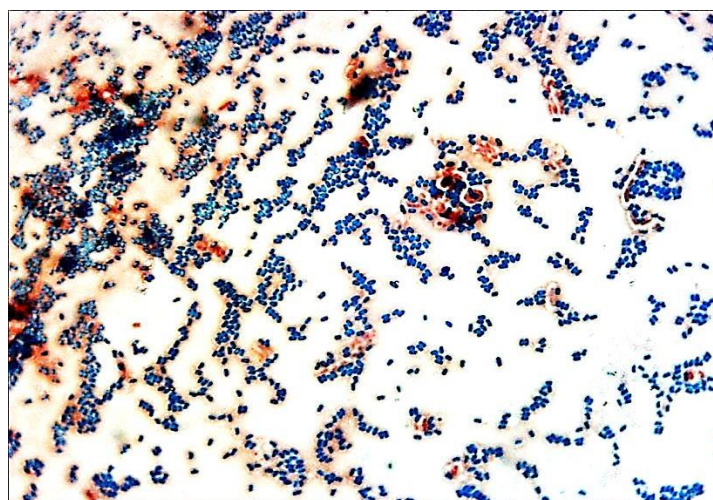


Figure 15: Phase contrast image of *Bacillus cereus* spores after 4 days at 37°C

DETERMINATION OF SURFACE CHARACTERISTICS OF ISOLATES

ADHESION OF SPORES TO HYDROCARBONS

For bacterial adhesion to hydrocarbons (BATH) the method of Ortega, Hagiwara, Watanabe and Sakiyama (2008), was used to determine the hydrophobicity of the isolates. The following hydrocarbons were used: chloroform (an electron acceptor solvent); hexane (a nonpolar solvent) and xylene (a polar solvent). BATH is based on comparing microbial cell affinity to a polar solvent and microbial cell affinity to a nonpolar solvent. The polar solvent can be an electron acceptor or an electron donor, but both solvents must have similar van der Waals surface tension components.

The suspensions were harvested by centrifugation at 3,000 x g for 15min (NF 400R Nuve Centrifuge, Ankara, Turkey). The bacterial cultures were washed twice in peptone saline: 1g/L peptone and 8.5g/L NaCl and re-suspended in peptone saline and 3ml of each sample of bacterial suspension in peptone saline (0.85%) was mixed with 1ml of hexane fraction, xylene and chloroform and then manually shaken for 20s followed by vigorous mixing with a vortex for 1min. The mixture was allowed to stand for 20min to ensure complete separation of phases. From the aqueous phase 1ml was transferred into spectrophotometer-cuvettes (A_{500nm}) of the aqueous phase was measured with a spectrophotometer and then compared with the A_{500nm} of the initial bacterial suspension. The percentage of bacterial suspension residing in chloroform, hexane and xylene was calculated by using the following equation (Basson *et al.*, 2008):

$$\%Adherence = \frac{(D_i - D_f)}{D_i} \times 100$$

Where (D_i) was the optical density of the bacterial suspension before mixing with the solvent and (D_f) the absorbance after mixing and phase separation. Each analysis was performed in duplicate.

A value of >50% indicates a strong hydrophobic cell surface, 20-50% indicates a moderate hydrophobic cell surface and <20% indicates hydrophilic cell surface.

ADHESION OF ISOLATES TO STAINLESS STEEL

PREPARATION OF STAINLESS STEEL STRIPS

Stainless steel strips (306), 45mm by 10mm (Metals 2 Steel, Pretoria, South Africa) were used as substrate to evaluate the ability of the selected isolates to form biofilms. The strips were treated with sand paper (grit: 120) increasing the degree of surface roughness. Stainless steel strips were sanded for 15min and rinsed three times with distilled water and allowed to dry at room temperature. The strips were finally autoclaved 121°C for 15min (Flint, Palmer, Bloemen, Brook and Crawford, 2001). They were stored in 70% ethanol for further use. Strips were oven-dried at 100°C for 30min before use.

LABORATORY BIOFILM REACTOR

Determination of initial concentration of bacterial suspensions

The bacterial cultures of *B. cereus*, *B. pumilus* and *Paenibacillus* spp. were heated at 63°C for 40min to kill all the vegetative cells. A spectrophotometer – absorbance and McFarland standard kit was used to estimate the density of bacterial suspension. The initial concentration of bacterial suspension was approximately 10^8 CFU/ml.

STATIC BIOFILM REACTOR

The stainless steel strips were immersed in Schott bottles with 40ml UHT skim-milk inoculated with 1ml of each bacterial suspension. Each inoculated skim-milk was incubated at 37°C (to create a favourable environment for the bacterial strains to form biofilm) in a shaking water-bath at 65rpm for 20h (Figure 16). The strips were removed from the milk after 20h and rinsed three times with sterile distilled water to remove unattached cells on the strips and immediately fixed with 2.5% glutaraldehyde in phosphate buffer for the microscopy.



Figure 16: Schott bottles with inoculated isolated from the nozzles of ESL milk filling machines, 40ml UHT skim-milk incubated at 37°C in a shaking water-bath at 65rpm for 20h

CONTINUOUS FLOW REACTOR SYSTEM

B. cereus, *M. luteus* and *S. epidermidis* were evaluated for their ability to form biofilms on stainless steel strips using a continuous flow system. These bacterial strains were selected because of their potential to form biofilms and cause contamination in dairy industry (Peng *et al.*, 2002; Dhillon, 2012). The system was continuous and designed to avoid recirculation of milk and to minimize contamination of the stainless steel strips. The system consisted of a vacuum-pump, rubber tubing, water-bath, medium-feed plastic bucket (10L capacity) and waste bucket. Components of the system were washed with a detergent solution and was sterilized by autoclave at 121°C for 15min and aseptically connected with silicone rubber tubing.

The feed container was filled with 5L of commercially sterile milk (UHT skim-milk) and inoculated with the spore suspension of *B. cereus*. Each stainless steel strip was inserted within the silicone rubber tubing (Figure 17) and immersed in a 37°C water-bath (to create a favourable environment for *B. cereus* to form biofilm on stainless steel strips). The tubing was connected to a vacuum-pump (Pall Life Science, Midrand, South Africa), through which UHT skim milk at ambient temperature was pumped at a flow rate of 8±1ml/min. After 20h, the outer surface of the silicone rubber tubing with stainless steel strip was disinfected with 70% ethanol. The strip was aseptically removed and rinsed three times with sterile distilled water to remove freely attached cells. The strips were fixed in 2.5% glutaraldehyde in phosphate buffer for microscopy and untreated stainless steel strip was used as a control. The milk after flowing through the system once was discarded (Flint *et al.*, 2001). This procedure was repeated with *M. luteus* and *S. epidermidis*.

ENUMERATION OF BACTERIAL CELLS ATTACHED TO STAINLESS STEEL STRIPS

STATIC REACTOR SYSTEM

The number of bacterial cells attached to stainless steel strips was determined on agar plate after 2, 4, 6, 8, 10, 15 and 20h of incubation at 37°C in a shaking water-bath. The strips were washed with distilled water to remove free cells attached. The bacterial cells attached to stainless steel strips were removed by using sterile cotton-tip swabs. The swabs were vortexed for 60s and incubated at 37°C for 24h.

CONTINUOUS FLOW BIOFILM REACTOR SYSTEM

The number of spores attached to stainless steel strips was determined after 2, 4, 6, 8, 10, 15 and 20h of incubation at 37°C. The strips were washed with sterile peptone water to remove free cells. The bacterial cells attached to the stainless steel strips were removed by using sterile cotton-tip swabs. The swabs were vortexed for 60s and incubated at 37°C for 24h. The procedure was repeated for *M. luteus* and *S. epidermidis*.



Figure 17: Laboratory biofilm reactor system used to evaluate the ability of the isolates to attach and form biofilm on the stainless steel strips

SCANNING ELECTRON MICROSCOPY (SEM)

SEM images were obtained using JEOL 8500LV scanning electron microscope (Tokyo, Japan). After treatment the stainless steel strips were observed by SEM. Prior to SEM observations, the biofilm sample on stainless steel strip was fixed in 2.5% glutaraldehyde in 0.075M phosphate buffer followed by a wash in 0.075M phosphate buffer for 15min and this was repeated three times. Then the biofilm sample was gradually dehydrated in ethanol from 50 to 100% (15min each 50, 70, 90 and 100% and dehydration in ethanol 100% was repeated three times), and the samples were air-dried for 1.5-2h, followed by coat with gold and examined with a scanning electron microscopy (Sarro, Garcia and Moreno, 2005).

STATISRICAL ANALYSIS

ANOVA was performed using Statistica software for Windows version 12 (Statsoft Inc, Tulsa, Oklahoma, USA, 2011) to determine if there was a significant difference between the isolates on adhesion to hydrocarbons at 5% level of significance.

3.3.3 RESULTS

ADHESION OF BACTERIAL STRAINS TO HYDROCARBONS

The degree of hydrophobicity of the spore formers ranged from 8-91% (Figure 18), while non-spore formers ranged from 6-67% (Figure 19). The hydrophobicity of *S. hominis*, *S. epidermidis* and *M. luteus* was similar for xylene. Hydrophobicity to chloroform differed significantly ($p \leq 0.05$) between *S. hominis*, *S. epidermidis*, *A. junii* and *A. castelli*. However, *S. hominis*, *S. epidermidis* and *M. luteus* showed greater affinity towards xylene in terms of hydrophobicity. *S. epidermidis* showed 67% affinity towards xylene. *A. castelli* and *A. junii* showed a low affinity towards all the hydrocarbons included in this study (Figure 19).

The degree of hydrophobicity of *B. pumilus*, *Paenibacillus* spp. and *B. cereus* were similar for chloroform. Hydrophobicity for hexane-fraction differed significantly between *B. pumilus*, *Paenibacillus* spp. and *B. cereus* ($p \leq 0.05$). High level of hydrophobicity towards chloroform and xylene ranging from 77-91% (Figure 18) for spore formers was observed. Low level of hydrophobicity towards hexane-fraction was observed for all spore formers ranging from 8-30% indicating hydrophilic character.

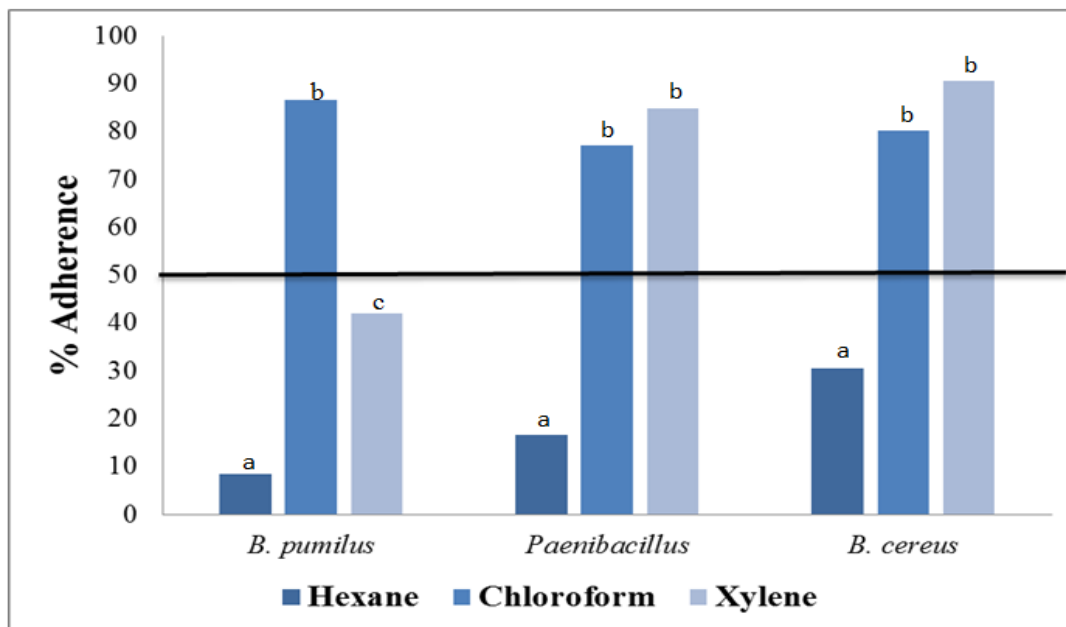


Figure 18: Hydrophobicity of *B. pumilus*, *Paenibacillus* spp. and *B. cereus* as measured by bacterial adhesion to hydrocarbons. Isolates were isolated from the nozzles of ESL filling machines [Bars with different letters differs significantly ($p \leq 0.05$). Horizontal line: values above 50% indicates strong hydrophobic cell surface (Basson *et al.*, 2008)]

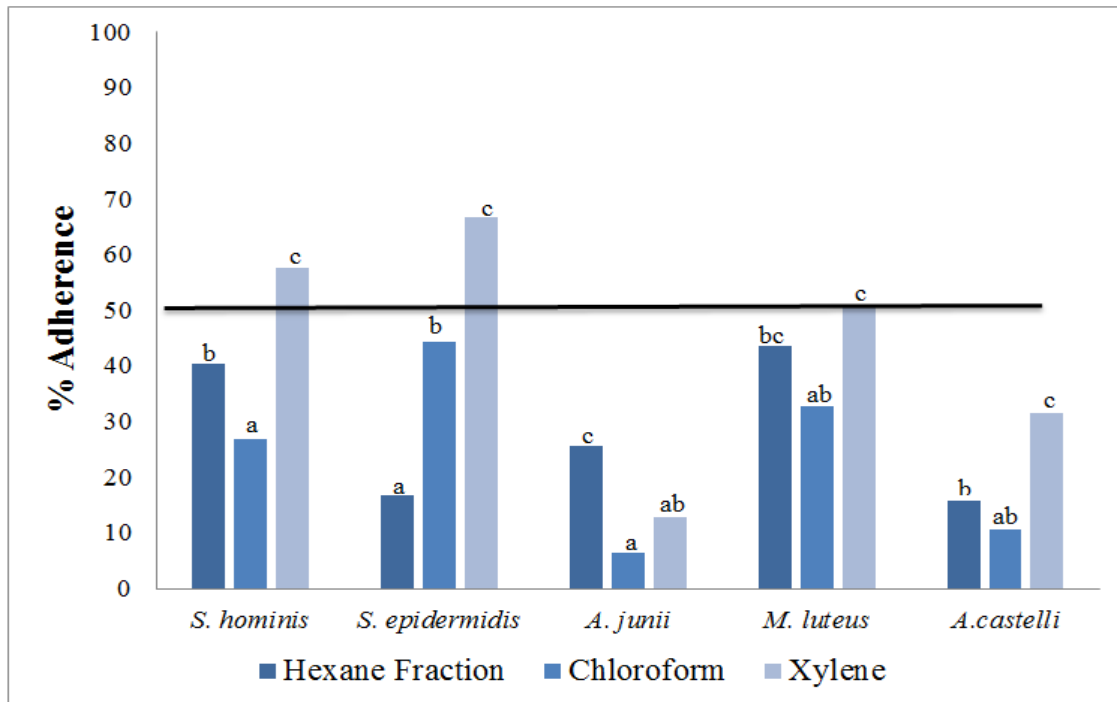


Figure 19: Hydrophobicity of *S. hominis*, *S. epidermidis*, *A. junii*, *M. luteus* and *A. castelli* as measured by bacterial adhesion to hydrocarbons. Isolates were isolated from the nozzles of ESL filling machines [Bars with different letters differs significantly ($p \leq 0.05$). Horizontal line: values above 50% indicates strong hydrophobic cell surface (Basson *et al.*, 2008)]

ADHESION OF BACTERIAL STRAINS TO STAINLESS STEEL SURFACE

STATIC RECTOR SYSTEM

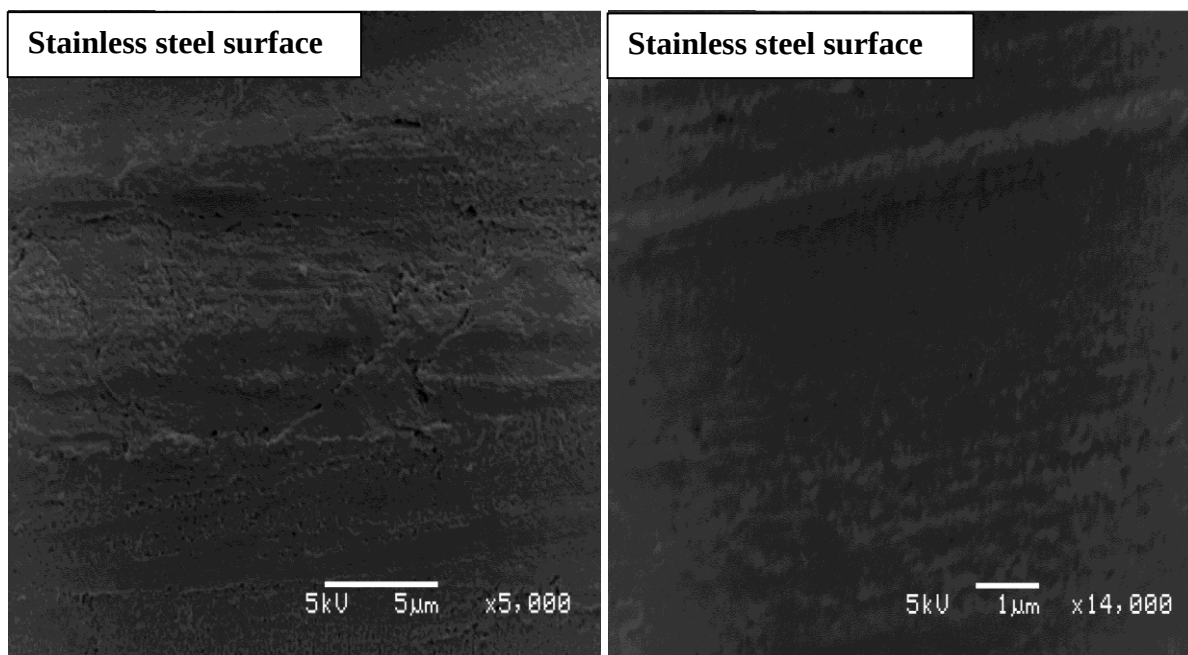


Figure 20: Sterile stainless steel strip surface (control)

SEM of *S. hominis* attached to stainless steel from the static reactor system (Figure 21a & b) showed cell aggregates of *S. hominis* were densely packed on the surface of the stainless steel strip, embedded by a layer which could be conditioning film. However, the cells of *S. hominis* also formed multilayer aggregates with small space between the intercellular networks (Figure 21a). At higher magnification of 14,000x (Figure 21b), it can be seen that a large number of aggregated bacterial cells of *S. hominis* connected together forming a thick matrix and consistent biofilm structure were observed. There were channels and pores between the aggregated bacterial cells.

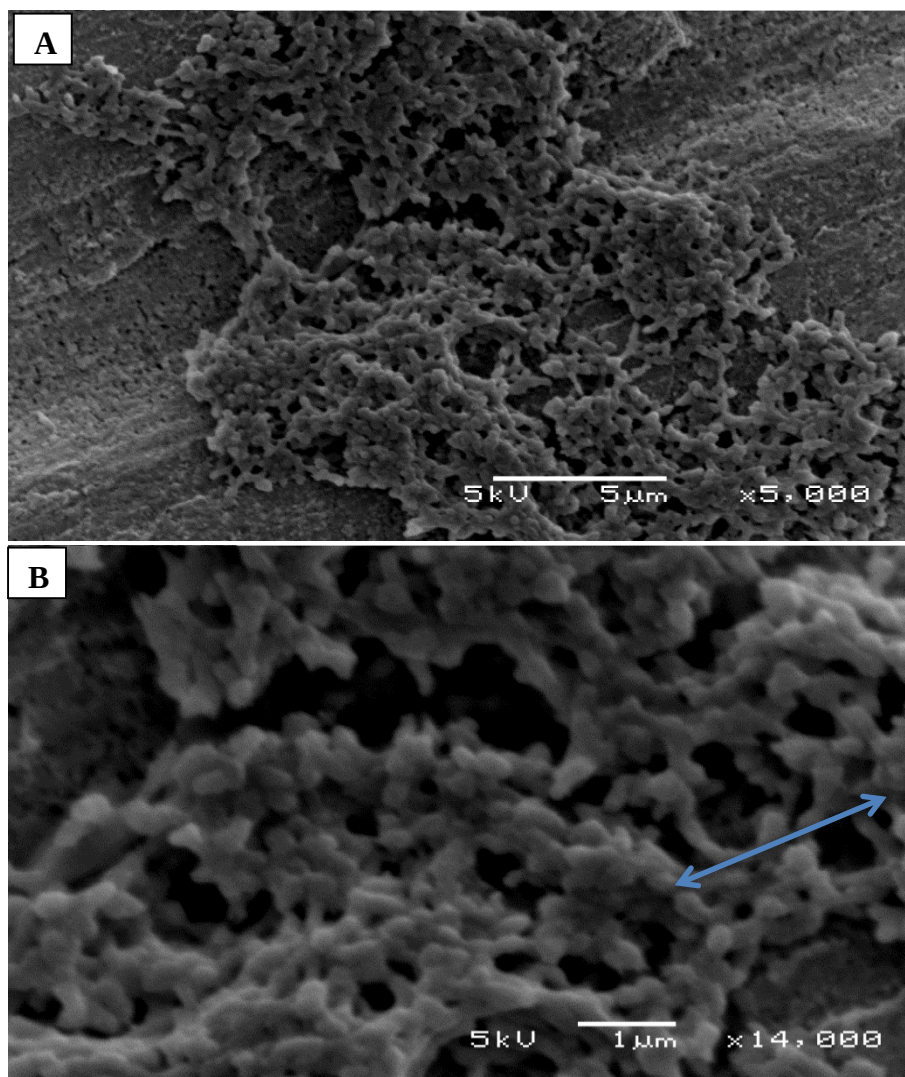


Figure 21: (A) *S. hominis* isolated from aseptic ESL filling nozzles attached on stainless steel under static reactor system after 20h of contact in skim milk at 37°C. (B) Channels and pores between the densely packed cells of *S. hominis* adhered to stainless steel and connected together forming a matrix at higher-magnifications (blue-arrows)

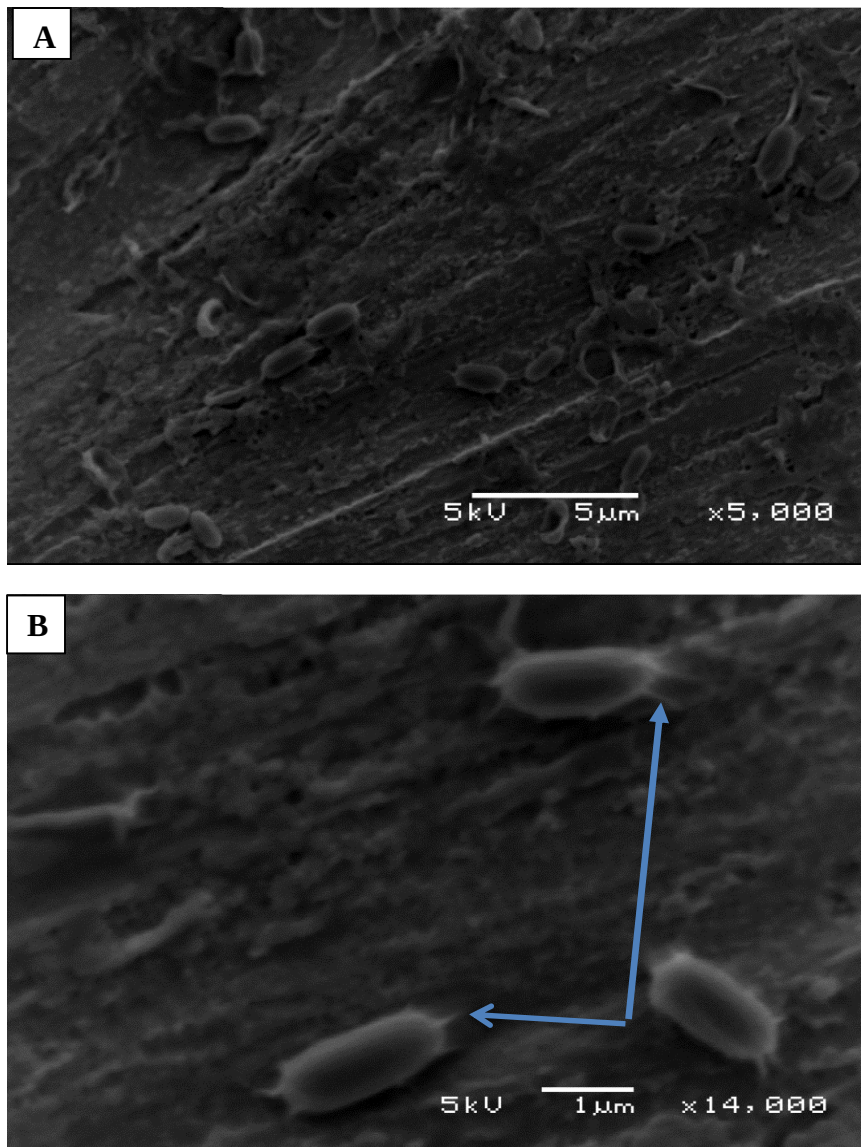


Figure 22: (A) Spores of *Paenibacillus* spp. isolated from aseptic ESL filling nozzles attached on stainless steel under static reactor system on stainless steel after 20h of contact in skim milk at 37°C. (B) Hair-like structures extending from the spore surfaces (blue-arrows)

The structures of *B. cereus*, *B. pumilus* and *Paenibacillus* spp. attached to the stainless steel were characterized by their unique features: rod shaped and hair-like appendages (Figures 22, 23 & 24). Single spores of *B. pumilus* and *Paenibacillus* spp. attached to stainless steel surfaces were observed and these spores were embedded in a layer which could be a conditioning film formed by organic molecules such as milk proteins (Figures 22 & 23). A thin white outer coat surrounding the spores of *B. pumilus* was also observed (Figure 23a). At higher magnification, hair-like structures extending from the spores of *B. pumilus* and *Paenibacillus* spp. were observed (Figures 22b & 23b).

Some of the *B. cereus* spores attached were arranged in chains and some attached as single spores attached to the stainless steel surface and there was no conditioning film covering the attached spores observed under static reactor system. Spores of *B. cereus* were surrounded by a thin white outer coat and this coat might be exosporium. Hair-like structures extending from the spores of *B. cereus* were observed (Figure 24). Cells of *A. junii* were also embedded by a layer which could be a conditioning film formed by milk residues such as milk proteins (Figure 25).

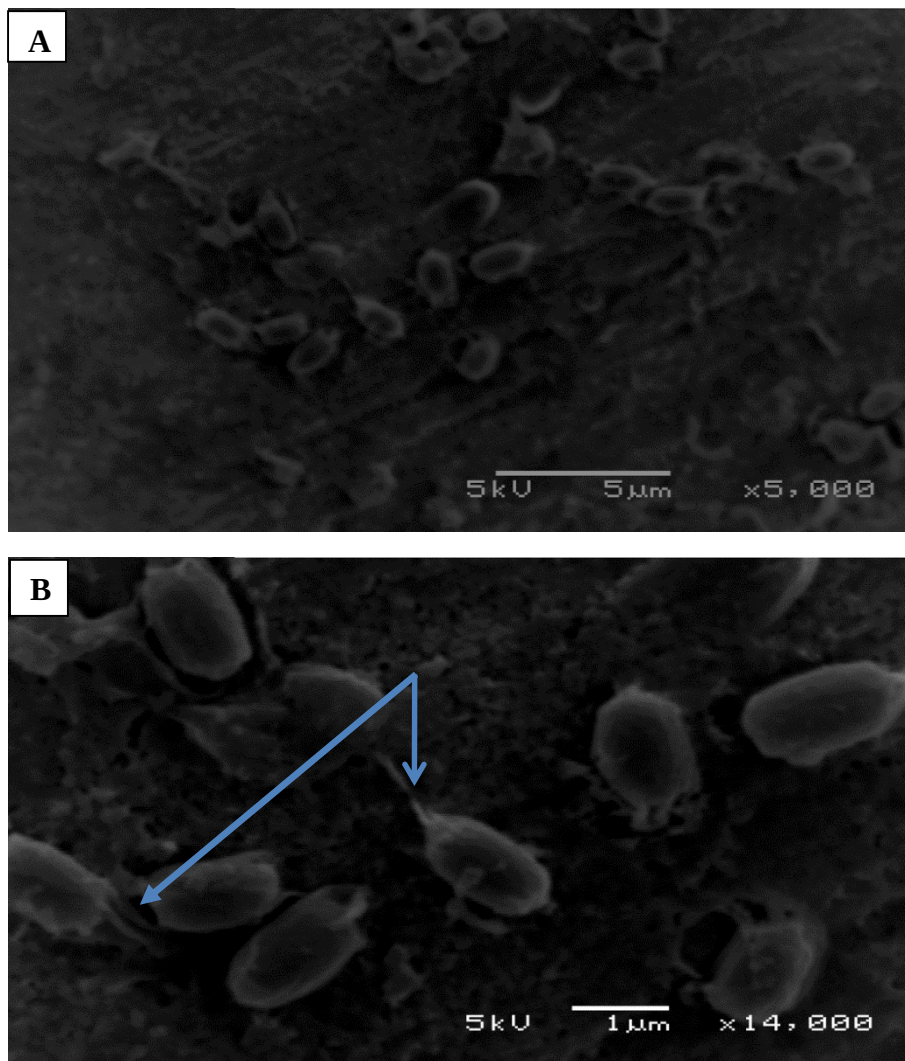


Figure 23: (A) Spores of *B. pumilus* isolated from aseptic ESL filling nozzles attached on stainless steel under static reactor system on stainless steel after 20h of contact in skim milk at 37°C. (B) Hair-like structures extending from the spore surfaces (blue-arrows)

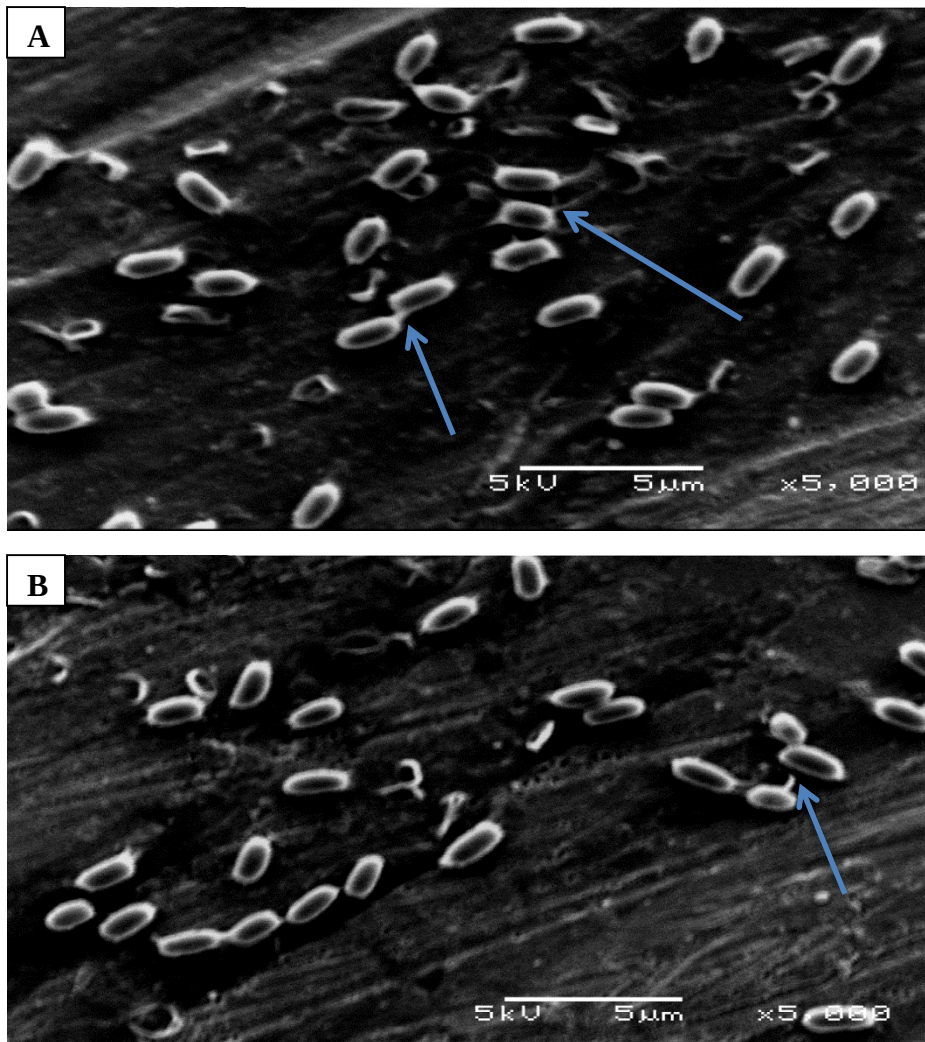


Figure 24: (A-B) Spores of *B. cereus* isolated from aseptic ESL filling nozzles attached on stainless steel under static reactor system on stainless steel after 20h of contact in skim milk at 37°C. Hair-like structures extending from the spores of *B. cereus* adhered on stainless steel surface (blue-arrows)

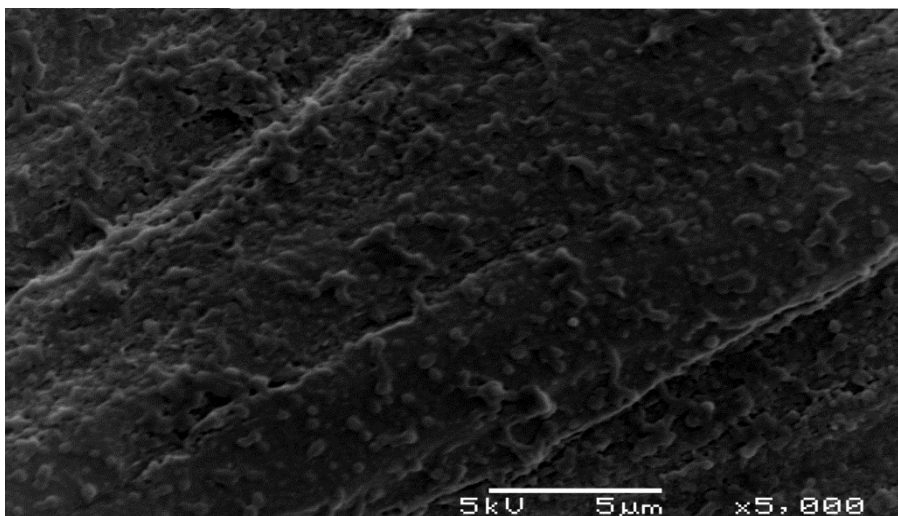


Figure 25: *A. junii* isolated from aseptic ESL filling nozzles attached on stainless steel under static reactor system on stainless steel after 20h of contact in skim milk at 37°C

CONTINUOUS FLOW REACTOR SYSTEM

A conditioning film was observed on the spores of *B. cereus* from continuous flow system (Figure 26a). Hair-like appendages on the edges of spores were observed and the core, inner membrane of the spores surrounded by exosporium was visible, which is the thin-white layer covering the *B. cereus* spores, as indicated by the arrows (Figure 26b). There was no multilayer of spores formed by *B. cereus* on the stainless steel surface. However, spores attached to the surface were widely distributed and some appeared in pairs with visible connection between them.

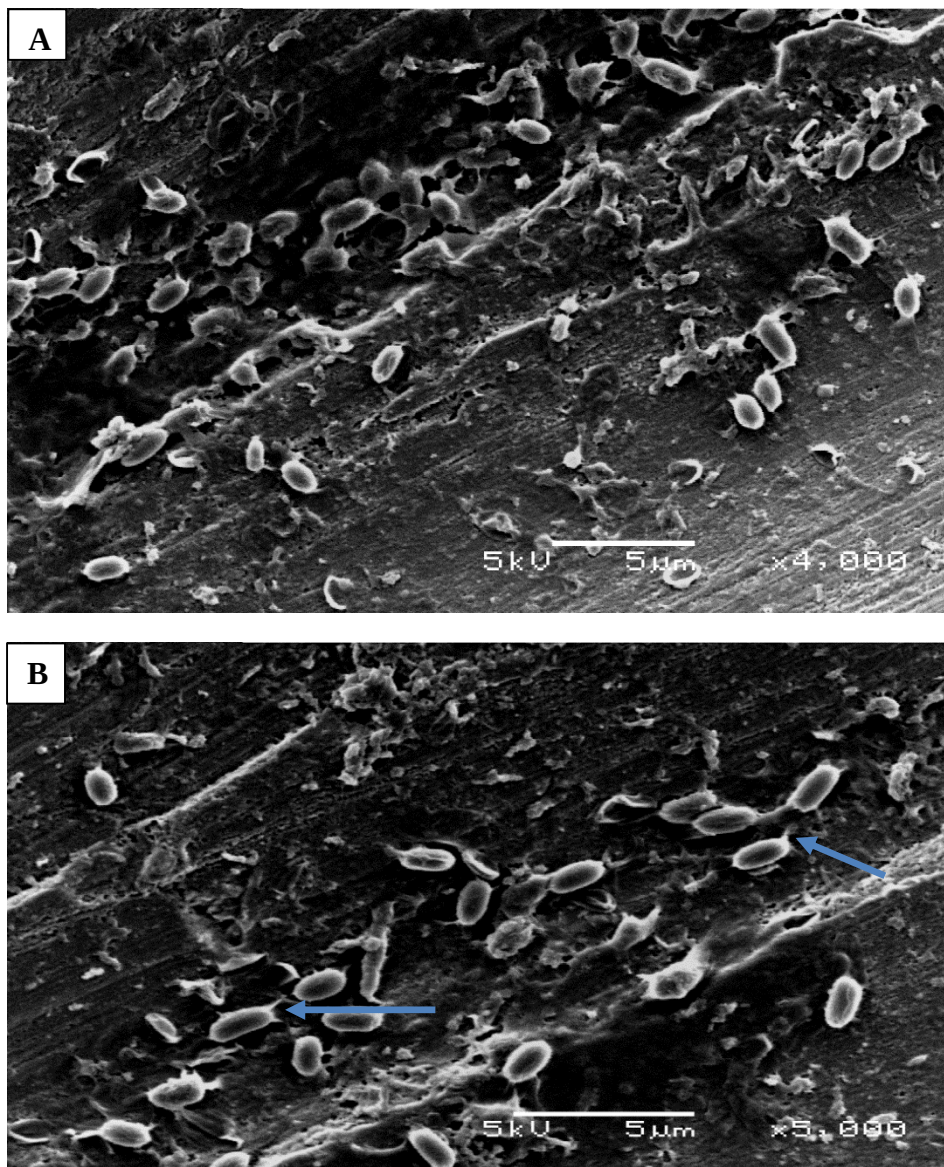


Figure 26: (A) Spores of *B. cereus* isolated from aseptic ESL filling nozzles attached on stainless steel under continuous flow system on stainless steel after 20h of contact in skim milk at 37°C. (B) Hair-like structures extending from the spore surfaces (blue-arrows)

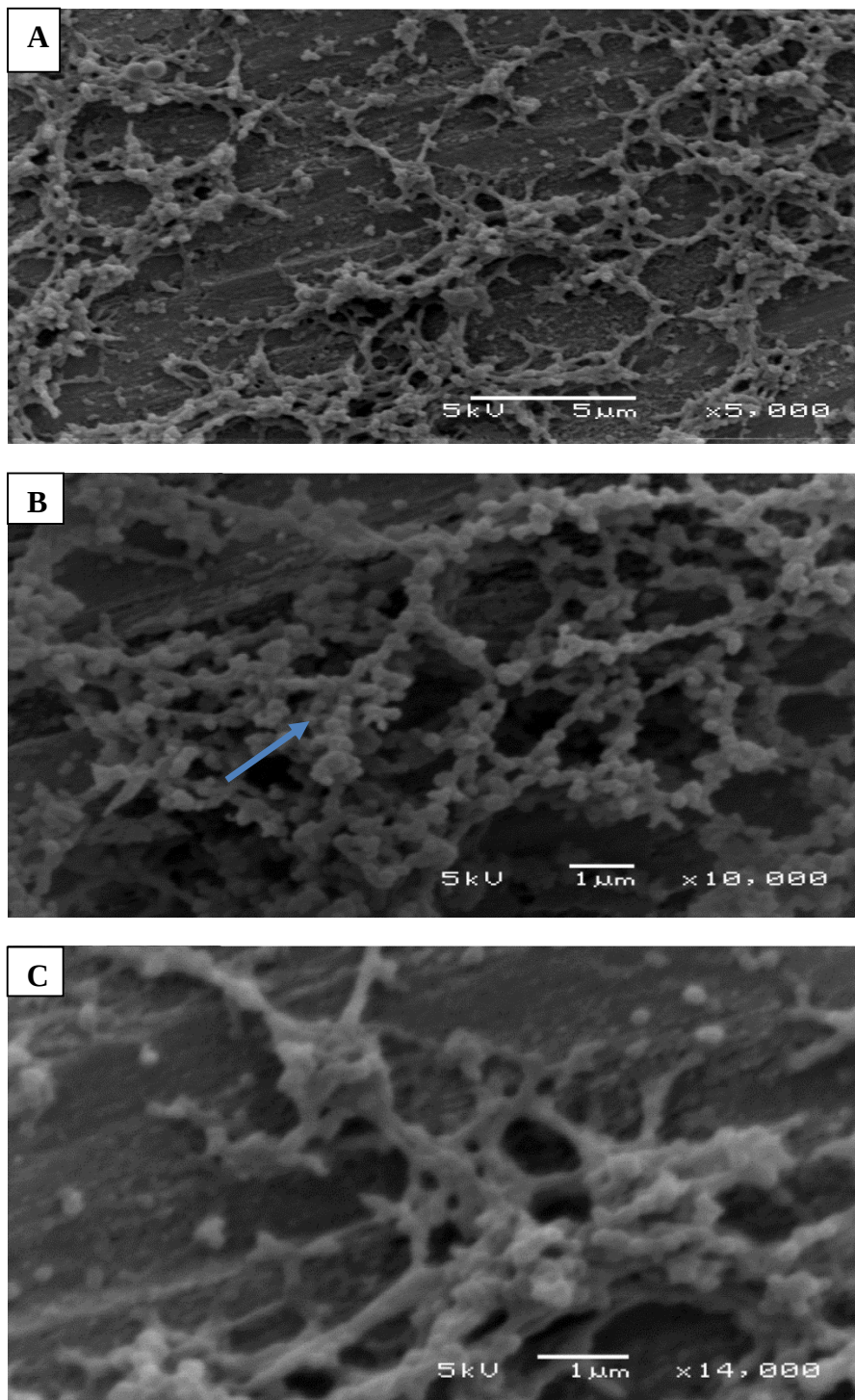


Figure 27: (A) *S. epidermidis* isolated from aseptic ESL filling nozzles attached on stainless steel under continuous flow system on stainless steel after 20h of contact in skim milk at 37°C. Clustered cells connected together forming a matrix (blue-arrow). (B-C) Cells of *S. epidermidis* adhered on stainless steel at higher-magnifications

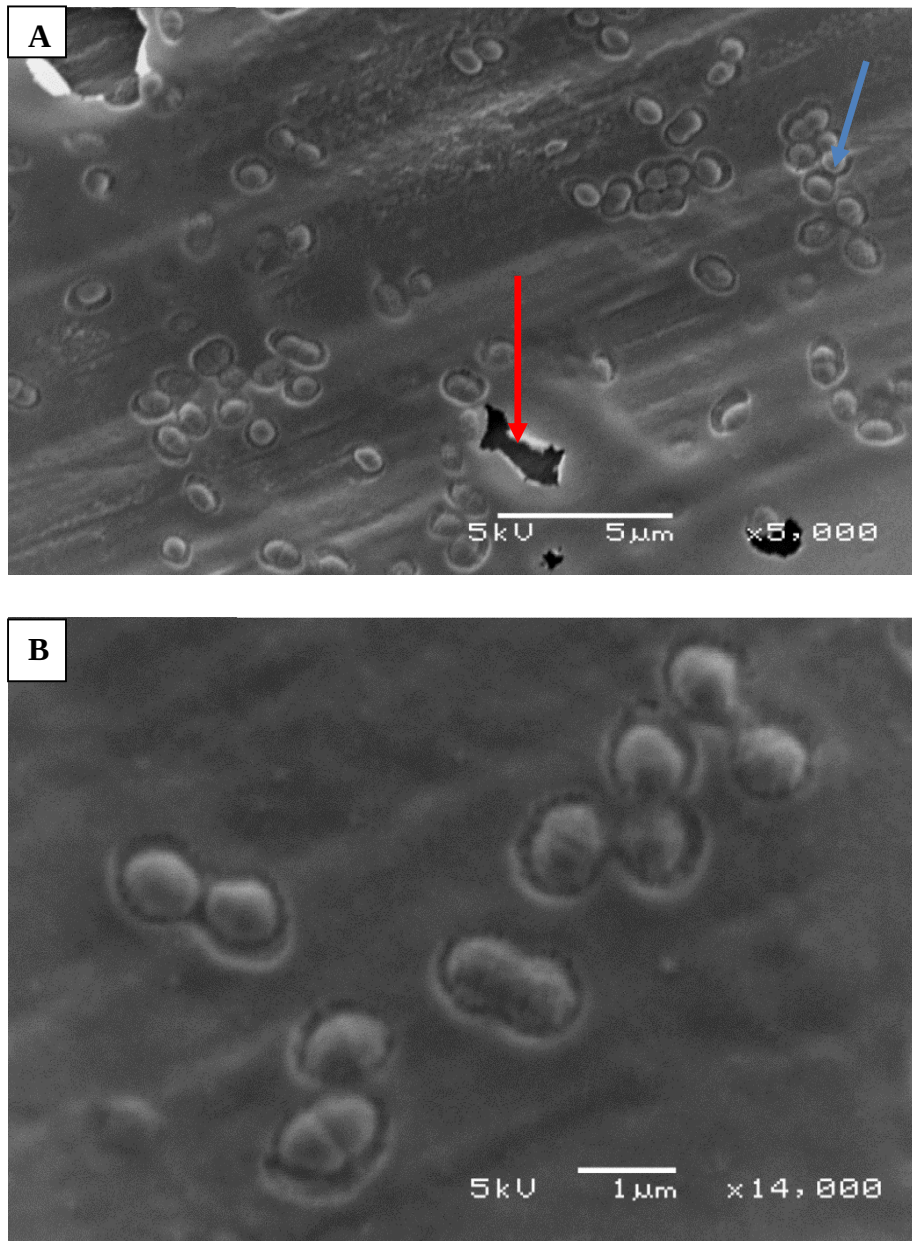


Figure 28: (A) *M. luteus* isolated from aseptic ESL filling nozzles attached on stainless steel under continuous flow system on stainless steel after 20h of contact in skim milk at 37°C. Clustered cells covered by a layer (blue-arrow) and a broken layer covering the cells revealing the surface of stainless steel (red-arrow). (B) Cells of *M. luteus* adhered on stainless steel at higher magnification.

Cells of *S. epidermidis* formed a thin and consistent multilayer of cells attached on the surface of stainless steel and large spaces between the intercellular networks was observed (Figure 27a). The aggregated cells of *S. epidermidis* formed a wall of cells with spaces between the networks resembling the basic honeycomb structure (Figure 27b). However, at higher magnification of 14,000x conditioning film connecting the cells of *S. epidermidis* was observed (Figure 27c).

SEM of cells of *M. luteus* and the surface of stainless steel strips were embedded by a conditioning film from the continuous flow system. The distribution of single and clustered surface-adhered cells of *M. luteus* and a broken conditioning film was observed (Figure 28a).

3.3.4 DISCUSSION

The variations noted in the degree of hydrophobicity of the bacterial strains in this study may have been an indicative of differences in physicochemical and morphological characteristics of the bacterial strains used in this study. It has been demonstrated that the physiochemical surface properties of microorganisms differ between Gram-negative and Gram-positive bacteria (Bellon-Fontaine *et al.*, 1996; Abdulla *et al.*, 2014). It is well known that bacterial cell walls differ and it is composed of complex molecules, in this regard, the isolates may have possessed different morphological properties, which may have played a role in the adhesion of the isolates to hydrocarbons leading to the variations in the degree of hydrophobicity.

Spores of *B. cereus* and *Paenibacillus* spp. were noted to have a high affinity for xylene, a polar solvent, demonstrating hydrophobic nature of these spores and also for chloroform, an electron acceptor, which means these spores were strong electron donors. These spores showed the highest hydrophobicity for both solvents, this can be attributed to their fundamental surface structures which may have enabled them to adhere strongly to the hydrocarbons and probably due to their similar physico-chemical properties such as appendages and exosporium which may have contributed to the hydrophobic nature of these spores. Similar results were observed by (Simmonds *et al.*, 2006; Marchand *et al.*, 2012), they attributed the hydrophobicity to the presence of exosporia which makes the spore very adhesive to hydrophobic surfaces.

It has been demonstrated by (Ronner *et al.*, 1990; Flint *et al.*, 2001; Marchand *et al.*, 2012) that the appendages extending from the surfaces of *Bacillus* spores enhance adhesion by overcoming the electrostatic repulsion forces. These hair-like structures of spores noted in this study could have facilitated the attachment of these spores to the stainless steel strips. This suggests that the appendages observed in this study and hydrophobic nature of the spores could have enhanced the attachment of *B. cereus*, *B. pumilus* and *Paenibacillus* spp.

It can be clearly seen from SEM images that the isolates were entrapped in a layer (Dhillon, 2012), this layer could be a conditioning layer formed by the organic molecules such as proteins from milk. It has been demonstrated that a conditioning layer is formed on a stainless steel

surface within 5 to 10 seconds on coming onto direct contact with milk (Mittelman, 1998; Dhillon, 2012). Therefore the formation of the conditioning layers entrapping the tested bacterial strains, may suggest that milk constituents may have played a role in the formation layers noted in this study. A higher attachment of *Bacillus* spp. to stainless steel strips coated with skim milk foulant was reported by Flint *et al.* (2001), Palmer (2008) and Dhillon (2012). The layers on stainless steel strips observed in this study could be conditioning layers formed after milk was brought into contact with the stainless steel surfaces.

The isolates of *S. epidermidis* and *S. hominis* isolated from the aseptic ESL filling nozzles showed the ability to attach and form biofilms on stainless steel strips and these bacteria showed similar biofilm developments in both continuous and static reactor methods. The SEM images of these isolates showed extensive aggregated bacterial cells forming biofilms on the stainless steel strips. It has previously been demonstrated by (Dunne, 2002; Ha, Chung and Ryoo, 2005; Michu, Cervinkova, Babak, Kyrova and Jaglic, 2011), that the more extensive adherence and biofilm formation on stainless steel by *S. epidermidis* was enhanced by the *icaA* operon. The large adherent aggregates of *S. epidermidis* with multilayers cell clusters noted in this current study could possibly be explained by the findings observed by Dunne (2002) and Ha *et al.* (2005). However, *S. epidermidis* and *S. hominis* also shown to possess hydrophobic properties.

From the above research, there is a link between hydrophobicity and adhesion capacity of the tested isolates. Furthermore, the isolates originating from the nozzles of aseptic ESL filling machines are capable of attaching and forming biofilm on stainless steel strips. The ability of these isolates to attach and form biofilm on stainless steel strip suggests that there might be a multispecies bacterial community adhered on the aseptic ESL filling nozzles, which might be the main source of contamination of ESL milk products. It is not surprising that the isolates of *B. cereus*, *B. subtilis*, *B. pumilus* and *S. epidermidis* demonstrated an ability to attach and form biofilms on stainless steel strip as noted in this study, as these isolates have been isolated from pasteurisers, filling machines and packed ESL milk products (Eneroth *et al.*, 2000; Mugadza and Buys, 2013).

3.3.5 CONCLUSION

This study demonstrated attachment and biofilm formation by the isolates originating from the aseptic ESL filling nozzles. The ability of the isolates to attach and form biofilm on the stainless steel strips may reflect that there might be biofilms formed on the aseptic ESL filling nozzles and ultimately these isolates may act as a potential source of bacterial contamination that may lead to spoilage of ESL milk products. There's limited information available on the rate of adhesion to hydrocarbons for *Paenibacillus* spp. isolated from the aseptic ESL filling nozzles. There's a need to understand the interactions involved in adhesion of the isolates to hydrocarbons and stainless steel surfaces. This information would be useful when designing dairy processing lines in such a way that the risk of colonization by bacteria is minimized or eliminated.

CHAPTER 4: GENERAL DISCUSSION

4.1 CRITICAL REVIEW OF METHODOLOGY

This study set out to isolate and characterise the biofilm associated with the nozzles of aseptic ESL filling machines. Additionally, the study aimed to determine whether isolates originating from the nozzles of aseptic ESL filling machines can attach and form biofilms on stainless steel strips. Swab samples were collected in duplicate from the nozzles of aseptic filling machines.

Swabbing method was used to detect biofilms from the nozzles of aseptic ESL filling machines and the recovered cells were subsequently plated on nutrient agar. The plating method provided some difficulties due to the fact that it required large amount of media and several days to detect the biofilms associated with aseptic filling nozzles. Techniques used to confirm biofilms problem are often inadequate in that these tests rely on the removal of bacteria from site by swabbing and bacterial enumeration by traditional dilution and plating techniques (Flint, 1998). Furthermore, there is a need for rapid detection methods to provide the dairy industry with a quick assessment of the hygiene of their manufacturing plant. These methods will also prevent transmission of diseases and hence minimising economic losses. There is no practical method for quantitative determination of biofilm microorganisms in the industry environment (Chmielewski and Frank, 2003). However, swab and sponge sampling provide useful information on the extent of microbial growth on a surface and on the extent to which cleaning has been effective. Techniques that show promise for the detection of bacteria in biofilms involve the detection of bacterial ATP, protein, or polysaccharide on surfaces or in water flushed through the lines (Flint, Bremer and Brooks, 1997). Such rapid techniques will assist in monitoring the effectiveness of cleaning procedures (Flint *et al.*, 1997; Flint, 1998). However, more detailed analyses should be conducted to prove that there are biofilms formed on the nozzles of aseptic ESL filling machines. Therefore a rapid method for biofilms detection in the dairy processing lines is crucial in order to be able to react fast and adequately to the arising microbial problems in the ESL milk and dairy products.

The use of MALDI Biotyper 3.0 software (Bruker Daltonics) may have helped identifying the isolates during the study hence saving time and other resources. Nonetheless some of the isolates originating from the aseptic ESL filling nozzles were not identified by MALDI Biotyper, this could be attributed to the fact that there were no reference spectra in the library

and hence they could not be identified. Furthermore, a wider range of bacterial strains should be investigated in order to establish a standard database for further identification. The isolates were identified as *Bacillus cereus*, *B. pumilus*, *Paenibacillus* spp., *S. epidermidis*, *S. hominis*, *M. luteus*, *A. junii* and *A. castelli*. The use of genotypic methods such as molecular methods involving PCR can be useful to confirm the isolates hence providing accurate and reliable results. The results can be obtained in shorter times as in a day such as in real-time multiplex PCR (de Boer, Ott, Kesztyus, Kooistra-Smid, 2010). Additionally, molecular methods give reliable, high throughput, reproducible and specific results (Lindstrom, Keto, Markkula, Nevas, Hielm and Korkeala, 2001; Klein, 2002). Besides, the molecular characterisation of the selected isolates using a PCR based method should be used to verify whether the aseptic ESL filling nozzles are the major source of ESL milk contamination.

The use of BATH as a means of determining cell surface hydrophobicity of the isolates may have provided more conclusive information relating to the hydrophobicity characteristics of the isolates. However, much debate has existed as to which is the best method to measure bacterial surface hydrophobicity (Palmer, Flint and Brooks, 2007; Sedlackova, Cerovsky, Horsakova and Voldrich, 2011).

The growth of biofilms in different conditions (temperature, aeration, turbulent/laminar flow and different milk types) may vary and requires investigation (Flint *et al.*, 2001). The experimental conditions such as the diameter of the tubing, vacuum pump, water-bath temperature, surface of the substratum and the isolates could have significantly altered the results noted on the laboratory biofilm reactor system used to evaluate the ability of the isolates to attach and form biofilm on the stainless steel strips and the ability of the isolates to attach to hydrocarbons even though the standardised protocols were used in this research. It is worth keeping in mind that different researchers use different bacteria and different methods in determining the surface hydrophobicity and bacterial attachment which may result in conflicting results (Hood and Zottola, 1995; Brooks and Flint, 2008; Dhillon, 2012). Therefore the bacterial attachment to hydrocarbons and stainless steel strips noted in this study may have been influenced by the experimental conditions.

4.2 RESEARCH FINDINGS AND FUTURE WORK

Adhesion of microorganisms to stainless steel surfaces used in the dairy industry, can result in contamination of ESL milk and milk products when the cleaning process in a dairy manufacturing plant is ineffective. Additionally, stainless steel surfaces have a tendency to attract bacteria that can develop into biofilms. The main objective of this study was to determine the ability of isolates originating from the nozzles of aseptic ESL filling machines to attach to stainless steel.

This study demonstrated that the isolates originating from the nozzles of aseptic ESL filling machines can attach on the stainless steel strips. Additionally, appendages were observed in spores of *B. cereus*, *B. pumilus* and *Paenibacillus* spp. It may be that these appendages could be associated with the ability of these spores to attach to stainless steel surfaces. These isolates could potentially form layers of spores on the stainless steel pipe surfaces making it difficult for cleaning detergents to penetrate during cleaning of the stainless steel pipes. An alternative explanation for the presence of the non-spore forming microorganisms isolated on the nozzles of aseptic ESL filling machines post cleaning process could be their intrinsic ability to withstand cleaning and disinfection process through acquired resistance, due to the presence of *Bacillus* spp., which dominated the community of multispecies adhered to the nozzles.

Studies have shown that spores of *B. cereus* adhere better to hydrophobic than hydrophilic surfaces (Simmonds *et.al*, 2003; Marchand *et.al*, 2012). It has been found that spores of *B. cereus* adhere to stainless steel surfaces found in dairy processing plants by increasing the hydrophobicity of the spore surface (Husmark and Ronner, 1990). In this study the degree of hydrophobicity and the presence of appendages have been demonstrated for *B. cereus*, *B. pumilus* and *Paenibacillus* spp. Additionally, as far as we could assess, this is the first study to demonstrate the degree of hydrophobicity and attachment of *Paenibacillus* spp. to stainless steel surfaces. Attached spores may lead to the formation of biofilms, thereby becoming more resistant to sanitizers. These biofilms are a menace in the dairy industry because the capacity of bacteria to form biofilms also contributes to the persistence of bacteria, as the secreted polymeric matrix provides them with protection from the surrounding environment, thus from cleansing agents (Cleto *et al.*, 2012). There is a need to determine the effect of combining enzymes, antibiotics and chemical agents on the bacteria present in the aseptic ESL filling

nozzles. Such information would help in designing specific cleaning procedures to target the spores of *Bacillus* spp. present in the dairy processing lines.

From the cluster analysis of *B. pumilus*, *Paenibacillus* spp. and *B. cereus*, it was found that these spores isolated from the nozzles of aseptic ESL filling machines were related to the spores isolated from the final packaged ESL milk product. It is not surprising that these spores were isolated from the nozzles of the ESL filling machines and the packaged ESL milk products, since it is unlikely that the presence of spores *Bacillus* spp. in the milk production and processing environment can be prevented. Previous studies found preliminary evidence for the persistence of *Paenibacillus* and *Bacillus* in dairy processing plants. Thus, contamination of fluid milk with endospore-forming spoilage microorganisms may also occur at the processing plant, either pre or post pasteurisation (Svennson, Eneroth, Brendehaug and Christiansson, 1999; Huck, Hammond, Murphy, Woodcock and Boor, 2007).

In recent years, the dairy industry progress towards extending the shelf life of conventionally pasteurised milk products. The presence of spore forming microorganisms in the dairy processing lines, which are capable of surviving pasteurisation, attaching to stainless steel surfaces and growing at refrigeration temperatures might be the hurdle limiting the extension of shelf life beyond 14 days. This study clearly showed that spore forming bacteria, particularly *B. cereus* and *Paenibacillus* spp. are present on the nozzles of aseptic ESL filling machines and they are capable of attaching to the stainless steel surfaces. These spore-forming bacteria might lead to poor keeping quality of ESL milk products. Studies have identified *Paenibacillus* spp. as an important contributor to pasteurised milk spoilage (Huck *et al.*, 2007). Ultimately, further extension of the shelf life of pasteurised milk products will require elimination of spore forming microorganisms. This study suggests that nozzles of ESL filling machines are likely to be the main source of the identified microorganisms in the final packaged ESL milk products. Therefore control of these microorganisms represents a considerable challenge that will require a comprehensive cleaning approach of stainless steel surfaces in the dairy industry.

The presence of *B. cereus* in packaged ESL milk may have the potential to cause an adverse health effect upon consumption of ESL milk products, considering that the end user of ESL milk consume the product containing $>10^5$ of *B. cereus* ml⁻¹ due to temperature abuse of packaged ESL milk. However, if the packaged ESL milk can be stored under proper storage conditions $<6^{\circ}\text{C}$ and consumed within the expiry date, the levels of *B. cereus* in ESL milk will

in general not exceed 10^5 /ml. On the contrary, factors that may contribute to the low rate of *B. cereus* infection and intoxication are, visible spoilage occurs before numbers are sufficiently high to cause problems, unsuitable conditions for growth and toxin production in milk (Te Giffet and Breumer, 1998).

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

Species of *S. hominis*, *S. epidermidis*, *M. luteus* and *Anaerococcus* spp. were isolated from the nozzles of aseptic ESL filling machines after CIP treatment, indicating that they might be resistant to heat treatment and cleaning chemical agents. Furthermore, *S. epidermidis*, *B.cereus*, *Paenibacillus* spp. were also found to be present in the packaged ESL milk products. Their presence in the final ESL milk products indicate that they might have gained entry into the final product through the nozzles of ESL filling machines. Spores of *Bacillus* and *Paenibacillus* spp. were shown to have appendages which may be involved in their attachment to stainless steel pipe surfaces resulting in post contamination of ESL milk products. With the potential of the appendages resulting in attachment to surfaces, it may be the main reason why it is so difficult to eradicate spores from the processing pipes. Therefore, we can say that *B. cereus* and *M. luteus* detached from the nozzles of aseptic filling machines and contaminated the final milk product as it passed through the filling machines. Our findings indicated that *B. cereus*, *S. epidermidis* and *M. luteus* can attach and form biofilm on stainless steel surfaces, indicating that these strains can be the main source of contamination of ESL milk.

5.2 RECOMMENDATIONS

- A further study would be interesting to determine the effect of combining enzymes with current cleaning chemicals on the bacterial biofilms adhered to stainless steel surfaces and also determine the actual mechanism of bacterial adhesion to stainless steel surfaces this may assist in formulating improved cleaning methods to control development of biofilm and prevention of cross contamination of dairy products.
- Further studies need to be performed to observe biofilm formation using model systems that closely simulate the dairy processing environment and also characterise the identified isolates using a PCR-RAPD based method to verify whether the nozzles of aseptic ESL filling machines are really the major source of ESL milk contamination.

CHAPTER 6: REFERENCES

- ABDULLA, A. A., ABED, T. A. AND SAEED, A. M. 2014. Adhesion, autoaggregation and hydrophobicity of six *Lactobacillus* strains. *British Microbiology Research Journal* **4**, 381-391.
- ANDERSSON, A., RONNER, U AND GRANUM, P. E. 1995. What problems does the food industry have with the spore-forming pathogens *Bacillus cereus* and *Clostridium perfringens*?. *International Journal of Food Microbiology* **28**, 145-155.
- ANSARI, I. A. AND DATTA, A. K. 2003. An overview of sterilization methods for packaging materials used in aseptic packaging systems. *Institution of Chemical Engineers* **81**, 57-65.
- AUGUSTIN, M., ALI-VEHMAS, T. AND ATROSHI, F. 2004. Assessment of enzymatic cleaning agents and disinfectants against bacterial biofilms. *Journal of Pharmaceutical Sciences* **18**, 55-64.
- AUSTIN, J.W. AND BERGERON, G. 1995. Development of bacterial biofilms in dairy processing lines. *Journal of Dairy Research* **62**, 509-519.
- BARNES, L.M., LO, M.F., ADAMS, M.R. AND CHAMBERLAIN, A.H.L. 1999. Effect of milk proteins on adhesion of bacteria to stainless steel surfaces. *Applied and Environmental Microbiology* **10**, 4543-4548.
- BASSON, L., FLEMMING, A. AND CHENIA, H. Y. 2008. Evaluation of adherence, hydrophobicity, aggregation characteristics and biofilm development of *Flavobacterium johnsoniae*-like isolates from South African aquaculture systems. *Microbial Ecology* **55**, 1-14.
- BELLON-FONTAINE, M. N., RAULT, J. AND VAN-OSS, C. J. 1996. Microbial adhesion to solvents: a novel method to determine the electron-donor/electron-acceptor or Lewis acid-base properties of microbial cells. *Colloid Surface B* **7**, 47-53.
- BREMER, P. J., FILLERY, S. AND MCQUILLAN, A. J. 2006. Laboratory-scale clean-in-place (CIP) studies on the effectiveness of different caustic and acid wash steps on the removal of dairy biofilms. *International Journal of Food Microbiology* **106**, 254-262.

BREMER, P., FLINT, S., BROOKS, J. AND PALMER, J. 2015. Introduction to Biofilms: Definition and Basic Concepts. In Teh, K. H., Flint, S., Brooks, J. and Knight, G. (ed.) Biofilms in the dairy industry. John Wiley and Sons, Chichester, UK. 5p.

BRIANDET, R., HERRY, J. AND BELLON-FONTAINE, M. 2001. Determination of the van der Waals, electron donor and electron acceptor surface tension components of static Gram-positive microbial biofilms. *Colloids and Surfaces B: Biointerfaces* **21**, 299-310.

BROOKS, J.D. AND FLINT, S.H. 2008. Biofilms in the food industry: problems and potential solutions. *International Journal of Food Science and Technology* **43**, 2163-2176.

BUNT, C. R., JONES, D. S. AND TUCKER, I. G. 1993. The effects of pH, ionic strength and organic phase on the bacterial adhesion to hydrocarbons (BATH) test. *International Journal of Pharmaceutics* **99**, 93-98.

BUSSCHER, H. J., VAN DER BELT-GRITTER, B. AND VAN DER MEI, H. C. 1995. Implications of microbial adhesion to hydrocarbons for evaluating cell surface hydrophobicity. 1. Zeta potentials of hydrocarbon droplets. *Colloids Surface B Biointerfaces* **5**, 111-116.

BUSZEWSKI, B., DZIUBAKIEWICZ, E., POMASTOWSKI, P., HRYNKIEWICZ, K., PŁOSZAJ- PYREK, J., **KRAMER, M., TALIK, E. AND ALBERT, K.** 2015. Assignment of functional groups in Gram-positive bacteria. *Journal of Analytical and Bioanalytical Techniques* **6**, 232-300.

BUYS, E. 2001. Good hygiene practices for extended shelf-life milk. South African Society of Dairy Technology: Dairy Symposium, Gordons Bay, Cape Province. 1-5.

CAIXETA, D. S., SCARPA, T. H., BRUGNERA, D. F., FREIRE, D. O., ALVES, E., DE ABREU, L. R. AND PICCOLI, R.H. 2012. Chemical sanitizers to control biofilms formed by two *Pseudomonas* species on stainless steel surface. *Food Science and Technology* **32**, 142-150.

CARLSON, B. 1998. Food processing equipment-Historical and modern designs. In: *Aseptic Processing and Packaging of Food* (ed. J.R.D. David, R.H. Graves and V.R. Carlson), CRC Press, Boca Raton, FL. Chavez, M., Negri. 143p.

CHAU, T. T., BRUCKARD, W. J., KOH, P. T. L. AND NGUYEN, A. V. 2009. A review of factors that affect contact angle and implications for flotation practice. *Advances in Colloid and Interface Science* **150**, 106-115.

CHMIELEWSKI, R. N. A. AND FRANK, J. K. 2003. Biofilm formation and control in food processing facilities. *Comprehensive Reviews in Food Science and Food Safety* **2**, 22-32.

CITAK, S., YUCEL, N. AND MENDI, A. 2005. Antibiotic resistance of enterococcal isolates in raw milk. *Journal of Food processing and Preservation* **29**, 183-195.

CLETO, S., MATOS, S., KLUSKENS, L. AND VIEIRA, M. J. 2012. Characterization of Contaminants from a Sanitized Milk Processing Plant. PLoS ONE 7(6): e40189. doi:10.1371/journal.pone.0040189 [Available online, accessed June 2015]

COSTERTON, J. W., LEWANDOWSKI, Z., CALDWELL, D. E., KORBER, D. R. AND LAPPINSCOTT, H. M. 1995. Microbial biofilms. *Annual Reviews of Microbiology* **49**, 711-745.

COSTERTON, J. W., STEWART, P. S. AND GREENBERG, E. P. 1999. Bacterial biofilms: a common cause of persistent infections. *Science* **284**, 1318-1322.

CROMIE, S. J. 1991. Microbiological aspects of extended shelf life products. *Australian Journal of Dairy Technology* **2**, 101-104.

DARDIR, H. A., ABA-ALKHAIL, N. A. AND ABDEL-ALL, A. A. A. 2011. Safety evaluation of enterococcal strains from dairy products and clinical samples using RT-PCR. *World Journal of Dairy and Food Science* **6**, 234-240.

DE BOER, R. F., OTT, A., KESZTYUS, B. AND KOOISTRA-SMID, A. M. D. 2010. Improved detection of five major gastrointestinal pathogens by use of a molecular screening approach. *Journal of Clinical Microbiology* **48**, 4140-4146.

DEPARTMENT OF HEALTH (DOH). 2002. Foodstuffs, Cosmetics and Disinfectant Act and Regulations. Act No. 54 of 1972, G.N.R. 1555/1997. Johannesburg, South Africa.

DHILLON, M. 2012. The effect of silver ion-implantation of stainless steel on bacterial adhesion and biofilm formation. MSc (Microbiology) dissertation: New Zealand. Massey University. 33-55p.

DHILLON, G. S. AND KAUR, S. 2014. Facile fabrication and characterization of chitosan-based zinc oxide nanoparticles and evaluation of their antimicrobial and anti-biofilm activity. *International Nano Letters* **4**, 107-118.

DOGAN B. AND BOOR, K. J. 2003. Genetic diversity and spoilage potentials among *Pseudomonas* spp. isolated from fluid milk products and dairy processing plants. *Applied and Environmental Microbiology* **69**, 130-135.

DOYLE, R. 2000. Contribution of the hydrophobic effect to microbial infection. *Microbiological Infections* **2**, 391–400.

DUMALISILE, P. 2004. Impact of processing temperatures on survival of microbial contaminants from pasteurized milk. MSc (Food Science) dissertation: Stellenbosch University of Stellenbosch. 2p.

DUNNE, W. M. 2002. Bacterial adhesion: Seen any good biofilms lately?. *Clinical Microbiological Reviews* **15**, 155-166.

ELDER, M.J., STAPLETON, F., EVANS, E. AND DART, J. K. 1995. Biofilm-related infections in ophthalmology. *Eye* (London, England) **9**, 102-111.

ELHARIRY, H. M. 2008. Biofilm formation by endospore-forming *Bacillus* on plastic surface under some food-related and environmental stress conditions. *Global Journal of Biotechnology and Biochemistry* **3**, 69-78.

ENEROTH, A., SVENSSON, B., MOLIN, G. AND CHRISTIANSSON, A. 2000. Contamination of pasteurized milk by *B. cereus* in the filling machine. [Available on-line: www.ncbi.nlm.nih.gov/pubmed/115043 , accessed 15 March 2015]

ENEROTH, A., AHRNE, S. AND MOLIN, G. 2000. Contamination routes of Gram-negative spoilage bacteria in the production of pasteurised milk, evaluated by randomly amplified polymorphic DNA (RAPD). *International Dairy Journal* **10**, 325-331.

FACCIA, M., MASTROMATTEO, M., CONTE, A., DEL NOBILE, M.A. 2013. Influence of the milk bactofugation and natural whey culture on the microbiological and physico-chemical characteristics of mozzarella cheese. *Journal of Food Process Technology* **4**, 218-224.

FALLIE, C., C. JULLIEN, F. FONTAINE, M. N. BELLON-FONTAINE, C. SLOMIANNY, AND T. BENEZECH. 2002. Adhesion of *Bacillus* spores and *Escherichia coli* cells to inert surfaces: role of surface hydrophobicity. *Canadian Journal of Microbiology* **48**, 728–738.

FITZGERALD, L. E. 2012. The application of microfiltration as a partial sterilisation technique for the reduction of psychrotrophic spore forming bacteria from viscous dairy feeds. PhD (Chemical Engineering) thesis: Bath University of Bath. 16p.

FLINT, S. H. 1998. Formation and control of biofilms of thermo-resistant Streptococci of stainless steel. PhD (Food Technology) thesis: New Zealand Massey University. 5p.

FLINT, S. H., BREMER, P.J. AND BROOKS, J. D. 1997. Biofilms in dairy manufacturing plants – description, current concerns and method of control. *Biofouling* **11**, 81-97.

FLINT, S. H., PALMER, J., BLOEMEN, K., BROOKS, J. AND CRAWFORD, R. 2001. The growth of *Bacillus stearothermophilus* on stainless steel. *Journal of Applied Microbiology* **90**, 151-157.

FLINT, S., BROOKS, J. D., ELZEN, H. V. AND BREMER, P. J. 1997. Biofilms in dairy manufacturing plant - a threat to product quality. *Food Technology* **27**, 61- 64.

FRANZ, C.M.A.P., HOLZAPFEL, W.H. AND STILES, M.E. 1999. *Enterococci* at the crossroads of food safety? *International Journal of Food Microbiology* **47**, 1–24.

GIMENEZ-PEREIRA, M. L. 2005. Enterococci in milk products. MSc (Veterinary Studies) dissertation: New Zealand. Massey University. 75p.

GRAUMANN, P. AND MARAHIEL, M. 1996. Some like it cold: response of microorganisms to cold shock. *Arch Microbiology* **166**, 293-300.

HA, K. Y., CHUNG, Y. G. AND RYOO, S. J. 2005. Adherence and biofilm formation of *Staphylococcus epidermidis* and *Mycobacterium tuberculosis* on various spinal implants. *Spine (Phila Pa 1976)* **30**, 38-43.

HARIMAWAN, A., ZHONG S., LIM, C. T. AND TING, Y. P. 2013. Adhesion of *B. subtilis* spores and vegetative cells onto stainless steel--DLVO theories and AFM spectroscopy. *Journal of Colloids Interface Science* **405**, 233-241.

HEIKENS, E., FLEER, A., PAAUW, A., FLORIJN, A., AND FLUIT, A. C. 2005. Comparison of genotypic and phenotypic methods for species-level identification of clinical isolates of coagulase-negative staphylococci. *Journal of Clinical Microbiology* **43**, 2286-2290.

HENYON, D.K. 1996. Extended shelf life milks in North America: a perspective. *International Journal of Dairy Technology* **52**, 95- 101.

HEYNDRIX, M. 2011. The importance of endospore-forming bacteria originating from soil for contamination of industrial processing. *Applied and Environmental Soil Science*, Article ID 56197, 1-11.

HOOD, S.K. AND ZOTTOLA, E.A. 1995. Biofilms in food processing. *Food Control* **6**, 9-18.
HUCK, J. R., HAMMOND, B. H., MURPHY, S. C., WOODCOCK, N. H. AND BOOR, K J. 2007. Tracking spore-forming bacterial contaminants in fluid milk-processing systems 90, 4872-4883.

HUSMARK, U. AND RONNER, U. 1990. Forces involved in adhesion of *Bacillus cereus* spores to solid surfaces under different environmental conditions. *Journal of Applied Bacteriology* **69**, 557–562.

IDF. 1994. Recommendation for the hygienic manufacturing of milk and milk based products. *Bulletin of the International Dairy Federation* No. 292, 1-32.

JANSTOVA, B. AND LUKASOVA, J. 2001. Heat resistance of *Bacillus* spp. spores isolated from cow's milk and farm environment. *Journal of Acta Veterinaria Brno* **70**, 179-184.

JEONG, D.K. AND FRANK, J. F. 1994. Growth of *Listeria monocytogenes* at 10°C in biofilms with microorganism isolated from meat and dairy processing environments. *Journal of Food Protection* **57**, 576-586.

KLEIN, D. 2002. Quantification using real time PCR technology: applications and limitations. *Trends in Molecular Medicine* **8**, 257-260.

KOKARE, C. R., CHAKRABORTY, S., KHOPADE, A. N. AND MAHADIK, K.R. 2009. Biofilm: Importance and applications. *Indian Journal of Biotechnology* **8**, 159-168.

KROLASIK, J., ZAKOWSKA, Z., KREPSKA, M. AND KLIMEK, L. 2010. Resistance of bacterial biofilms formed on stainless steel surface to disinfecting agent. *Polish Journal of Microbiology* **59**, 281-287.

LESLIE, A. 2011. Preventing biofilm formation using microbes and their enzymes. *Basic biotechnology* **7**, 6-11.

LEWIS M.J. AND DEETH, H.C. 2009. *Heat treatment of milk*. A.Y. Tamime (Ed.), *Milk Processing and Quality Management*, Blackwell Publishing Ltd., Chichester, UK. 168-204p.

LINDSAY, D., BROZEL, V. S., MOSTERT, J. F. AND VON HOLY, A. V. 2000. Physiology of dairy associated *Bacillus* spp. over a wide pH range. *International Journal of Food Microbiology* **54**, 49-62.

LINDSAY, D. AND VON HOLY, A. 2006. "What food safety professionals should know about bacterial biofilms". *British Food Journal* **108**, 27-37.

LINDSTROM, M., KETO, R., MARKKULA, A., NEVAS, M., HIELM, S. AND KORKEALA, H. 2001. Multiplex PRC assay for detection and identification of *Clostridium botulinum* types A, B, E and F in food and faecal material. *Applied and Environmental Microbiology* **67**, 5694-5699.

LU, T. K AND COLLINS, J. J. 2007. Dispersing biofilms with engineered enzymatic bacteriophage. *Proceedings of the National Academy of Science* 104, 11197-11202.

MALEK, F., MOUSA-BOUDJEMAA, B., KHOUANI-YOUSFI, F., KALAI, A. AND KIHIL, M. 2012. Microflora of biofilm on Algerian dairy processing lines: An approach to improve microbial quality of pasteurized milk. *African Journal of Microbiology Research* **17**, 3836-3844.

MATTILA, T., MANNINEN, M. AND KYLASIUIROLA, A. L. 1990. Effect of cleaning-in-place disinfectants on wild bacterial strains isolated from a milking line. *Journal of Dairy Research*. **57**, 33-39.

MARCHAND, S., DE BLOCK, J., DE JONGHE, V., COOREVITS, A., HEYNDRIKX, M. AND HERMAN, L. 2012. Biofilm formation in milk production and processing environments; influence on milk quality and safety. *Comprehensive Reviews in Food Science and Food Safety* **11**, 133-147.

MAYR, R., GUTSER, K., BUSSE, M. AND SEILER, H. 2004. Indigenous aerobic spore-formers in high heat treated (127°C for 5 s) German ESL (Extended Shelf Life) milk. *Milk Science International* **59**, 143-146.

MCAULEY, C. M., GOBIUS, K. S., BRITZ, M. L. AND CRAVEN, H. M. 2012. Heat resistance of thermotolerant *Enterococci* isolated from milk. *International Journal of Food Microbiology* **154**, 162-168.

MICHU, E., CERVINKOVA, D., BABAK, V., KYROVA, K. AND JAGLIC, Z. 2011. Biofilm formation on stainless steel by *Staphylococcus epidermidis* in milk and influence of glucose and sodium chloride on the development of *ica*-mediated biofilms. *International Dairy Journal* **21**, 179-184.

MINH, H. N. T., DANTIGNY, P., PERRIER-CORNET, J. AND GERVAIS, P. 2010. Germination and inactivation of *B. subtilis* spores induced by moderate hydrostatic pressure. *Biotechnology and Bioengineering* **107**, 876-883.

MITTELMAN, M. 1998. Structure and functional characteristics of bacterial biofilms in fluid processing operations. *Journal of Dairy Science* **81**, 249-253.

MOGHA, K. V., SHAH, N. P., PRAJAPATI, J. B. AND CHAUDHARU, A. R. 2014. Biofilm –a threat to dairy industry. *Indian Journal of Dairy Science* **67**, 459-466.

MUGADZA, D. AND BUYS, E. 2013. Diversity of spore formers and non-sporeformers in Extended Shelf Life Milk. South African Society of Dairy Technology: Dairy Symposium, Lagoon Beach, Cape Town.

MUNSCH-ALATOSSAVA, P., GHAFAR, A. AND ALATOSSAVA, T. 2013. Potential of nitrogen gas (N₂) flushing to extend the shelf-life of cold stored pasteurized milk. *International Journal of Molecular Science* **14**, 5668-5685.

MYSZKA, K. AND CZACYK, K. 2011. Bacterial biofilms on food contact surfaces- a review. *Polish Journal of Food and Nutritional Science* **61**, 173-180.

OLIVEIRA, R. AZEREDO J., TEIXEIRA P. AND FONSECA A. P. 2001. “The role of hydrophobicity in bacterial adhesion,” In *Biofilm Community Interactions: Chance or Necessity*, (Ed.) Gilbert, I. P., Allison, D., Brading, M., Verran, J. and Walker, J. Cardiff, UK. 11-22p.

OOSTHUIZEN, M. C., B. STEYN, D. LINDSAY, V. S. BROZEL, AND VON HOLY, A. 2001. Novel method for the proteomic investigation of a dairy-associated *Bacillus cereus* biofilm. *FEMS Microbiology letters* **194**, 47-51.

ORTEGA, M. P., HAGIWARA, T., WATANABE, H. AND SAKIYAMA, T. 2008. Factors Affecting Adhesion of *Staphylococcus epidermidis* to Stainless Steel Surface. *Japan Journal of Food Engineering* **9**, 251-259.

PALMER, J., FLINT, S., AND BROOKS, J. 2007. Bacterial cell attachment, the beginning of a biofilm. *Journal of Industrial Microbiology and Biotechnology* **34**, 577–588.

PALMER, J., SEALE, B. AND FLINT, S. 2015. Significance of Bacterial Attachment: A Focus on the Food Industry. In Teh, K. H., Flint, S., Brooks, J. and Knight, G. (ed.) *Biofilms in the dairy industry*. John Wiley and Sons, Chichester, UK. 21-28p.

PEMBREY, R. S., MARSHALL, K. C. AND SCHNEIDER, R. P. 1999. Cell surface analysis technique: What do cell preparation protocols do to cell surface properties? *Applied and Environmental Microbiology* **65**, 2877-2894.

PENG, J. S., W. C. TSAI, AND C. C. CHOU. 2002. Inactivation and removal of *Bacillus cereus* by sanitizer and detergent. *International Journal of Food Microbiology* **77**, 11–18.

PETRUS, R. R., FREIRE, M. T., SETOGUTE, L. C. AND HIGAJO, V. M. 2011. Effect of pasteurisation temperature on aseptically filled milk shelf-life. *Journal of Food and Nutrition* **22**, 531-538.

POULSEN, V. L. 1999. Microbial biofilm in food processing. *Food Science and Technology* **32**, 321-326.

PRETORIUS, J. M. 2010. Identification and characterization of genes involved in *Bacillus cereus* biofilm formation. MSc (Microbiology) dissertation: Pretoria. University of Pretoria. 7p.

PRUTHI, V. AND CAMEOTRA, S. S. 1997. Short communication of a biosurfactant exhibiting excellent emulsification and surface active properties by *Serratia marcescens*. *World Journal of Microbiology and Biotechnology* **13**, 133-135.

KAPLAN, J. B. 2010. Biofilm dispersal: mechanisms, clinical implications, and potential therapeutic uses. *Journal of Dental Research* **89**, 205-218.

KUMAR, C. G. AND ANAND, S. K. 1998. Significance of microbial biofilms in food industry: A Review. *International Journal of Food Microbiology* **42**, 9-27.

- REID, G., BIALOWSKA-HOBRZANSKA, H., VAN DER MEI, H. C. AND BUSSCHER, H. J. 1999. Correlation between genetic, physic-chemical surface characteristics and adhesion of four strains of *Lactobacillus*. *Colloids and Surfaces B: Biointerfaces* **13**, 75-81.
- ROBINSON, R. K. 2005. Dairy microbiology: The microbiology of milk and milk products, 3rd Ed. John Wileys and Sons. New York. 95p.
- RONNER, U., HUSMARK, U. AND HENRIKSSON, A. 1990. Adhesion of *Bacillus* spores in relation to hydrophobicity. *Journal of Applied Bacteriology* **69**, 550-556.
- RYSSTAD, G. AND KOSTAD, J. 2006. Extended shelf life milk—advances in technology. *International Journal of Dairy Technology* **59**, 58-96.
- SALUSTIANO, V.C., ANDRADE N.J., SOARES, N.F.F., LIMA, J.C., BERNARDES, P.C., LUIZ, L.M.P. AND FERNANDES, P.E. 2009. Contamination of milk with *Bacillus cereus* by post-pasteurisation surface exposure as evaluated by automated ribotyping. *Food Control* **20**, 439-442.
- SARRO, M., GARCIA A. AND MORENO, D. 2005. Biofilm formation in spent nuclear fuel pools and bioremediation of radioactive water. *International Microbiology* **8**, 223-230.
- SCHMIDT, V. S.J., KAUFMANN, V., KULOZIK, U., SCHERER, S., WENNING M. 2012. Microbial biodiversity, quality and shelf life of microfiltered and pasteurized extended shelf life (ESL) milk from Germany, Austria and Switzerland. *International Journal of Food Microbiology* **2**, 1-9.
- SEALE, B., BREMER, P., FLINT, S., BROOKS, J. AND PALMER, J. 2015. Overview of the problems resulting from biofilm contamination in the dairy industry. In Teh, K. H., Flint, S., Brooks, J. and Knight, G. (ed.) *Biofilms in the dairy industry*. John Wiley and Sons, Chichester, UK. 50p.
- SEDLACKOVA, P., CEROVSKY, M., HORSAKOVA, I. AND VOLDRICH, M. 2011. Cell Surface Characteristic of *Asaia bogorensis* – Spoilage Microorganism of Bottled Water. *Czech Journal of Food Science* **29**, 457-461.

SEN, R. 2010. *Biosurfactants*. Lands bioscience, Texas, USA. 9-10p.

SHARMA, M. AND ANAND, S. K. 2002. Characterization of constitutive microflora of biofilms in dairy processing lines. *Food Microbiology* **19**, 627–636.

SHARMA, M., ANAND, S.K. AND PRASAD, D. N. 2003. *In vitro* propagation of mixed species biofilms using online consortia for dairy processing lines. *Milk Science International* **58**, 270-273.

SIMMONDS, P., MOSSEL. B. L., INTARAPHAN, T. AND DEETH, H. C. 2003. Heat resistance of *Bacillus* spores when adhere to stainless steel and its relationship to spore hydrophobicity. *Journal of Food Protection* **66**, 2070-2075.

SIMOES, M., SIMOES, L. C., MACHADO, I., PEREIRA, M. O. AND VIEIRA, M. J. 2006. Control of flow-generated biofilms with surfactants evidence of resistance and recovery. *Food and Bioproducts Processing* **84**, 338–345.

SIMOES, M., SIMOES, L. C. AND VIEIRA, M. J. 2010. A review of current and emergent biofilm control strategies. *LWT-Food Science and Technology* **43**, 573-583.

STOODLEY, P., SAUER, K., DAVIES, D. G. AND COSTERTON, J. W. 2002. Biofilms as complex differentiated communities. *Annual Reviews of Microbiology* **56**, 187-209.

SUTTON, G. H. C. 2004. Enumeration of total airborne bacteria, yeast and mold contaminants and identification of *Escherichia coli* O157:H7, *Listeria* spp., *Salmonella* spp., and *Staphylococcus* spp. in a beef and pork slaughter facility. PhD (Food and Agricultural Science) thesis: Florida, University of Florida. 53p.

SVENSSON, B., ENEROTH, A., BRENDEHAUG J. AND CHRISTIANSSON, A. 1999. Investigation of *Bacillus cereus* contamination sites in a dairy plant with RAPD-PCR. *International Dairy Journal* **9**, 903-912.

TE GIFFET, M. C. AND BEUMER, R. R. 1998. *Bacillus cereus*: A Review. *The Journal of Food Technology in Africa* **4**, 7-13.

TEH, K. H., FLINT, S. AND FRENCH, N. 2010. Biofilm formation by *Campylobacter jejuni* in controlled mixed-microbial populations. *International Journal of Food Microbiology* **143**, 118-124.

THOMAS, A. AND PRASAD, V. 2012. Thermotolerant bacteria in milk - A review. *International Journal of science and Research* **3**, 2438-2442.

VALIK, L., GORNER, F. AND LAUKOVA, D. 2003. Growth of *Bacillus cereus* and shelf life of pasteurised milk. *Czech Journal of Food Science* **21**, 195-202.

VAN HOUTT, R. AND MICHIELS, C. W. 2010. Biofilm formation and the food industry, a focus on the bacterial outer surface. *Journal of Applied Microbiology* **109**, 1-15.

VAN OSS, C. J. AND GIESE, R. F. 1995. The hydrophilicity and hydrophobicity of clay minerals. *Clays and Clay Minerals* **43**, 474-477.

VLKOVA, H., BABAK, V., SEYDLOVA, R., PAVLIK, I. AND SCHEGELOVA, J. 2008. Biofilms and hygiene on dairy farms and in the dairy industry: sanitation chemical products and their effectiveness on biofilms – a review. *Czech Journal of Food Science* **26**, 309-323.

VON BOCKELMANN, B. AND VON BOCKELMANN, I. 1998. Aseptic packaging. *Long-Life Products: Heat treated, Aseptically Packed: A Guide to Quality*, Bernhard von Bockelmann Publisher, A° karp, Swede. 9 -13p.

WALTER, V., SYLDATK, C. AND HAUSMANN, R. 2010. Screening concepts for the isolation of biosurfactant producing microorganisms. *Advances in Experimental Medicine and Biology* **672**, 1-13.

WIEDMANN, M., WEILMEIER, D., DINEEN, S. S., RALYEA, R., AND BOOR, K. J. 2000. Molecular and phenotypic characterization of *Pseudomonas* spp. isolated from milk. *Applied Environmental Microbiology* **66**, 2085-2095.

WIJMAN, J. G. E., DE LEEUW, P. L. A., MOEZELAAR, R., ZWIETERING, M. H. AND ABEE, T. 2007. Air-Liquid Interface Biofilms of *Bacillus cereus*: Formation, Sporulation, and Dispersion. *Applied and Environmental Microbiology* **73**, 1481-1488.

XU, H., ZOU, Y. AND LEE, H. 2010. Effect of NaCl on the biofilm formation by foodborne pathogens. *Journal of Food Science* **75**, 580-585.

ZOTTOLA, E. A. 1994. Microbial attachment and biofilm formation: a new problem for the food industry. *Food Technology* **48**, 107-114.