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# Studying Microflora of Semi-hard Cheese, and Sporulation within Contact Surface Biofilms

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# STUDYING MICROFLORA OF SEMI-HARD CHEESE, AND SPORULATION WITHIN CONTACT SURFACE BIOFILMS

By

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This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not comply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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This thesis is dedicated to my mother Aishah Mazi, my father Ishaq Khan, my siblings Shahd, Ahmad, Layan, and especially my oldest sister Abrar Khan who keeps pushing us forward. All my hard work here is a partial payback for what my family has done and their support. I always want them to be proud of me and I am hoping they will always be.

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CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ATCC | American type culture collection |
| BHI | Brain heart infusion |
| CFU | Colony forming units |
| CIP | Clean-in-place |
| EPS | Exopolysaccharides |
| EPSs | Extracellular polymeric substances |
| LAB | Lactic acid bacteria |
| LBS | Lactobacillus selective agar |
| MALDI-TOF | Matrix Assisted Laser Desorption Ionization mass spectrometry - Time of technique |
| MRS | Man, Rogosa, and Sharpe agar |
| NFDM | Non-fat dry milk |
| NSLAB | Non-starter lactic acid bacteria |
| PCA | Plate count agar |
| PBS | Phosphate buffer saline |
| RCM | Reinforced clostridial agar |
| SC | Spore counts |
| SEM | Scanning Electron Microscopy |
| SDSU | South Dakota State University |
| SPC | Standard plate count |
| SP | Spore |
| SS | Stainless steel |
| TVC | Total viable count |

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**Figure 7**: Scanning electron micrographs of *Bacillus sporothermodurans* DSMZ 10599$^{T}$ and its spores within biofilm formed on stainless steel coupons. a) Vegetative cells in 12 hours old biofilm. b) Spores in 12 hours old biofilm.

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# ABSTRACT

## STUDYING MICROFLORA OF SEMI-HARD CHEESE, AND SPORULATION WITHIN CONTACT SURFACE BIOFILMS

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The first study was conducted on commercial Italian semi-hard cheese samples that were analyzed microbiologically to understand the effect of fast and slow rate of cooling on cheese microflora during ripening process at different temperatures. A cheese plant noticed an active growth of non-starter lactic acid bacteria, especially the heterofermentative bacteria, in ripened cheese blocks, which weren't fully cooled. For that, three cheese sample sets, each having 8 samples, were received from the cheese plant for microbiological analysis. The first set included a group of fast cooled cheese samples to 38°F, and the other group was slow cooled to 50°F. The second set was cooled as the first set but further ripened for 2 months at either 38°F or 50°F, while the third set was ripened for 6 months after cooling. We were targeting the desired and undesired non-starter lactic acid bacteria (NSLAB), the total aerobic viable count for mesophiles / thermophiles, the thermoduric mesophiles / thermophiles, gas producers, *Leuconostoc* spp., *Lactobacillus wasatchensis*, and *Clostridium spp*. Non-selective and different types of selective media were used in order to enumerate and isolate each type of microorganism. The media were incubated aerobically or anaerobically at different temperatures depending on the targeted isolate's optimum growth conditions. Random colonies on the selective media were picked and sent to another analytical laboratory for further analysis using Matrix Assisted Laser Desorption Ionization mass spectrometry -

Time of flight technique MALDI-TOF. The total aerobic counts showed, in general, a declining trend during 6 months of ripening. Initial NSLAB counts in the 12 days old samples cooled to 38°F started at 5.87±0.13 logs CFU/g, and 6.19±0.13 logs CFU/g in the samples cooled to 50°F, and the two were significantly different. The fast-cooled samples thus showed lower counts than the slow cooled samples. According to MALDI-TOF identification, the added adjunct cultures were dominating in the whole study, which were *Pediococcus acidilactici, Lactobacillus paracasei,* and *Lactobacillus rhamnosus*. None of the samples had any of the *Clostridium* spp., undesired *Lactobacillus* spp. or *Leuconostoc* spp. Our results suggest that if accelerated ripening at 50°F is the goal, the rate of cooling to 38°F or 50°F will not make a difference from the bacterial count aspect in the presence of *Pediococcus acidilactici* as adjunct. However, bacterial distribution in cheese due to different rate of cooling might matter the most.

The second study was aimed to understand the sporulation within biofilm matrix that formed on stainless steel surface by common sporeformers in the dairy industry. Undisturbed biofilm works as a reservoir for spore forming contaminants that might produce spores within the biofilm matrix itself. The results of this study showed that the duration of the biofilm formation and sporulation is variable among the different species and their strains. A strain of *B. licheniformis* ATCC®6634™ formed biofilm within 4 hours, but formed no spores even after an extended duration of incubation up to 21 days. However, the second strain of *G. stearothermophilus* ATCC® 15952™ not only formed biofilms in 4 hours, but also sporulated within the biofilm matrix in 48 hours. On the other hand, *B. licheniformis* ATCC®14580™ took 7 days to produce spores within the NFDM and the biofilm

Keeping in mind the application of our research for future projects, the first study was conducted with cheese samples that contained high levels of *Pediococcus acidilactici* as a part of the adjunct cultures, which did not allow true predominance to emerge. It may be more useful to conduct this study without added *Pediococcus*, so that actual microbial interactions in cheese matrix could show up. The second study provided a proof of concept on the sporulation behavior of sporeformers within biofilm matrix. This would be useful to design cleaning strategies for resilient biofilms formed on plate heat exchangers and other equipment in dairy plants.

# INTRODUCTION

Cheese making process is a long procedure that includes many steps, starts with pasteurization of milk and ends with cooling of the cheese curds for ripening. The range or rate of cooling is regulated by the type of the cheese, and its characteristics (Tamime, 2011). Decreasing the temperature of cooked cheese helps to maintain fat crystallization that gives cheese its texture (Fox *et al*., 2004), especially if the cheese was fully and properly cooled. It also affects the growth of the starter lactic acid bacteria, added non-starter lactic acid bacteria (NSLAB), and the adjunct cultures. The major purpose of adding NSLAB to the milk cheese is accentuating the cheese flavor by enhancing proteolysis and other biochemical reactions. In addition, NSLAB could be part of the adjuncts culture, which is a mixture of certain microorganisms added for improving the final cheese products, especially when added to the milk during the cheese making process (El Soda et al. 2000). Ripening the cheese is an essential step that helps determining the cheese quality. The humidity, temperature level, and duration during the ripening process are critical in inducing the starter and non-starter lactic acid bacteria's biological activities (Wehr and Frank, 2004). Increasing the historical ripening temperature could decrease the ripening duration by accelerating the proteolysis, which is the most significant reaction that accelerates ripening and enhance the cheese flavor (Law, 1999; Fox, 2004). However, uncontrolled elevated temperature may lead to increase the risk of microbial spoilage (Fox et al. 1996). In addition, if we accelerated the proteolysis by elevated temperature, all other biochemical reactions are equally accelerated, which can result in an unbalanced flavor, and off-flavored cheese (Fox et al. 2000). Elevated temperatures besides other factors can enhance the contaminants growth,

or change the bacterial counts, which could cause cheese slits. In fact, maintaining the microbial content of cheese could be difficult sometimes, especially when external factors are hard to control. The examples of cheese defects by microorganisms are late blowing of cheese, early blowing of cheese, cracks, and off-flavor. The objective of this study was to understand the effect of the rate of cooling and ripening temperatures on the NSLAB counts and their distribution within cheese during the ripening process.

The sporeformers and their spores can be isolated from raw milk and dairy products such as dried milk powders. Composition of dried milk inhibits the vegetative cells growth due to the low water activity (≤0.3) (Wewala, 1990), which helps in the preservation of dried milk powder. However, reconstituting this milk is the start point of germination of the dormant spores that survived the milk processing. Yet, dormant spores are not the only source of milk contamination; the reconstitute could be another source of contamination. The adherence of spoilage bacteria in the dairy plants is a major concern for dairy products cross contamination, since they can create contamination that leads to lowering the shelf life or cause defects in the final products. The presence of contaminants that adhered to the processing lines will lead to early or late defects in the final products by releasing vegetative cells or germinating spores that were embedded within the biofilm. The spore-former bacteria form biofilm mostly in the heat exchange, evaporator, and the downstream of pasteurized milk (Sharma and Anand, 2002), which make these places the major source of contaminants (Seale *et al*., 2008). Even though Cleaning In Place (CIP) systems run frequently in dairy plants, the spores' ability to resist harsh conditions like chemicals, temperatures, surrounding stress, low water activity, and high acidity or alkaline, allow them to survive (Sharma and Anand, 2002, Wong, 1998,

Miller et al., 2015). Unremoved biofilm in hard to clean areas could be comprised of spores and sporeformers that germinate to vegetative cells, once the environment is suitable (Anand et al, 2014, Setlow, 2003). The objective of this study was to understand the formation of biofilms and sporulation within the biofilm matrix by three dairy sporeformers; *Geobacillus stearothermophilus, Bacillus licheniformis,* and *Bacillus sporothermodurans*.

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# CHAPTER 1

## LITERATURE REVIEW

### PART I: The influence of the rate of cooling and ripening temperatures on semi-hard Italian cheese microflora

Cheese making process varies from one manufacturer to another, but the basic steps of the typical cheese making processes remain the same. The process requires high quality of the incoming raw milk. Since the raw milk is the source of contaminants, it is pasteurized at either low temperature (63°C) for long time (30 minutes) or at high temperature (72°C) for short time (Rysstad and Kolstad, 2006, Khanal et al., 2014) to destroy the pathogens, molds, and yeast, too. Later, milk transfers through stainless steel pipes to a centrifuge that separates solids in the milk and filter them (filtration). During this step, milk protein and fat are tested for standardizing the milk. After that, milk is sent to troughs and the rennet and starter cultures are added. The rennet is a mixture of enzymes that curdle the milk protein. There are two types of rennet, commercial/ microbial rennet or animal rennet (chymosin). The animal rennet is extracted from the mammal's stomach while the commercial one is made of genetically modified fungi or bacteria which is the most used one within the cheese industry. Moreover, the added starter cultures are combination of different microorganisms that are selected depending on the cheese variety. These starter cultures control curdling the milk during the cheese making process, and converting lactose to lactic acid that give cheese its unique flavor and characteristics. However, starter culture involvement in producing flavors is not as prominent as nonstarter cultures. The common composition of the starter cultures is the lactic acid bacteria such as *Lactococcus* spp. and *Lactobacillus* spp. (Crow *et al*., 1995).

The addition of rennet and starter cultures, curdle the milk and separated it into semi-solid curd and liquid whey. Later, the extracted whey is drained, and curd is cut, stirred, cooked, cut into smaller pieces, and lastly salted. Fresh cheese would be ready after this step but, aged cheese need further processing. For aging cheese, the curd is transferred and pressed into molds that give the cheese its shape. The cheese may be removed from the mold and coated with wax, bacteria, oil or water to help the cheese to further ripen and uphold the flavor. In a controlled room environment, with consistent temperature and humidity, cheese molds are stored for ripening. The ripening temperatures and the duration are controlled based on the type of cheese and the sharpness of its flavor that cheese makers seek.

The ripening process is a very complicated biochemical procedure that includes chemical and physical changes of the cheese between the times of the curd processing until getting final desired cheese characteristics. The development of microbial action during the cheese ripening enhances and accentuates the cheese flavors.

There are many factors that play a role during cheese ripening, first, the physical factors such as the ripening temperature that ranges between 6-18˚C, humidity, and ripening duration that varies between days to several years. Secondly, the chemical factors that include rennet and its enzymes. Finally, the microbiological factors that include the starter lactic acid bacteria SLAB, non-starter lactic acid bacteria NSLAB -originate from the industry environment or as milk contaminants- molds, and yeasts (Fox *et al*., 1996; Tamime, 2011). The ripening process comprises of different biochemical reactions such as glycolysis, which breaks glucose to lactose and produces lactic acid. This reaction also known as lactose fermentation and it contributes in cheese flavor. The second

biochemical reaction is lipolysis that breaks down the fats and lipids to get free fatty acids and contribute to the cheese quality. The most critical reaction during ripening, which influences texture and flavor, is proteolysis, which breakdowns the protein to amino acids. The concentration of free amino acids influences the strength of the cheese flavor later.

***Role of lactic acid bacteria in cheese making***

Using lactic acid bacteria is essential in cheese making process. In fact, starter bacteria plays the main role in the formation of small peptides and amino acids that develop cheese flavor (Smit at al., 2005) by metabolizing the protein and fat. Their metabolic actions give the cheese its unique characteristics. Lactic acid bacteria breakdown proteins, produce aroma compound, and produce lactic acid by metabolizing lactose (Urbach, 1995). The process of breaking carbohydrates down in cheese into pyruvate is known as glycolysis. They also produce antimicrobials such as bacteriocins that help in preserving cheese by inhibiting the growth of undesirable microorganisms and pathogens. In fact, increasing the amount of added LAB could accelerate the cheese ripening process and give desired characteristics faster. On the other hand, high numbers may lead to bitterness and other undesirable features. There are two types of lactic acid bacteria, starter lactic acid bacteria (SLAB) and non-starter lactic acid bacteria (NSLAB), and both play a fundamental role in cheese making process. The number of non-starter lactic acid bacteria in fresh cheese is lower than starter LAB, and starts increasing during the ripening process.

***Starter lactic acid bacteria (SLAB)***

Starter lactic acid bacteria could include *Leuconostoc sp., Lactobacillus* sp., *Lactococcus* sp., and *Streptococcus* sp. Starter LAB starts the proteolysis by degrading casein into peptides and free amino acids. Starter LAB favorably contributes to protein hydrolysis into peptidase in presence of both plasmin and chymosin (Gobbetti et al., 2015). The addition of starter lactic acid bacteria could include primary and secondary SLAB depending on the cheese type. Each one of these SLAB groups contribute differently during the cheese making process. The first one improve the acidity balance and production in the cheese, balancing the pH, and produce antibiotics for contaminants (El Soda, 2000). The secondary SLAB is mostly selected for specific roles like cheese surface pigmentation and $CO_2$ production, and enzymes secretions.

***Non-starter lactic acid bacteria (NSLAB)***

The major purpose of adding NSLAB to the milk cheese is accentuating the cheese flavor by enhancing proteolysis and other biochemical reactions. The type of non-starter lactic acid bacteria in the cheese products varies between the cheese types. However, the common NSLAB species used for cheese making are mostly *Pediococci, Micrococci* and mesophilic *Lactobacilli,* (De Angelis et al., 2001), and especially *Lactobacillus sp. like Lactobacillus casei, Lactobacillus plantarum, Lactobacillus rhamnosus* and *Lactobacillus helveticus* (Law, 1997, Law, 1999). The NSLAB isolates that are found on ripened cheese are from survival of pasteurization, contaminant of industrial equipment, or added intentionally. The other factor that plays a role in the NSLAB counts is the source of them, and there are multiple sources of NSLAB in the cheese (Antonsson et al. 2001) such as added NSLAB or contaminants. According to

Crow et al (1995) that added NSLAB starts with 1-2 logs CFU/g in the first day, while El Soda et al (1999) reported that the added NSLAB counts starts with 3-4 logs CFU/g in the unripened cheddar cheese, and reach up to 9 logs CFU/g after 90 days of ripening. In ripened cheese, the NSLAB counts increase and predominate compared to SLAB (De Angelis et al., 2001). NSLAB predominant in cheese due to their ability to bear the aggressive cheese environment during the ripening process that includes a high content of salts, high acidity, low moisture, low nutrients, and low temperatures. These NSLAB's characteristics make the lipolytic and proteolytic enzymes released by NSLAB greater compared to enzymes released by SLAB when it comes to cheese flavor enhancement and texture development (Jokovic et al., 2011). Crow at al. (2001) analyzed cheddar cheese content of predominant strains in ripened New Zealand cheddar cheese. They found out that a mixture of the right adjunct NSLAB (ex. *Lb. rhamnosus* and *Lb. paracasei)* could improve the flavor and quality of aged cheddar cheese by 90%. The mechanisms of the flavor accentuating in that study were still under investigation. Interestingly, many articles discussed the important role of NSLAB in cheese, although they could be a reason of cheese defects. When NSLAB counts reached over 8-9 logs CFU/g in the ripened cheese, it may resulted in undesirable features.

***Adjunct cultures***

Pasteurizing the cheese milk leads to inactivation of lactic acid bacteria; hence, the addition of the microorganism is fundamental to make cheese. Using adjunct cultures would enhance flavor and texture of the cheese. Adjunct culture is a mixture of certain microorganisms that are known to improve the final cheese when added to the milk during the cheese making process (El Soda et al. 2000). Another advantage of using

adjuncts culture is to accelerate the cheese ripening process in order to produce more cheese in shorter time and reduce the cost of storing the cheese for ripening (Fox, 1996, El soda, 1993). According to Kocaoglu-Vurma et al (2008), a mixed culture of *Lactobacillus* spp. was added to a cooked Swiss cheese at low heat, they found out that these cultures were beneficial in improving cheese flavor and reducing undesirable characteristic like concentrated citrate. The reduction in citrate concentration was a reason for having *Lactobacillus casei* as a part of the adjunct culture that metabolized the citrate during the ripening process. In addition, *Pediococcus acidilactici* was used as a part of the adjunct culture to control other undesirable nonstarter lactobacilli during the ripening process of hard cheese. In fact, according to a recent study *Pediococcus* spp. produce pediocin, which is a bacteriocins that has recently been categorized as a safe bio-preservative that inhibits the growth of pathogens such as *Listeria* in food products (Papagianni and Anastasiadou, 2009).

***Effect of temperature on cheese microbiology***

The rate of cooling cheese after the cooking step has a significant impact on cheese texture, flavor, and microbial content. Decreasing the final temperature of cooked cheese to specific temperatures helps to maintain fat crystallization that gives cheese its texture (Fox *et al*., 2004). It also affects the growth of added Non-starter lactic acid bacteria (NSLAB) or adjunct cultures. Controlling NSLAB growth by using rate of cooling was achieved by keeping cheese cooled to (<10°C) for more than 10 days before ripening process (Fox *et al*., 1996; Tamime, 2011). The type of the cheese and its characteristics regulate the range or rate of cooling (Tamime, 2011). During the ripening process, temperature levels and duration are critical in inducing biological activities by

starter and non-starter lactic acid bacteria (Wehr and Frank, 2004). However, increasing the actual ripening temperature could decrease the ripening time by accelerating the most significant reaction that accelerates the ripening, and proteolysis (Law, 1999; Fox, 2004). In fact, cheese that is made under a very sterile environment, including making cheese out of pasteurized milk; do not need to be ripened under historical average low temperatures 6°C (El Soda et al. 1991; Law, 1999). However, the ripening temperature should not be increased to 20°C, since 20°C and higher can release fat on the cheese surface, which is an undesirable characteristic in the final product. El Soda (1993) conducted the first successful study using higher than actual temperatures on cheddar cheese. The cheddar cheese is typically ripened at 6-8 ˚C and may take up to 12 months depending on the type of cheddar being made to get a ripened cheddar (Fox et al., 1996). Whereas, if the ripening temperature is increased to 16˚C, this will give a full ripened cheddar cheese in 4 months without any defects in flavor and quality. The advantages of this technique are the simple and efficient technology, and there are no legal barriers. Even more, it does not involve additional costs, and in fact, there are savings from reducing refrigeration and storage costs when we store the cheese at a higher temperature or store them for a shorter length of time. However, increasing the temperature may lead to increase the risk of microbial spoilage (Fox et al. 1996). In addition, if we accelerated the proteolysis by elevated temperature, all biochemical reactions will be equally accelerated, which can result in an unbalanced flavor or an off-flavored cheese (Fox et al. 2000).

The growth of the cheese market in the United States shows an increase by 48 million pounds from 2016 to 2017. The total production of cheese reached up to 3.049

billion pounds (USDA, 2017). This expansion is one of the reasons of trending cheese acceleration techniques as discussed above. The cheese industry aims to produce more cheese and reduce the production cost with shorter ripening time.

***Common cheese defects / microbial aspects***

The first source of microorganisms in cheese is the raw milk, for that pasteurization is applied to control pathogens growth and coliforms (Law, 1999). However, many microorganisms are heat-resistant that can survive pasteurization, which leads to some cheese defects including slits. Other source of spoilage microbes in cheese are industrial contaminants during the cheese making process. In fact, maintaining the microbial content of cheese can be difficult at times. Especially when external factors are hard to control such as time and temperatures that support the changing cheese microflora. Uncontrolled lactic acid bacteria especially non-starter lactic acid bacteria could lead to slits due to their ability to produce $CO_2$ during sugar or citrate fermentation in hard and semi-hard cheeses (Law, 1999). *Streptococcus thermophilus* can cause early blowing on semi-hard cheddar cheese defects by producing gas and urease (Rehn et al., 2011). *Leuconostoc* has been reported as a causative organism for early blowing, too (Hull *et al*. 1992, Ledenbach and Marshall, 2009). The NSLAB contributing to cheese defects are mostly heterofermentative lactobacilli. Crow et al. (2001) found that some of the NSLAB caused cheese defects during extended ripening duration up to 20 months that led to undesirable cheese texture and flavor. Thermoduric thermopiles, which survive the pasteurization, are another cause of cheese slits. Their types and counts in pasteurized milk affect the cheese quality. These thermoduric are mostly *Bacillus* spores, *Clostridium* spores, *Lactobacillus*, and *Streptococcus* (Marth and Steele, 2001). Their survival during

pasteurization leads to cheese cracks, softening, color change, and off flavors. *Lactobacillus. casei* and *Lb. casei subspecies rhamnosus* caused flavor defects in Cheddar cheese when counts reached up to 5 logs CFU/g during ripening duration (2-6 months) (Lehmann, 1992).

In addition, coliforms cause an early blowing in cheese under uncontrolled conditions. On the other hand, cheese made of pasteurized milk hardly has coliforms (Law, 1999, Ledenbach and Marshall, 2009). Late blowing is another common cause of slits due to *Clostridial* spp. (contaminants) during the ripening process (Law, 1999; Morandi et al., 2015). Slow acid production in cheese is another cheese defect that could be a result of bacteriophage, which will slow down the activity of starter bacteria (Law, 1999). Bacteriophages are more likely to be found in cheese made of unpasteurized milk, since pasteurization destroys bacteriophage. Other cheese defects could be due to the growth of molds and yeast. While controlled yeast growth contributes to cheese flavor enhancement, extreme growth would influence cheese texture like softening or slime development (Law, 1999). Having a mold growth on cheese is predictable during cheese ripening due to manufactures or handling contaminations as undesirable features if it was not a mold-ripened type of cheese.

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**PART II: Understanding population within the biofilm matrix formed by common dairy sporeformers**

The adherence of spoilage bacteria in the dairy plants is a major concern for the quality of dairy products, since they can create contamination that leads to lowering of the shelf life or cause defects in the final products. Biofilms can be found in dairy plants where processing lines allow passing of raw and pasteurized milk, and where it is hard to clean and disinfect adequately. Biofilm formation mostly leads to cross contamination of dairy product resulting in their spoilage. Microorganisms that form biofilms or attach to a formed biofilm are commonly thermodurics, which survive pasteurization (Buehner et al., 2014). The presence of contaminants that adhered to the processing lines will lead to early or late milk defects in the final products by releasing vegetative cells or germinating spores embedded within the biofilm. The spore-former bacteria make the biofilm mostly in the heat exchange, evaporator, and the downstream of pasteurized milk (Sharma and Anand, 2002), which make these places the major source of cross contaminants when temperature is suitable for germination of bacterial spores (Seale *et al*., 2008). Other bacteria such as *Lactobacillus, Bacillus, Streptococcus,* and *Clostridium,* may also be heat-resistant (thermoduric) and thermophilic; grow at high temperatures. However, when conditions are not favorable, they tend to form spores for protection, and the presence of spores can affect the quality of the product undesirably. Even though Cleaning in place (CIP) systems run frequently in dairy plants, the ability of spores to resist harsh conditions like chemicals, temperatures, surrounding stress, low water

activity, and high acidity or alkaline, will allow them to survive (Sharma and Anand, 2002, Wong, 1998, Miller et al., 2015). Unremoved biofilms in hard to clean positions comprised of spores and sporeformers that germinate to vegetative cell once the environment is suitable (Anand et al, 2014, Setlow, 2003).

***Common spores and sporeformers in milk***

In raw and dried milk, the three species of aerobic sporeformer commonly isolated are *Geobacillus stearothermophilus* that was known as *Bacillus stearothermophilus*, *Anoxybacillus flavithermus* and *Bacillus* spp. such as *B. licheniformis* and *B. sporothermodurans* (Scheldeman et al., 2006; Miller et al., 2015). These genera have the capacity to produce highly resistance spores that can survive heat treatment processes. These heat-resistance sporeformers could lead to milk spoilages such as flat sour in milk by *B. licheniformis* and *G. stearothermophilus* or off flavor by *A. flavithermus* (Scheldeman *et al*., 2006, Yuan *et al*. 2012, Kent et al., 2016). They are considered as thermoduric bacteria, and most of the studies came to agreement that *Geobacillus* and *Bacillus* are the most prevalent strains in dairy industry that survive pasteurization and have the ability to grow at elevated temperatures (+ 50°C) (Rueckert et al. 2004). Miller *et al*., (2015) declared that 69% of the isolated bacteria in raw milk are *Bacillus* spp., while *Geobacillus* occupied more than 11% of the isolates. However, *Geobacillus* spp. spores are mostly isolated in the final products such as dry milk (Miller *et al*., 2015); while they are rarely isolated from raw milk (Kent at al., 2016). On the other hand, *Bacillus* spp. (ex. *Bacillus licheniformis*) could be isolated from the milk at all processing steps (Miller *et al*., 2015). In a study conducted by Yuan *et al*. (2012), dry milk samples were analyzed for contamination and more than 27% of the isolates were *B.*

*licheniformis.* They also found that more than 36% of 801 isolates were *B. licheniformis*, and 20% were *G. stearothermophilus*. Their predominance is due to the ability of their dormant spores to tolerate rough physical treatments like high temperatures and high pressures. In fact, the dry milk composition inhibits the vegetative cells growth due to the low water activity (≤0.3) (Wewala, 1990), which helps in preservation of dried milk. However, reconstituting this milk is the start point of germination for the dormant spores that survived the milk processing. Yet, dormant spores are not the only source of milk contamination; the reconstitute could be another source of contamination. The temperature at which the reconstitution occurs is significant, too. According to Raso et al. (1994), *B. licheniformis* starts sporulation at 52°C during the sterilization process of canned vegetables. Their outcome was that increasing heat-resistance of spores could fail the sterilization process. Thus, that is why *B. licheniformis* is a common isolate in dairy plants, where pasteurization done over 52°C, which is the favorable temperature for *B. licheniformis* to form spores. Watanabe *et al.* (2003) analyzed the effect of different treatments on *Bacillus* spp. spores and *G. stearothermophilus* spores and found that among all tested species, *G. stearothermophilus* spores were most resistant to heat, pressure, and $CO_2$ treatments. The treatment duration (2 hours), temperature (95°C), and pressure (30 MPa) with $CO_2$ were the most effective factors that inactivated *G. stearothermophilus* spores; however, these are not the appropriate treatments for dairy products.

***Biofilm forming ability of common sporeformers***

A biofilm is an accumulation of individual strain cells or different strains that colonize or their spores survive pasteurization and attach to milk residues (Flemming *et*

*al*., 2010, Seale *et al*., 2008). These organisms adhere to stainless steel surface by producing extracellular polymeric substances (EPS) and enclosing cells within it (Marchand *et al*., 2012). The extracellular polymeric substances are important for forming biofilms, which is a key characteristic of many biofilm-forming organisms. The EPS are high molecular weight molecules like extracellular polysaccharide materials, lipids, nucleic and amino acids (Anand et al. 2014, Kumar and Anand 1998, Flint et al., 1997). These layers of EPS isolate the microorganisms and protect them from surrounding hostile conditions like the changing in the nutrient contents or its absence, elevated temperatures or fluctuating acidity. Other factors that help microorganisms attach to the surfaces besides EPS secretion are bacterial cell surface charge, or the pili in some bacteria (Melaugh et al. 2015). In fact, the plant surfaces play significant role in influencing the strength of attachment mechanisms (Wong, 1998). The rough surfaces are more likely to support the biofilm formation. Jindal et al. (2016) studied the mechanisms of the biofilm formation by aerobic sporeformers and the bacterial adherence using stainless steel coupons with surface modifications. They found that modified stainless steel surface with electrolytic deposition of nickel and co- deposition of polytetrafluoroethylene (PTFE) elements is promising for reducing biofilm attachment in dairy industry. This coating eliminates the adherence of biofilm and milk residues. However, since this stainless steel modification is still under study, most of the current stainless steel in the food industry that is in use are cleanable, corrosion-resistant stainless steel that is made of chromium and nickel. In a dairy plant in New Zealand, Seale et al. (2008) studied the bacterial attachment to stainless steel surface from a milk powder production system, and they found up to $10^4$ spores per $cm^2$ of *Geobacillus* spp. In

another study, it took 7 days for *Geobacillus* spp. to form spores. This suggest that the detected spores were formed in areas where the CIP could not reach properly, since the CIP runs repeatedly during the production process. However, there are other studies that show that even with frequent cleaning-in-place, use elimination of the biofilms is challenging. A study conducted on different dairy industries in Algeria aimed to analyze the efficiency of CIP in cleaning formed biofilm that formed in the pasteurization track. Malek et al (2012) observed low reduction on the bacterial counts after running CIP from swabbed biofilms. However, their counts remained significant, which ranges between 3-8 logs CFU/$cm^2$. Most of the isolates were gram-positive strains, rarely gram-negative, and they suggested that the unclean industry equipment and environment are the main source of contamination.

***Spore and spore germination process***

Most of the production lines dairy plants run up to 20 hours per day, the time between each production line seems to be sufficient for sporulation (Alvarez et al., 2010). In general, *Bacillus* spp. tends to sporulate during the lack of nutrients when the production is stopped. Their dormant spores are mostly resistant to high temperatures, changing pH, and chemical detergents. The changes in the microorganisms' optimum conditions lead to the sporulation in order to protect the vegetative cells. The spore former bacteria can produce coats around the core for protection. However, once the environment becomes suitable, they germinate and become vegetative cells that are able to utilize the milk nutrients and can cause milk spoilage (Setlow, 2003). The spore formation and composition are complicated but the germination requires nutrient sources and just a short duration. In the dairy plants, thermoduric sporeformers attach to the

stainless-steel surfaces during the production process of the milk after surviving the pasteurization. By the end of the process, when the nutritional source (milk) is gone, starving will lead the microorganisms to sporulation. The CIP runs frequently in dairy industries to try to combat this issue. However, these spores are resistant to the heat treatment and chemical detergents during the cleaning process. The dormant spores within the biofilm matrix would be further protected until the next production run, which would provide these spores with the required nutrition for germination. It takes few seconds for germination and the contamination of the new product. Many studies were performed on pasteurized and dried milk samples to prove the ability of sporeformers to survive pasteurization and form spores. The main purpose of pasteurization is eliminating pathogens and spoilage bacteria and this process can be applied by two methods, high-temperature short time-treatment (HTST) and Low-temperature long time-treatment (LTLT) (Rysstad and Kolstad, 2006). Both techniques apply high temperatures which is 72°C or 63°C, however, these temperatures are not efficient to remove at least for two of the most common sporeformers in dairy plants *G. stearothermophilus* and *Anoxybacillus flavithermus* (Burgess et al., 2010, Khanal et al., 2014). In fact, these temperatures are not effective in inactivating spores, which make their elimination difficult. A recent study conducted, which aimed to understand the appropriate temperature that inactivate *G. stearothermophilus* spores, reported that 95°C is the most effective temperature when applied for 2 hours under specific pressure (Watanabe et al., 2003). This method could be promising for cooked food preservation, but not for dairy. As discussed previously, the common dairy processing temperatures currently used to treat milk are not sufficient to inactivate spores. Although, studies are available about the ability of sporeformers to

form biofilms in dairy processing environment, there is hardly any information on the ability of vegetative cells to convert to spores within biofilm matrices, as the biofilms grow older. In view of this, the current study was conducted to understand the population dynamics of common dairy sporeformers, and their ability to form spores within biofilm matrices.

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# CHAPTER 2

## THE INFLUENCE OF RATE OF COOLING AND RIPENING TEMPERATURES ON SEMI-HARD ITALIAN CHEESE MICROFLORA


## ABSTRACT

The rate of cooling and ripening temperature may influence the growth and survival of cheese microorganisms including lactic acid bacteria, sporeformers, and others. The starter and non-starter lactic acid bacteria (NSLAB) play a major role in influencing cheese flavor and quality. The increase of NSLAB is predictable during the ripening process, but active heterofermentative metabolism could lead to some typical cheese defects. To study this, a set of semi-hard Italian cheese samples was fast cooled to (38°F) in 40 hours, and compared with the samples that were slow cooled to (50°F) in 10 days. These two treatments were each ripened at 38°F and 50°F for 6 months. The samples were drawn at 2 and 6 months intervals, and were analyzed using standard microbiological techniques. For total aerobic viable count, plate count agar (PCA) was used. The other selective media used included Rogosa selective *Lactobacillus* agar for non-starter lactic acid bacteria (NSLAB), deMan Rogosa and Sharpe (MRS) agar for *Lactobacillus* spp., MRS-V agar with 20 μg/ml of vancomycin for *Leuconostoc* spp., MRS-VR agar with 2 μg/ml vancomycin and 1.5% ribose for *Lactobacillus wasatchensis*, MRS broth was used for detection of gas producers, and M17 agar for lactic streptococci. Isolated colonies on the selective media were further identified using Matrix Assisted Laser Desorption Ionization mass spectrometry - Time of Flight (MALDI-TOF). The total aerobic counts showed, in general, a declining trend during 6 months of ripening. Initial NSLAB counts in the 12 days old samples cooled to 38°F started at 5.87±0.13 logs

CFU/g, and 6.19±0.13 logs CFU/g in the samples cooled to 50°F, and the two were significantly different. The fast-cooled samples thus showed lower counts than the slow cooled samples. At the end of two months of ripening, the samples that were fast cooled to 38°F, and ripened at 38°F showed lower NSLAB counts (6.0 logs) than those that were ripened at 50°F (6.33 logs). On further ripening to 6 months, the trend remained the same with a slight decline. However, the samples that were slow cooled to 50°F and ripened at 38°F for 6 months didn't follow predicted trends, presumably due to the presence of the adjunct culture. Hence, the predominance of *Pediococcus,* as an adjunct, influenced the counts of NSLAB population in an unpredicted manner, an observation that needs to be studied further. The predominant isolates included *Lactobacillus paracasei*, *Lb. rhamnosus, Pediococcus acidilactici, Streptococcus salivarius* ssp. *Thermophilus,* and *Lactococcus lactic*. None of the samples, however, showed the presence of *Lb. wasatchensis* and *Leuconostoc* spp. The distribution pattern based on randomly picked colonies indicated a change in species distribution pattern, as the ripening period progressed. The addition of *Pediococcus acidilactici* appeared to control the overall the NSLAB population during ripening even under the accelerated ripening at 50°F. It may be more useful to conduct this study without added *Pediococcus*, so that actual microbial interactions in cheese matrix could show up.

***Keywords:*** cheese, NSLAB, ripening, cooling.

## INTRODUCTION

Cheese making process is a long procedure that includes milk pasteurization, standardization, and the addition of rennet and microbial culture to curdle milk. The last step of the cheese production process after cooking, cutting, salting, and pressing curd is

cooling cheese for ripening. This step has a significant impact on cheese texture, flavor, and microbial content. Decreasing the temperature of cooked cheese helps to maintain fat crystallization that gives cheese its texture (Fox *et al*., 2004). It also affects the growth of added non-starter lactic acid bacteria (NSLAB) or adjunct cultures. This step could be achieved by cooling cheese to <50°F or (<10°C) for more than 10 days before ripening process (Fox *et al*., 1996; Tamime, 2011). The type of the cheese and its characteristics regulates the range or rate of cooling (Tamime, 2011). During the ripening process, temperature levels and duration are critical in inducing biological activities by starter and non-starter lactic acid bacteria (Wehr and Frank, 2004). The non-starter lactic acid bacteria (NSLAB) play a critical role in influencing cheese flavor and quality. The microflora content of cheese changes with time during the ripening process. However, NSLAB remain the predominant microflora in ripened cheese (Jokovic *et al*., 2011). The NSLAB include both facultative and obligate heterofermentative lactobacilli that produce proteolytic and lipolytic enzymes. These enzymes influence the development of the cheese texture and flavor. The NSLAB have genes that are encoded for proteases enzyme, peptides transporters, and peptidase, which catabolize free amino acids (FAA) like leucine, lysine, proline, valine, and aspartic acid. These free amino acids accentuate the flavor and develop the texture of cheese (Gobbetti *et al*., 2015). In fact, increase in NSLAB during the ripening process is anticipated, but high counts (> 8 logs CFU/g) of undesired strains of NSLAB, especially heterofermentative lactic acid bacteria could lead to some typical cheese defects (Rapposch *et al*., 1999). Examples of cheese defects by either facultative or obligate heterofermentative are flavor defects, slits due to gas production, lactate crystals formation, and over acidification (Gobbetti *et al*., 2015).

*Leuconostoc* sp. is one of the NSLAB that is known to produce $CO_2$, ethanol, acetate, and diacetyl, which can lead to undesired features during the cheese ripening process (Mathot *et al*., 1994). The cheese with the proper levels of *Leuconostoc* sp. and *Lactococcus* sp. should be beneficial for cheese quality. However, having only *Leuconostoc* sp. might lead to cheese defects (Mathot *et al*., 1994). *Leuconostoc* sp. is resistant to Vancomycin and by adding it to MRS agar, growth of the contaminant background microflora such as *Bacillus* sp. could be inhibited (Benkerroum *et al*., 1993). Another common, naturally occurring, microorganism that may lead to cheese defects during ripening at elevated temperatures is *Lactobacillus wasatchensis* (Ortakci *et al*., 2015). *Lactobacillus wasatchensis* is an obligatory heterofermentative NSLAB that is known for utilizing cheese sugars to produce $CO_2$ that can cause late blowing of cheese. Many *Lactobacillus* sp. resists vancomycin in selective media, but the addition of ribose to MRS agar make it selective for *Lb. wasatchensis*. As stated by Ortakci *et al*., (2015), *Lb. wasatchensis* does not grow in MRS-RV within 2 days when incubated at optimal growth temperature (37°C) of common *Lactobacillus* sp. It takes about 5 days to grow at room temperature (23°C) (Ortakci *et al*., 2015). Besides, *Clostridium* spp. cause gassy defects that affect cheese quality (Mullan, 2003). For that, the rate of cooling to different temperatures and the corresponding ripening temperatures could affect NSLAB population in cheese. The objective of this study was to understand the effect of the rate of cooling and ripening temperatures on the NSLAB counts and their distribution within the cheese during the ripening process.

## MATERIAL AND METHODS

### *Sourcing of cheese and cheese types*

Semi-hard Italian cheese samples were obtained in sealed polybags, from a commercial dairy plant. Three batches of sealed cheese samples were transported in Styrofoam cooler and were stored at 4°C until further analysis. All the samples had no visible defects or slits. However, cheese plant noticed an active growth of non-starter lactic acid bacteria, especially the heterofermentative bacteria, in ripened cheese blocks, which were not fully cooled. For that, the first set of cheese cooled for 8-10 days to two different temperatures. The first group that had 8 samples fast cooled to 38°F (3.3°C), and the other group (8 samples) was slow cooled (traditionally) to 50°F (10°C) (Figure 1). The fast cooling was achieved in a special cooler by lowering the cheese temperature from 90-100°F to 38°F (final packaging temperature) in 40 hours. However, the slow cooling is meant to let the cheese reach 50°F in 8-10 days, and that was achieved by lowering the cheese temperature from the starting temperature of 90-100°F. After cooling the cheese samples, they were stored in either a 38°F or 50°F warehouse for ripening up to 6 months. The exact duration to reach the ripening temperatures that were not the same as the cooling temperatures was not available. The following cultures were all part of the starter cultures: *Lactobacillus helveticus, Lactobacillus casei, Streptococcus thermophilus, Lactococcus sp., Lactobacillus sp., Lactobacillus rhamnosus*, and *Lactobacillus lactis* subsp. *cremoris*. *Pediococcus acidilactici* was added as a special starter adjunct.

***Cheese sample preparation***

Cheese extract was prepared by softening 11g of cheese sample in 99ml of Phosphate Buffer Saline (PBS). The samples were macerated in a Seward Stomacher 400 C Circulator Lab Blender Mixer (California, USA) for 3 minutes at 250 RPM in a strainer

stomacher bag. Then, serial dilutions out of the original solution were made up to $10^{-6}$ for plating. Duplicate plates of each sample were made for each plating medium. Each treatment represents the mean and standard deviation of 8 samples. Standard pour and spread plates methods were used for bacterial enumeration (Wehr and Frank, 2004).

***Enumeration of total viable counts***

Plate count agar (PCA) was used for total aerobic mesophilic and thermophilic counts, and the plates were incubated aerobically at 37°C for mesophilic counts and 55°C for thermophilic counts. For thermoduric mesophilic and thermoduric thermophilic counts, heat treatment at 63°C was applied on all the samples for 30 minutes prior to plating, using pour plate method (Wehr and Frank, 2004). All plates were done in duplicate. All thermoduric mesophilic plates were incubated aerobically at 37°C for 48 hours, and thermoduric thermophilic plates were incubated at 55°C for 48 hours.

***Isolation of lactic acid bacteria***

The non-starter LAB were enumerated on Rogosa Lactobacillus Selective (RSL) agar (Rogosa et al., 1951, Somers et al., 2001), and *Lactobacillus* spp. were enumerated on deMan Rogosa and Sharpe Agar (MRS) (Morandi et al., 2015; Corry et al., 2003). For *Leuconostoc* spp. isolation, modified MRS agar with 20 µg/ml of Vancomycin solution was used. Vancomycin was prepared in distilled water, sterilized through 0.22 um pore filter, and then added to MRS after autoclaving (Mathot et al., 1994). Another 2 µg/ml of vancomycin solution plus 1.5% ribose was added to the autoclaved MRS agar to isolate *Lactobacillus wasatchensis* (Ortakci et al., 2015). The RSL, MRS, and MRS-V agars were incubated anaerobically using anaerobic jars and gas packs to generate anaerobic

atmosphere (GasPak™ EZ anaerobe container system, BD) at 32°C for 48 h. Further, the MRS-RV plates were kept under extended incubation for 5 d at room temperature (23°C).

For lactic streptococci, M17 agar (Morandi et al., 2015), with 10% of Lactose solution (Lowrie and Pearce,1971) was used. This analysis was conducted only for the ripened samples. There were a total of 64 samples were plated in duplicate, and plates were incubated aerobically at 32°C for 48h.

***Isolation of anaerobic sporeformers***

The incidence of late blowing of cheese, during accelerated ripening under elevated temperatures, by *Clostridium* spp. is undesired and unpredictable. Thus, anaerobic sporeformers presence was examined using selective media. Reinforced clostridial medium (RCM) was used to enumerate *Clostridium* spp. (Morandi et al., 2015; Morandi et al., 2014; Atlas, 2004). Isolation of vegetative clostridial cells was done directly on RCM agar in duplicate from the serial dilutions. For thermoduric endospore counts, the macerated cheese samples in PBS were heated in a water bath to 80°C for 10 min (Morandi et al., 2015), cooled immediately after heat treatment to 3±1°C (Wehr and Frank, 2004), followed by pour plating 1.0mL of the respective dilution using RCM agar. All RCM plates were incubated anaerobically at 32°C for 48 h. All the plates were sent for further colony identification using MALDI-TOF.

***Detection of gas production by selected isolates***

The ability of heterofermentative bacterial isolates to form gas was examined via Durham tubes that were inserted upside down in test tubes containing MRS broth. The media tubes were autoclaved at 121°C for 15 minutes. Each sterilized tube was inoculated with an isolated NSLAB colony from MRS plates (Pyar et al., 2014; Nikita

and Hemangi, 2012). All tubes were incubated in anaerobic jar, with gas pack system, at 32°C for 72 hours, and examined for any gas entrapment in the inverted tubes.

***Species identification for studying changes in bacterial population***

All colonies between 25-250 were counted for enumeration purposes, and the results were expressed as the means and standard deviations of 8 samples under each treatment as $Log_{10}$ colony forming units (CFU) per gram. Isolated colonies on the selective media were further identified using Matrix Assisted Laser Desorption Ionization mass spectrometry - Time of Flight (MALDI-TOF) (Animal Disease Research and Diagnostic Laboratory, SDSU, Brookings, SD, USA). Selected colonies from RCM, MRS-V, and MRS-RV plates for all samples were sent for identification. All Rogosa agar and MRS plates had the same colony morphologies, hence, random isolates were sent for identification.

***Statistical analysis***

The effect of cooling and ripening temperatures on the bacterial counts was evaluated from the collected data, which analyzed by a T-test and using R-program. The significance was declared at $P < 0.05$.

## RESULTS AND DISCUSSION

***Changes in total viable counts***

All total aerobic counts data are presented in Tables 1 and 2. The comparative of the two treatment groups and sampling points are presented in Figure 1. It was observed that the total viable mesophilic counts start with 7.26±0.28 logs cfu/g in samples fast cooled to 38°F, and 7.19±0.15 logs cfu/g in samples slow cooled to 50°F. No significant difference was recorded at this stage (*P-value 0.6*). No thermoduric mesophilic or

thermoduric thermophilic isolates were detected. However, the overall mesophilic counts decreased slightly after 2 months when ripening temperatures differed than cooling temperatures ($P< 0.05$), no significant differences were recorded when cooling and ripening temperatures remain the same. After 6 months of ripening, as expected due to NSLAB predominance, a reduction in the mesophilic counts was observed in samples that were cooled and ripened at 38°F, from 7.30±0.18 to 6.53±0.24 logs cfu/g ($P< 0.01$). On the other hand, samples ripened at 50°F (after cooling to 38°F), showed a count reduction from 6.76±0.33 logs (2 m) to 6.04± 0.34 logs (6m) at $P< 0.05$. Also, the counts of samples that ripened at 50°F after cooling to 50°F dropped from 7.25±0.38 to 6.08±0.24 logs cfu/g ($P< 0.001$). Lower enumeration of total aerobic mesophilic counts in cheese during ripening is normal in a typical ripened cheese (Jokovic *et al*., 2011). Ripening samples at 38°F after cooling them at 50°F showed no count differences. It is known that predominant NSLAB are effective in inhibiting the growth of other bacteria that may spoil the cheese and help to lower the total viable counts, too. Some of isolated aerobic organisms on PCA agar were *Bacillus licheniformis* and *Lactococcus lactic.*

In order to understand the treatments effects on cheese samples, another comparison was done that presented the case when samples cooled to different temperatures and ripened at the same. The bacterial counts on the selective and non-selective media initiated with comparable numbers when cooled to different temperatures. The differences in the counts of ripened samples were noticed after two months of ripening. However, the final counts of the ripened cheese under different treatment after six month reached comparable numbers with the predominance of the adjunct *Pediococcus acidilactici*.

***Isolation of Lactic acid bacteria***

Enumeration was done in duplicate from each sample under each treatment. The mean and standard deviations of 8 samples are shown in Tables 1 and 2. All data for the isolated *Lactobacillus* spp. on both agars, RSL and MRS were categorized depending on the cooling temperatures. Table 1 shows the data of cheese samples cooled to 38°F and ripened at two different temperatures (38°F and 50°F). While Table 2 presents data of samples cooled to 50°F and further ripened either to 38°F or 50°F. This compares with the previous studies indicating that NSLAB population in typical cheese making process started with 4-6 logs CFU/ml and reached to 7.5-9 logs during ripening (Gobbetti *et al*., 2015; Ong *et al*., 2006; Swearengin *et al*. 2001). In contrast, cheeses made with pasteurized milk and inoculated with only starter bacteria showed less than 1 log cfu/g of NSLAB (Swearengin *et al*. 2001), and their growth could be controlled by cooling the cheese to < 50°F for 10 days (Fox *et al*., 1996). In our case, the average initial count of *Lactobacillus* spp. on RSL agar was 5.87 ±0.13 logs cfu/g on samples cooled to 38°F, and 6.19 ±0.13 logs cfu/g on samples cooled to 50°F, which increased after 2 months of ripening under all treatments**.** Significant differences ($P<0.01$) were observed between samples cooled to 50°F and ripened at 38°F for 6 months (7.67±0.31) logs CFU/g to the samples cooled to 50°F (6.19±0.13) logs CFU/g, and samples ripened for 2 months (6.69±0.24) logs CFU/g. The total counts of NSLAB in all samples varied between 5.44±0.59 to 7.67 ±0.31 log CFU/g, which is typically comparable to cheese content during ripening (De Angelis *et al*., 2001). Also, it was observed that the counts on MRS agar were generally higher than the counts on Rogosa agar, even though both media are selective for *Lactobacillus* spp. and similar species were isolated on both agars according

to MALDI-TOF results. *Pediococcus acidilactici* and *Lactococcus lactis* were detected in most of the cheese samples and were isolated on both RSL and MRS, which are known for supporting the growth of *Pediococcus, Lactococcus,* and *Streptococcus* species (Rogosa *et al*., 1951; Somers *et al*., 2001; Corry *et al*., 2003.). *Pediococcus acidilactici,* were predominant in cheese samples ripened for 2 and 6 months, but this was expected as they were added as starter adjuncts during cheese production. Fitzsimons *et al*. (2001) studied the NSLAB distribution within cheddar cheese aged for 2-5 months and proved that *Lactobacillus paracasei* predominate even over *Lactobacillus rhamnosus*. Their predominance could be from the environment of the ripening cheese such as high acidity, low moisture, and high content of fermented carbohydrates, which is favorable for *Lb. paracasei* over *Lb. rhamnosus.* Figure 2 and 3 shows the strains distribution in different stages and as presented *Lb. rhamnosus* was isolated at some stages and not at others, which could be a reason of its low counts during that stage. From these results, it is most likely that predominant species might not be distributed uniformly throughout the cheese, which is why some species were not isolated at some stages.

The isolates on MRS-V and MRS-RV were similar to the isolates on MRS agar, which were lactobacilli, pediococci, and lactococci, due to their resistant to vancomycin (Tharmaraj and Shah, 2003). *Leuconostoc* spp. and *Lactobacillus wasatchensis* were not detected in any of the 80 samples according to MALDI-TOF identification of selected colonies observed on the selective agar plates. This minimizes the probability of having cheese defects or slits in the final product. According to Ortakci *et al.* (2015) it also might indicate that content of galactose or ribose in cheese was not sufficient to support *Lactobacillus wasatchensis* growth.

The total counts on M 17 agar and the means and standard deviations of 8 samples from each group of ripened cheese are presented in Tables 1 and 2. These counts showed variable trend indicating the possible non-uniform distribution of these organism and the small sample size that might have influenced the results. The starter adjuncts could also be reason for these trends. Nevertheless, the species distribution (Figures 2 and 3) showed the presence of pediococci, streptococci and lactococci, almost throughout the ripening period. Tharmaraj and Shah (2003) reported that *Streptococcus salivarius* subsp. *thermophilus* showed no growth in medium with sugar base, however, it was isolated on M17 agar with 10% of lactose. In fact, *Streptococcus salivarius* subsp. *thermophilus* could be useful in accelerating cheese ripening (Tharmaraj and Shah, 2003). Especially, when it was a part of the added cultures during the cheese making process. In our study, *Streptococcus salivarius* subsp. Thermophilus isolates were observed in cheese samples cooled to 38°F but none in samples cooled to 50°F, and it was observed to predominate in the ripened samples.

***Isolation of anaerobic sporeformers***

For *Clostridia* species identification, the suspect colonies from the RCM plates, for each sample, were sent for MALDI-TOF. None of the plates showed *Clostridia* spp. The samples that were heat treated at 80°C for 12 min, prior to plating, showed no growth after incubation, indicating the absence of any anaerobic endospores. This limits the probability of occurrence of cheese slits (gassy defects) because of the lack of anaerobic sporeformers.

***Screening of the isolates for gas formation***

The presence of FHL (facultative heterofermentative *Lactobacilli*) NSLAB such as *Lactobacillus rhamnosus,* and *Lactobacillus paracasei* would increase the possibility of heterofermentation; yet, some of them are added as starter cultures during cheese making (Rapposch *et al*., 1999). The NSLAB produce $CO_2$ during fermenting of the carbohydrates in cheese that might lead to cheese slits, if their growth is uncontrolled. However, in our study, none of the tested isolates showed any gas formation under the lab testing conditions. One of the reasons could be the low availability of fermentable substrate in the MRS broth in which the isolates were tested, which might have led to their low metabolic activity and less gas production, not detected by the method used. Although, methods based on similar principles have previously been also used to detect gas production by lactobacilli gave similar results.

***Changes in the bacterial population***

Direct observation methods were not suitable for the determination of the population in this study, since the colony morphologies were quite similar for all the isolates, and they were not easy to differentiate. In addition, some isolated microorganisms were only shown in samples ripened at 50°F, either cooled to 38°F or 50°F such as *Kocuria varians, Solibacillus silvestris,* and *Acidovorax delafieldii.* Figures 2 and 3 show the changes in microbial distribution within cheese samples under different treatments. The percentage distribution of organisms were assigned depending on how many times they show up on MRS, Rogosa, and M17 plates out of the total plates that were sent to MALDI-TOF for species identification. These percentages may not reflect the exact distribution of the microorganisms in the samples, and should only be used for an overall indication of the presence or absence of any species. As shown in Figures 2

and 3, *P. acidilactici* and *L. lactis* dominated samples cooled to 38°F as compared to the samples that were cooled to 50°F. The differences were also noticed in the presence of *L. paracasei*, only in fast cooled samples (Figure 2), and absence of *L. rhamnosus*, which also was not detected in samples that were cooled and ripened at 38°F for 2 months (Figure 2 a). Moreover, *L. paracasei* was not detected initially in the samples that were slow cooled to 50°F (Figure 3), but were observed in all the ripened samples that were cooled originally to 50°F (Figures 3 a, b, c and d)). Both *L. paracasei* and *L. rhamnosus* were a part of the adjunct cultures, their absence in only cooled cheese samples is likely due to their low initial counts, which could be affected by their optimum growth temperatures. In fact, *L. paracasei* is known to be a predominant culture in most of the ripened Italian cheese (De Angelis *et al*., 2001). In addition, *Streptococcus salivarius* subsp. *thermophilus* was not detected in cheese samples that were slow cooled to 50°F or further ripened to 50°F for 2 months.

As mentioned earlier, *Lactobacillus helveticus*, *Lactobacillus casei*, *Streptococcus salivarius* subsp. *thermophilus*, *Lactobacillus rhamnosus*, *Lactobacillus lactis subsp. cremoris,* and *Pediococcus acidilactici* were starter and starter adjuncts in the cheese samples. Swearingen et al. (2001) reported that the increases in nonstarter LAB counts is beneficial in improving cheese flavour and texture. He stated that cheddar cheese inoculated with NSLAB resulted in higher sensory and quality due to their proteolysis activity. Banks *et al*. (1998) revealed that *Lactobacillus paracasei* showed highest productivity in utilizing sulphur in cheddar cheese that led to improved final product characteristics.

The intention of *Pediococcus acidilactici* culture addition in the current cheese samples was to control other undesirable nonstarter lactobacilli during accelerated ripening of cheese. The pediococci produces pediocin, which is a bacteriocins that has recently been categorized as a safe biopreservative that inhibits the growth of pathogens such as *Listeria* in food products (Papagianni and Anastasiadou, 2009). In view of the above, the distribution pattern based on randomly picked colonies indicated a change in species distribution pattern, as the ripening period progressed. The high prevalence of *Pediococcus acidilactici* (the added starter adjunct) in all samples did not allow true predominance to emerge. It may be more useful to conduct this study without added *Pediococcus*, so that actual microbial interactions in cheese matrix could show up. Also, in our study, the limitation in the identification method we used was that isolates selected for MALDI-TOF were based on colony morphology, which limited our analyses to presence or absence but not for quantification of the isolated species in each sample. For future studies, analyzing Italian hard cheese with adjunct cultures excluding *P. acidilactici* should be considered for comparing with the addition of this culture as an adjunct culture.

## CONCLUSIONS

This study implies that adjunct cultures dominated throughout all the ripening phases. It also reveals that changing rate of cooling and ripening temperatures influence bacterial counts diversity within cheese samples. The cheese samples that were ripened for two months had more strain variability and higher counts compared to only cooled cheese or cheese ripened for 6 months, which indicates higher biological activity that could lead to cheese slits. Predominant isolates remained the same in all samples, which

are *Pediococcus acidilactici*, *Lactobacillus rhamnosus*, *Lactobacillus paracasei*, and *Streptococcus salivarius* subsp. *Thermophilus*, respectively. In addition, their predominance led to inhibit the growth of other added cultures such as *Lactobacillus helveticus* and *Lactobacillus casei.* Six months of ripening of the cheese samples that were fast cooled to 38°F and ripened at 50°F lead to control NSLAB counts on cheese, which might help improve cheese quality due to the predominance of *Pediococcus acidilactici* which was added as adjuncts. In general, if accelerated ripening at 50°F is the goal, our results suggest that the rate of cooling cheese to 38°F or 50°F will not make a difference from the bacterial count perspective, especially if *Pediococcus acidilactici* has been added as an adjunct. A better perspective can be obtained by conducting this study without the *Pediococcus acidilactici* adjunct. However, bacterial types and their distribution pattern as affected by different rates of cooling might matter the most. The results present a useful tool for determining the appropriate rate of cooling and ripening temperatures during cheese making process in order to improve and standardize cheese quality.

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## TABLES AND FIGURES

**Table 1.** Bacterial counts ($log_{10}$ cfu/g) of cheese samples cooled to 38°F and ripened at 38°F or 50°F.

| Cooling temp | Ripening temp | PCA agar | RSL agar | MRS agar | M17 |
|---|---|---|---|---|---|
| **Only cooled samples for 12 days** | | | | | |
| 38 $^{O}$F | | $7.26^{a} \pm 0.28$ | $5.87^{a} \pm 0.13$ | $6.26^{a} \pm 0.21$ | - |
| **Cooled and ripened samples 2 months** | | | | | |
| 38 $^{O}$F | 38 $^{O}$F | $7.30^{a,A} \pm 0.18$ | $6.00^{a,A} \pm 0.28$ | $7.32^{b,A} \pm 0.51$ | $7.09^{A} \pm 0.81$ |
| 38 $^{O}$F | 50 $^{O}$F | $6.76^{b,A} \pm 0.33$ | $6.33^{b,A} \pm 0.47$ | $7.46^{b,A} \pm 0.21$ | $7.47^{A} \pm 0.22$ |
| **Cooled and ripened samples 6 months** | | | | | |
| 38 $^{O}$F | 38 $^{O}$F | $6.53^{b,B} \pm 0.24$ | $5.70^{a,B} \pm 0.27$ | $6.86^{b,B} \pm 0.19$ | $7.22^{A} \pm 0.20$ |
| 38 $^{O}$F | 50 $^{O}$F | $6.04^{b,B} \pm 0.34$ | $5.48^{b,B} \pm 0.36$ | $6.26^{a,B} \pm 0.25$ | $6.46^{B} \pm 0.39$ |

*Mean and standard deviation of 8 samples

Different alphabet indicates statistical differences ($p < 0.05$)

$^{a,b}$ a comparison between only cooled cheese and cooled and ripened cheese

$^{A, B}$ compare between cooled and ripened cheese

**Table 2.** Bacterial counts ($log_{10}$ cfu/g) of cheese samples cooled to 50°F and ripened at 38°F or 50°F.

| Cooling temp | Ripening temp | PCA agar | RSL agar | MRS agar | M17 |
|---|---|---|---|---|---|
| **Only cooled samples for 12 days** | | | | | |
| 50 $^{O}$F | | 7.19 $^{a}$ ± 0.15 | 6.19 $^{a}$ ± 0.13 | 6.09 $^{a}$ ± 0.17 | - |
| **Cooled and ripened samples 2 months** | | | | | |
| 50 $^{O}$F | 38 $^{O}$F | 6.75 $^{b,A}$ ± 0.51 | 6.69$^{b,A}$ ± 0.24 | 7.39$^{b,A}$ ± 0.22 | 7.13$^{A}$ ± 0.13 |
| 50 $^{O}$F | 50 $^{O}$F | 7.25 $^{a,A}$ ± 0.38 | 6.48$^{b,A}$ ± 0.28 | 7.12$^{b,A}$ ± 0.13 | 6.53$^{A}$ ± 0.76 |
| **Cooled and ripened samples 6 months** | | | | | |
| 50 $^{O}$F | 38 $^{O}$F | 6.71$^{b,A}$ ± 0.33 | 7.67 $^{b,B}$ ± 0.31 | 7.24 $^{b,B}$ ± 0.15 | 7.21$^{A}$ ± 0.18 |
| 50 $^{O}$F | 50 $^{O}$F | 6.08 $^{b,B}$ ± 0.24 | 5.44$^{b,B}$ ± 0.59 | 6.28 $^{a,B}$ ± 0.47 | 6.13$^{A}$ ± 0.40 |

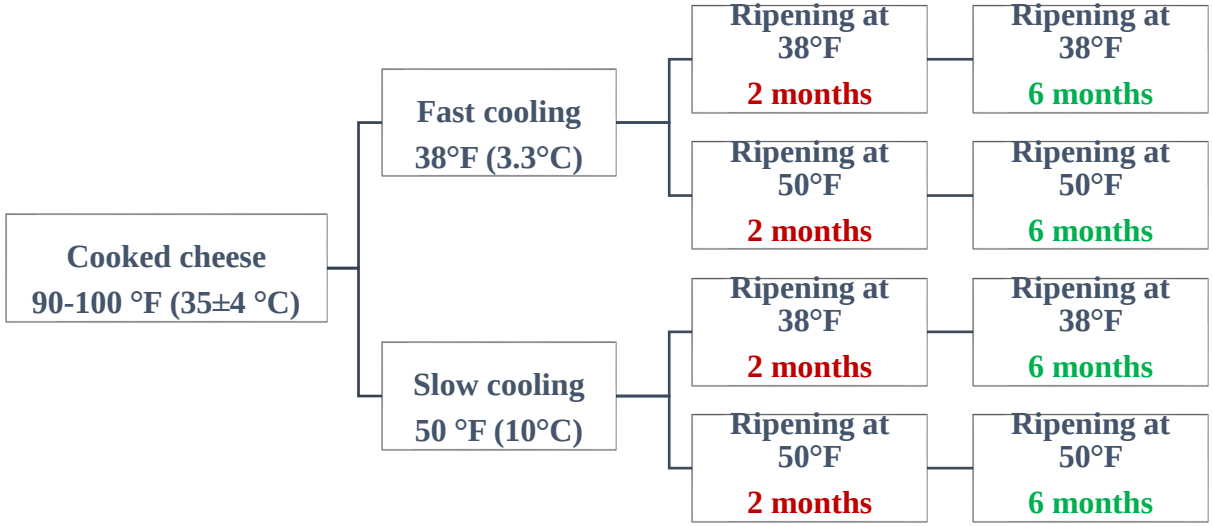
*Mean and standard deviation of 8 samples

Different alphabet indicates statistical differences ($p < 0.05$)

$^{a,b}$ a comparison between only cooled cheese and cooled and ripened cheese

$^{A, B}$ compare between cooled and ripened cheese

**Figure 1:** Description of treatments and cheese sampling points.



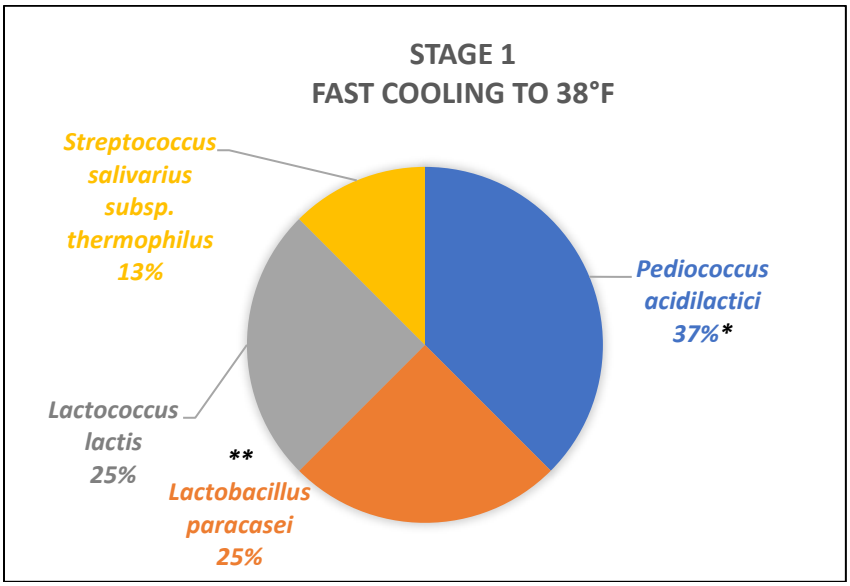



**Figure 2:** Isolated bacteria on selective media from only slow cooled cheese to 38°F that identified using MALDI-TOF.

Note: * The percentages of organisms were assigned based on how many times were observed in the in plates that were sent to MALDI-TOF to be identified, and may not reflect the exact percentage of the microorganisms in the samples

**Absence of *Lactobacillus rhamnosus* in fast cooling stage or absence of *Lactobacillus paracasei* in some samples could be due to their low counts, which were not detected.

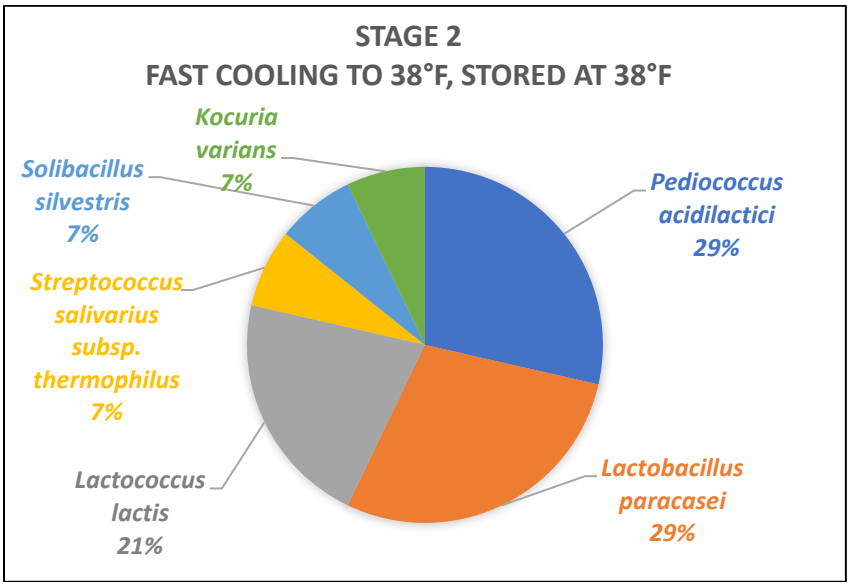



**Figure 2 (a):** Isolated bacteria on selective media from fast cooled cheese samples stored at 38°F for 2 months.

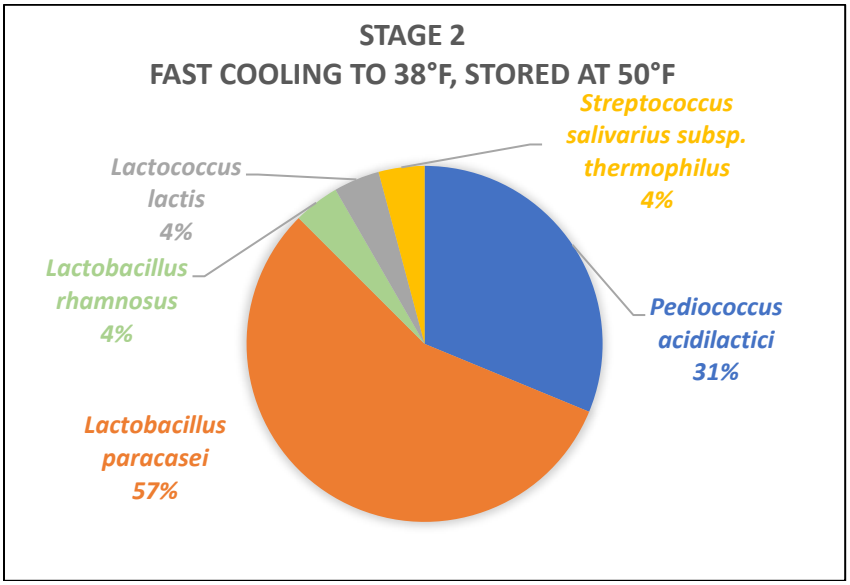



**Figure 2 (b):** Isolated bacteria on selective media from fast cooled cheese samples stored at 50°F for 2 months.

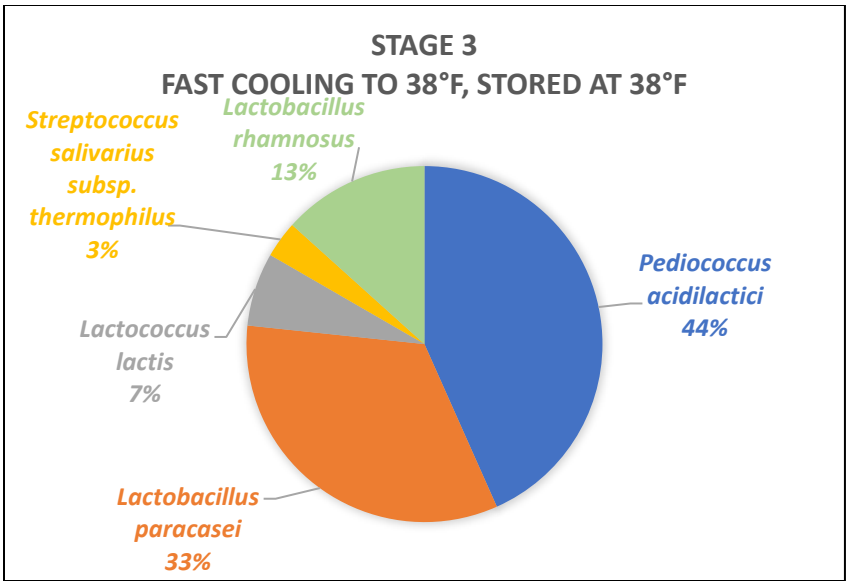



**Figure 2 (c):** Isolated bacteria on selective media from fast cooled cheese samples stored at 38°F for 6 months.

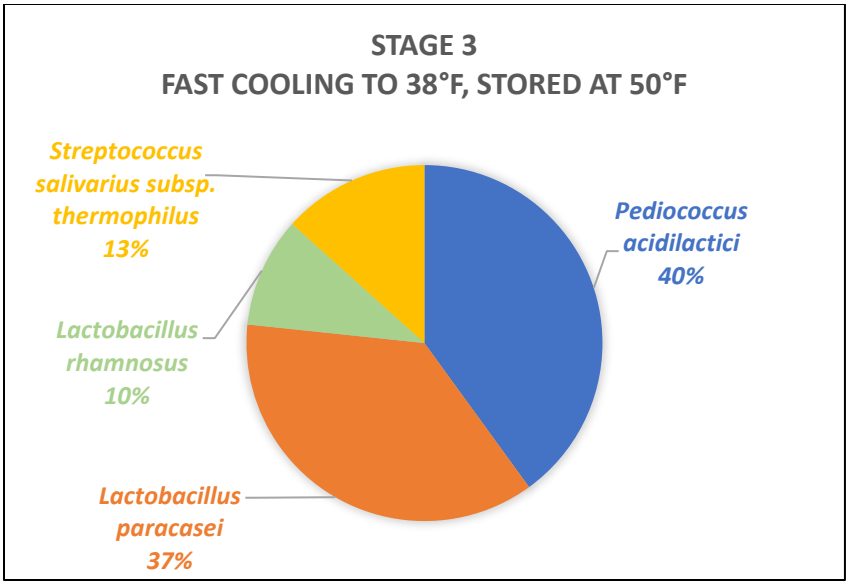



**Figure 2 (d):** Isolated bacteria on selective media from fast cooled cheese samples stored at 50°F for 6 months.

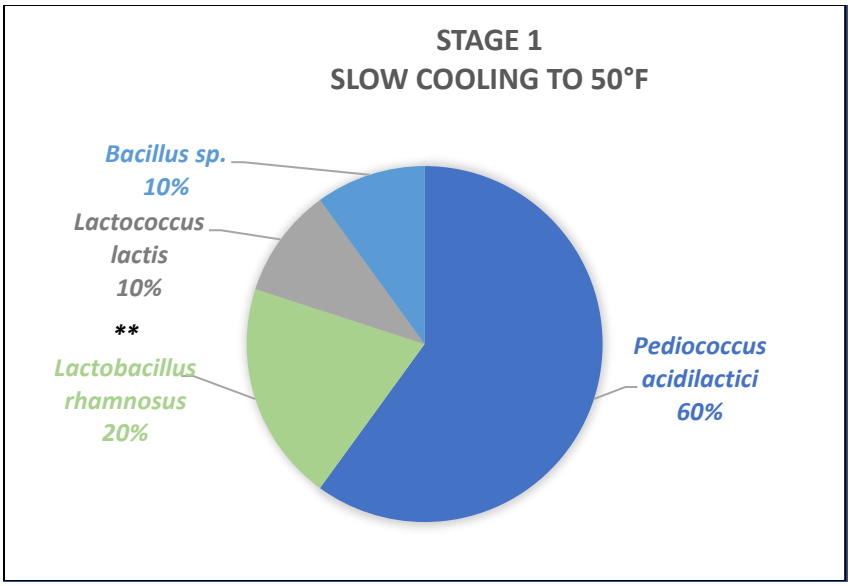



**Figure 3:** Isolated bacteria on selective media from only fast cooled cheese to 50°F that identified using MALDI-TOF.

Note: * The percentages of organisms were assigned based on how many times were observed in the in plates that were sent to MALDI-TOF to be identified, and may not reflect the exact percentage of the microorganisms in the samples

**Absence of *Lactobacillus rhamnosus* in fast cooling stage or absence of *Lactobacillus paracasei* in some samples could be due to their low counts, which were not detected.

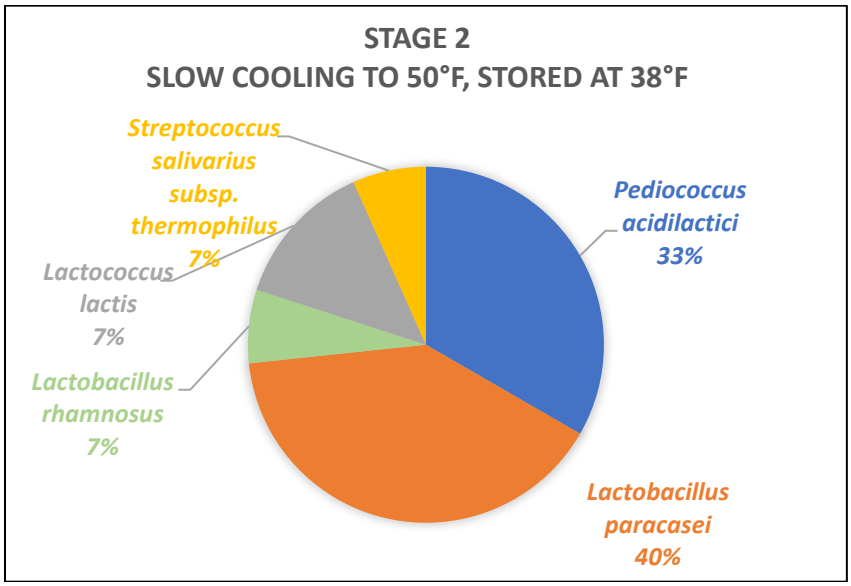



**Figure 3 (a):** Isolated bacteria on selective media from slow cooled cheese samples stored at 38°F for 2 months

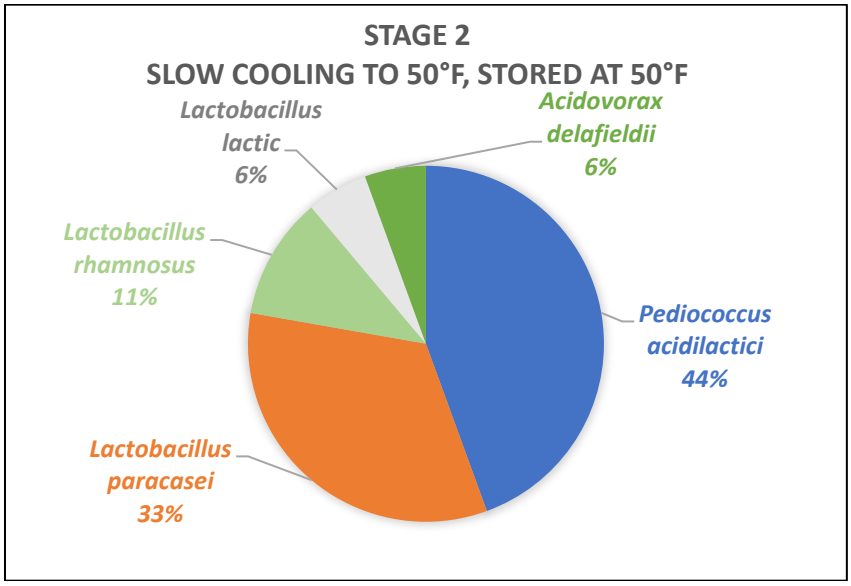



**Figure 3 (b):** Isolated bacteria on selective media from slow cooled cheese samples stored at 50°F for 2 months

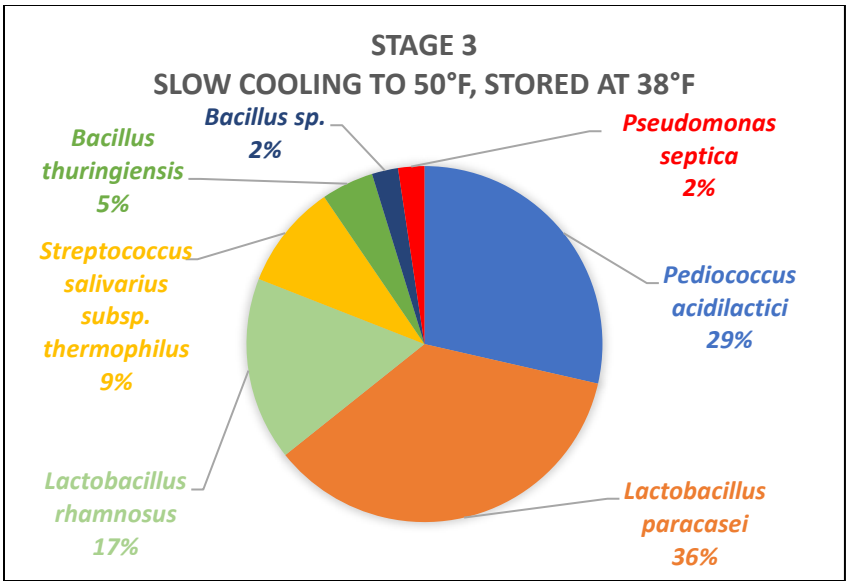



**Figure 3 (c):** Isolated bacteria on selective media from slow cooled cheese samples stored at 38°F for 6 months

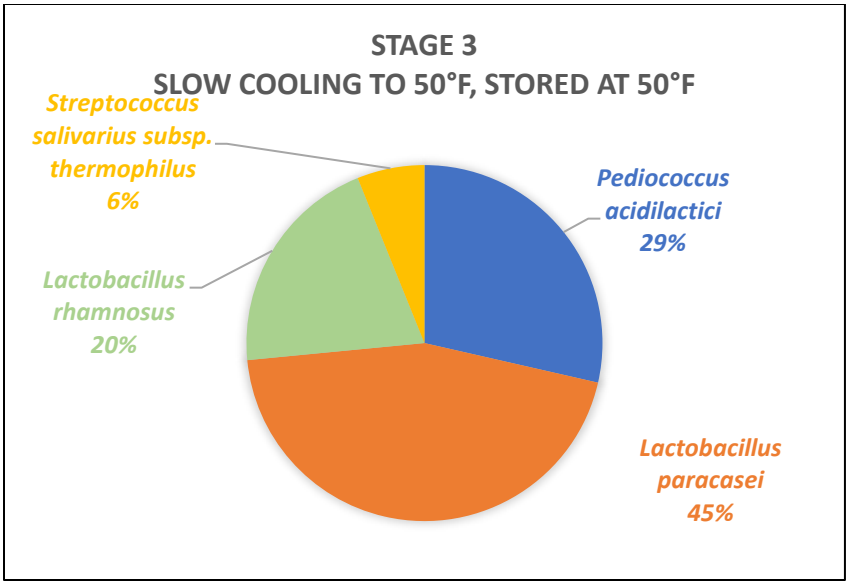



**Figure 3 (d):** Isolated bacteria on selective media from slow cooled cheese samples stored at 50°F for 6 months.

# CHAPTER 3

## UNDERSTANDING SPORULATION WITHIN CONTACT SURFACE BIOFILMS BY COMMON SPORE FORMER

## ABSTRACT

The purpose of this study was to understand the formation of biofilms and sporulation within biofilm matrix. Stainless steel coupons grade 316 were immersed in inoculated skim milk with common sporeformers in dairy plants namely *Bacillus licheniformis* (ATCC®6634™, and ATCC®14580™), *Geobacillus stearothermophilus* (ATCC®15952™), and *Bacillus sporothermodurans* (DSMZ 10599$^{T}$) to develop biofilms. Standard plate counts method was used to enumerate the vegetative cells and spores within biofilm matrix developed on stainless steel coupon surfaces. All species were observed to generate biofilms on stainless steel coupon surfaces in a short time (~4 hours) when immersed in spiked skim milk. Three species *Geobacillus stearothermophilus* ATCC®15952™, *Bacillus sporothermodurans* DSMZ 10599$^{T}$, and *Bacillus licheniformis* ATCC®14580™ formed spores within the biofilm matrix. For *B. sporothermodurans*, it took 12 hours to form log 1.62 CFU/cm$^{2}$ of spores on the SS surface. While *G. stearothermophilus* ATCC®15952™ formed 3.10 logs CFU/cm$^{2}$ spores within the biofilm matrices in 3 days. For *B. licheniformis*, there was a strain variability between ATCC®6634™ and ATCC®14580™ for sporeforming ability. *Bacillus licheniformis* ATCC®6634™ did not form spores even with prolonged incubation

up to 21 days. On the other hand, *B. licheniformis* ATCC®14580™ took 7 days to form 2.89 logs CFU/cm$^2$ spores within the biofilms formed on the SS coupons.

***Keywords:*** biofilm, sporulation, sporeformer, skim milk

## INTRODUCTION

The adherence of spoilage bacteria to processing equipment in dairy plants is a major concern for cross contamination of dairy products. Since cross contamination affects the shelf life or causes defects in the final products. Biofilms can be found in dairy plants where processing lines allow passing of raw or pasteurized milk, and where it is hard to clean and disinfect adequately. Biofilm formation can lead to dairy product spoilage, and microorganisms that form biofilm or attach to a formed biofilm are commonly known as milk contaminant, which may produce off-flavors in milk, and may survive pasteurization (Buehner et al., 2014). The presence of contaminants that adhered to the processing lines will lead to early or late defects in the final products by releasing vegetative cells or germinating spores embedded within the biofilm. Even though Cleaning In Place (CIP) systems run frequently in dairy plants, the ability of spores to resist harsh conditions like chemicals, temperatures, surrounding stress, low water activity, and high acidity or alkaline, will allow them to survive (Sharma and Anand, 2002, Wong, 1998, Miller et al., 2015). Unremoved biofilms in hard to clean positions comprised of sporeformers and spores that may germinate to vegetative cell once the environment is suitable (Anand et al, 2014, Setlow, 2003). In raw and dried milk samples, the three species of aerobic sporeformer commonly isolated are *Geobacillus stearothermophilus* that was previously known as *Bacillus stearothermophilus*,

*Anoxybacillus flavithermus* and *Bacillus* spp. such as *B. licheniformis* and *B. sporothermodurans* (Scheldeman et al., 2006; Miller et al., 2015). These genera have the capacity to produce highly resistant spores that can survive heat treatment processes. Miller *et al*., (2015) declared that 69% of the isolated bacteria in raw milk are *Bacillus* spp., while *Geobacillus* occupied more than 11% of the isolates. However, *Geobacillus* spp. spores are mostly isolated in the final products such as dry milk (Miller *et al*., 2015); while they are rarely isolated from raw milk (Kent at al., 2016). On the other hand, *Bacillus* spp. (ex. *Bacillus licheniformis*) could be isolated from the milk at all processing steps (Miller *et al*., 2015). Most of the production in dairy plants runs up to 20 hours per day, the time between each production line seemed to be sufficient for sporulation (Alvarez et al., 2010). In general, *Bacillus* spp. tend to sporulate during the lack of nutrients when the production is stopped. Their dormant spores are mostly resistant to high temperatures, changing pH, and chemical detergents. The changes in the growth conditions lead to the sporulation in order to protect the vegetative cells. The spore forming bacteria can produce coats around the core for protection. However, once the environment becomes suitable, they germinate and become vegetative cells that are able to utilize the milk nutrients and can cause milk spoilage (Setlow, 2003).

We hypothesize that the biofilm matrix could be a reason for bacterial sporulation. It protects the bacteria from the surrounding environment and provide nutrients for a certain time. However, the thick and old formed biofilms might isolate the bacteria from the nutrients source, and keep toxins within it, as well.

Our objective was to understand the biofilm formation on SS coupons by common sporeformers, and the process of sporulation within biofilm matrix. The pH of the milk (nutrient source) was monitored, and the temperatures were held constant for each microorganisms depending on their optimum temperature.

## MATERIAL AND METHODS

### *Sourcing of Spore-forming bacteria and their propagation*

The three strains of spore-former bacteria that were used in this study were *Bacillus licheniformis* (ATCC®6634™, and ATCC®14580™), *Geobacillus stearothermophilus* (ATCC® 15952™), and *Bacillus sporothermodurans* (DSMZ 10599$^{T}$) (Table 1). The first three strains were procured from the American Type Culture Collection (ATCC, Manassas, VA), and *B. sporothermodurans* was obtained from Deutsche Sammlung von Mikroorganisem und Zellkulturen (DSMZ), Germany. Preparation of the strains was performed using recommended media for these strains, which was Brain Heart Infusion (BHI) Broth (Oxoid, USA), and the incubation conditions were as recommended by ATCC and DSMZ. All the strains were incubated aerobically for 24 hours for vegetative cells enumeration, and 48 hours for spores. *Bacillus* spp. were incubated at their optimum temperature (32°C) and *G. stearothermophilus* was incubated at 55°C. A recently procured *G. stearothermophilus* was also compared with a two year old strain that was used in previous studies conducted in our lab by Khanal et al. (2014), and Jindal et al. (2016). All the previous cultures were kept in 1.8-mL cryogenic vials and stored at -75°C for further studies.

The cultures were activated in BHI broth by incubating them aerobically at the organism's optimum temperatures, and sub-cultured twice before performing the study. The media were centrifuged at 3,500 RPM for 10 min at 4°C to harvest the cells in a pellet form (Khanal et al., 2014, Jindal et al., 2016). Sterile distilled water was used to wash harvested spores by vortexing followed by centrifuging at 3,500 RPM for another 10 minutes. The supernatant was discarded and sterile distilled water was added to make adjusted suspension to get 8-9 logs CFU/mL. The turbidity of the suspension was measured at $OD_{600nm}$ = 0.3 by visible spectrophotometer (Spectronic 200, V 2.06). To determine the initial counts, serial dilutions were made, and plated on BHI agar, followed by incubation for 24 hours.

***Non-Fat Dry Milk preparation for inoculation***

Non-fat dry milk was obtained from (Davis Dairy Plant, South Dakota State University, Brookings, USA) to make 11% total solids sterile skim milk. A 113 g sample of the dry milk was mixed with 887 ml of distilled water then sterilized by autoclaving at 121°C for 15 minutes, then cooled immediately for further use.

***Biofilm formation studies***

Stainless steel (SS) coupons, type 316 (6.25 $cm^2$), were used in this study. In petri dishes, sterile SS coupons were placed and immersed under 26±1 ml of autoclaved skim milk that was inoculated with about 7 logs CFU/ml of 24 hours old activated culture as described above. Plates were incubated aerobically at 37°C for *Bacillus licheniformis* strains and *Bacillus sporothermodurans,* and at

55°C for *Geobacillus stearothermophilus*. At approximately 12h intervals, the milk in the plates was replaced with the same volume of autoclaved skim milk without any inoculum.

***Enumeration of sporeformers and spores within biofilm matrix during a shorter incubation***

The first step in this study was to estimate the duration necessary for biofilm to be formed and the number of embedded vegetative cells and spores. Three microorganisms were used at this step; *Bacillus licheniformis* (ATCC®6634™), *Geobacillus stearothermophilus* (ATCC® 15952™), and *Bacillus sporothermodurans* (DSMZ 10599$^{T}$). The inoculation count was determined by making serial dilutions from the spiked milk at 0 hour. The first coupon was pulled after 4 hours, under aseptic environment, and rinsed gently with sterile distilled water. After discarding the excess water, the coupon surface was swabbed by 3M quick swabs (3M center, St. Paul, MN). Serial dilutions were made and plated on BHI agar plates. Plates were incubated aerobically for 24 hours. This step was repeated at 8, and 12 hours, too. After 12 hours of incubation, pH was measured and sporulation was tested by heat-treating 1/10 dilution of the swabbed coupons at 80°C for 10 minutes, prior to plating. A 1ml of the heat-treated suspension was transferred to a petri dish and pour plated with BHI agar. The plates were incubated at each organism optimum temperature for 48-72 hours.

***Prolonged incubation for population dynamics changes within biofilm matrix***

The aim of this stage was to understand the sporulation behavior of sporeformer bacteria within the biofilm under extended incubation (21 days). This step was only applied for *Bacillus licheniformis* ATCC®6634™ and *Bacillus sporothermodurans* DSMZ 10599$^{T}$. The incubation time continued to 504 hours (21 days). Fresh sterile milk was replaced at approximately 12h intervals. The coupons were pulled out at regular intervals (1, 2, 3, 4, 7, 14, and 21 days) for each organism. Each coupon was examined for the total counts of vegetative cells and spores within the biofilm matrix. As explained previously, 3M quick swabs were used to swab the coupon surface after rinsing it with sterile distilled water. Serial dilutions were made and plated on BHI agar plates. For counting spores, the first dilution was heat treated at 80°C for 10 minutes, cooled and pour plated with BHI agar. The plates were incubated at 32°C for 48-72 hours.

**Comparing population dynamics of the two strains of *Bacillus licheniformis* ATCC®6634™ and ATCC®14580™**

Two strains of *B. licheniformis* were handled under similar propagation and biofilm development conditions. The skim milk was inoculated with 8-9 logs CFU/ml of the microorganisms. The sterile SS coupons were submerged under the spiked milk, and aerobically incubated up to 7 days at 32°C. Vegetative cells and spores were enumerated as above. All the results were expressed as log CFU/mL for counts in the milk medium, and log CFU/cm$^2$ for counts in biofilms developed on the SS coupon surfaces.

***Scanning electron micrographs SEM of biofilms***

A scanning electron microscope (SEM) was used to scan the embedded vegetative cells and spores within the biofilm matrix. The scanning procedures was completed at Daktronics Engineering Hall, South Dakota State University, Brookings, SD. Stainless steel coupons washed with distilled water were dried in oven for 2-3 hours at 40±5 °C to fix the biofilm, then prepared for scanning immediately. Coupons were coated with a thin layer of gold before observation. The parameter used with the SEM was 10.0kV 7.7mm x 10.0k SE.

## RESULTS AND DISCUSSION

Since biofilm is the biggest concern in a milk production line, we used the three most common sporeformers associated with dairy plants and biofilm formation. We generated individual biofilm matrices on stainless steel coupons in our lab, using three common sporeformers, and analyzed their behavior to remain as vegetative cells or form spores. Sterile skim milk (by autoclaving at 121°C for 15 min) was used as the growth medium for biofilm formation in this study.

### *Population dynamics change during short-duration biofilm formation*

This study was conducted using three species *G. stearothermophilus* ATCC® 15952™, *B. licheniformis* ATCC®6634™, and *B. sporothermodurans* DSMZ 10599$^{T}$. When the coupon surfaces were exposed to the skim milk that was inoculated with 7-9 logs CFU/ml of the selected sporeformers, they adhered quickly, resulting in about 5 logs CFU/cm$^2$ on the coupons surface, and resulted in ~3 logs CFU/cm$^2$ when inoculated with 5 logs CFU/ml after 4 hours of incubation. In about four hours, there were many vegetative cells within the biofilm matrix formed by the different species that were tested in this study.

*Geobacillus stearothermophilus* ATCC®15952™, had the lowest count at four hours (3.19 logs CFU/cm$^2$) that decreased to 2.80 logs CFU/cm$^2$ after 12 hours. The vegetative cells that were attached to the SS coupon surface were 5.21 logs CFU/cm$^2$ and 4.37 logs CFU/cm$^2$ in 4 hours of incubation for *B. licheniformis* and *B. sporothermodurans*, respectively. Their counts increased slightly with the time and reached up to 5.57 Logs CFU/cm$^2$ of *B. licheniformi*s and 4.77 logs CFU/cm$^2$ of *B. sporothermodurans* after 12 hours (Figure 2). According to Flint et al. (1997) biofilm formation is a fast process that occur in dairy plants in less than 12 hours, which is confirmed in this study. However, among the three species *B. sporothermodurans* was the only species that formed spores on the coupon surface within 12 hours, which was detected at 1.62 logs CFU/cm$^2$ (Figure 7). The lower count of the bacterial attachment of *G. stearothermophilus* might be due to the low inoculation count that started with 5.9 logs CFU/ml. When the pH was measured after 12 hours, *Geobacillus stearothermophilus* showed no changes in the milk pH, when a reduction in pH was observed with the other two strains (Table 2). The high growth of the organisms and their metabolic activity led to lowering the pH, which could be a reason for starting the sporulation of *B. sporothermodurans*. The pH of the skim milk inoculated with *G stearothermophilus* remained unchanged and no spores were detected in 12 hours (Table 2). During this phase we provided an estimated time for biofilm formation, where we found changes in the vegetative cells within biofilm matrix within the short-duration of biofilm formation. We also observed a variability in species as *B. sporothermodurans* was the only species that formed spores within a short

duration, but the others did not. The possible reasons for species variability are; first; some of these species have a longer generation time and they do not multiply very rapidly and consequently they do not exhaust nutrients and convert to spores. The second reason could be that these strains do not readily form spores under a short duration of incubation. The third possibility is that the biofilm at early stages keeps providing some nutrients to the embedded organisms because we were changing the suspension medium at regular intervals.

This finding led us to do the next stage of our study where we prolonged the duration for biofilm formation by enhancing the incubation time.

***Population dynamics change under prolonged biofilm forming conditions***

Since the biofilm formation and sporulation for *B. sporothermodurans* was determined within 12 hours, while other organisms did not show biofilm formation during this period. The next study with extended incubation for up to 21 days, was conducted in order to understand the sporulation dynamics during prolonged biofilm formation. In this case, we also kept changing the suspension medium approximately every 12 hours. In this study, we used *B. licheniformis* ATCC®6634™, *G. stearothermophilus* ATCC® 15952™, and *B. sporothermodurans* DSMZ 10599$^{T}$. As shown in Table 3, the vegetative cell counts of *B. sporothermodurans* increased on the SS coupons during the 4 days incubation. The spore counts that started with 1.0 log CFU/cm$^2$ after 24 hours fluctuated over time between 1.0 to 2.8 logs CFU/cm$^2$ (Table 3). Changes in cell within biofilms occurred commonly. In a previous study, similar changes were observed in a biofilm formed by *A. flavithermus* under prolonged incubation up to

4 days (Parkar et al. 2003). Parkar et al. further stated that the counts changed from 2 logs cfu/$cm^2$ to more than 6 logs. Moreover, the chances of germination, during replacing the spent milk with fresh milk, could be an explanation for the fluctuating counts. In a similar case, Hinton et al. (2002) noticed the changes in spores counts after running chemicals for cleaning that affected the spores and were likely a reason of spore germination. In our study, sporulation might have happened within 48h, since the vegetative cell decreased by 1 log CFU, while the spores counts increased. Sporulation and germination play role in changing the vegetative cells counts and the spore counts. Malek et al. (2012) suggested that germination could be a factor that leads to spores count reduction if the goal was to eliminate the spores in the biofilm. *Geobacillus stearothermophilus* formed spores on the SS coupon in 3 days (Figure 4); yet, the spores were detected in the spent milk after 24 hours. Also, a reduction in spore counts was observed in the spent milk and the biofilm indicating the potential germination (Table 4).

Hinton et al. (2002) compared the bacterial attachment and persistence of biofilms and foulant in dried milk manufacture, and concluded that biofilm matrix is a favorable medium for bacterial growth in dairy plants. Another hypothesis is that the biofilms provide nutrients for bacteria, which maintain their growth (Burgess et al. 2010). However, if the biofilms layer protects the bacteria, which are inside the biofilm, from the external environment (Vu et al., 2009), it could block the continuous nutrient source as the biofilm gets thicker and older. The protection from the outside environment will also keep the metabolism residue and toxins within the biofilm, which would be another reason for sporulation. In

fact, cleaning systems in dairy plants tend to eliminate mostly the formed biofilms by pathogenic bacteria (Parkar et al., 2004). This elimination may not be sufficient for common sporeformer and biofilm formation. Also, during the elimination of the outer layers of biofilm, nutrients could be absorbed by spores as they germinate and thus causing the spores to become vegetative cells that contaminate the new milk.

In this part of our study, we extended the incubation of biofilms, because the hypothesis was that by increasing the time duration of incubation the other sporeformers will start forming spores. Thus, our hypothesis was proven that if you kept the formed biofilm for longer duration, all the organisms will likely start forming spores at some stages or other. Also, because *B. sporothermodurans* already formed spores, and *Geobacillus stearothermophilus* ATCC® 15952™ and *B. licheniformis* ATCC®6634™ did not form spores in the previous short duration (12 hours). However, *G. stearothermophilus* formed spores within the biofilm in three days and *B. licheniformis* ATCC®14580™ in 7 days. Which raised another question, would be there a difference in the strain variability to form spores? Which led to the next study.

***Comparing population dynamics for different strains of the same species***

In this study, we also compared between a new and a two-years-old strain of *G. stearothermophilus* ATCC® 15952™ that was kept frozen (-75°C) and sub cultured previously to examine their sporulation ability. Both strains were prepared and incubated with the same conditions. The older strain formed no spores even at the extended incubation (21 days), while the new strain showed the

ability to form spores in milk in 24 hours (6 logs CFU/ml) and within biofilm matrix in 72 hours. In a study conducted by Seale et al. (2008), they determine that sporulation process take 7 days to succeed, however, it is species based and under favorable condition microorganisms could form spores within 12 hours.

In the second study, a comparison of two strain of *B. licheniformis* ATCC®6634™ and *B. licheniformis* ATCC®14580™was performed, under similar conditions of growth. *Bacillus licheniformis* ATCC®14580™formed spores after 7 days within biofilm matrix on the SS coupons (SEM is not provided for this stage), while *B. licheniformis* ATCC®6634™ showed no sporulation even after long-term duration of biofilm formation (21 days).

Figure 1 shows the pH changes during 11 days of the experiment. A reduction on the pH after 11 days was observed with three microorganisms. The pH was 6.5±0.2, 5.9±0.8, and 6.3±0.9 for *G. stearothermophilus*, *B. licheniformis* ATCC®6634™, and *B. licheniformis* ATCC®14850™, respectively. The decrease in the pH induce the microorganisms' attachment within the biofilm on the SS coupon. According to Dat et al. (2012), the pH reduction is predictable during the bacterial multiplication, and controlling milk pH boost the bacterial growth in the milk but reduces the biofilm formation. In addition, the significant reduction in the pH lead to faster coagulation of the milk and thickness the biofilm by adherence of the milk residue. Further studies need to be done on controlled pH and its influence on sporulation.

***Generating visual evidence by SEM for the population dynamics***

In addition to vegetative cell count and spore counts, we used visual evidence for the population dynamics within the biofilm matrix for different sporeformer (see Figures 4-6).

This is the first study to discuss the sporulation within biofilm matrices formed on stainless steel surfaces by common sporeformers in dairy industry. Most of the previous studies reported biofilms as a reason for spore contamination, and were conducted in raw milk (Kent at al., 2016), pasteurized milk (Heyndrickx, and Scheldeman, 2002), and dried milk (Yaun et al., 2012). In addition, the heat-resistance of isolated spores from dairy plants is higher compared to the spores generated in vitro (Hill, 2004).

## CONCLUSIONS

This study indicates that biofilms on stainless steel surfaces in dairy plants are reservoirs for contaminants, especially dormant spores that can affect the final product quality. Bacterial attachment on plant equipment surfaces happens quickly, but sporulation process and timing is species based. The common isolates that we worked with had the ability to survive and multiply within the biofilm even under prolonged incubation times. Understanding the interfaces between the sporeformers and stainless steel surfaces will help provide effective elimination protocols. The information in this study will also help dairy industry adjust their cleaning systems in order to deal with and eliminate the spores within formed biofilm in the production lines since the CIP primarily targets the vegetative cells.

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## TABLES AND FIGURES

**Table 1.** The microorganisms used in the study and their sources.

| **Microorganism** | **Source** |
|---|---|
| *Bacillus sporothermodurans* | DSMZ 10599$^{T}$ |
| *Bacillus licheniformis* | ATCC®6634™ |
| *Bacillus licheniformis* | ATCC®14580™ |
| *Geobacillus stearothermophilus* | ATCC®15952™ |

**Table 2.** Determining the biofilm formation (log CFU/cm$^2$) on SS coupons and sporulation during short duration.

| | *Geobacillus stearothermophilus* ATCC® 15952™ | *Bacillus licheniformis ATCC®* 6634™ | *Bacillus sporothermodurans* DSMZ 10599$^T$ |
|---|---|---|---|
| Incubation (hours) | | Vegetative cells | |
| **0** | 5.90 ± 0.4[1] | 7.33 ± 0.7 | 8.41 ± 0.3 |
| **4** | 3.19 ± 0.1[2] | 5.21 ± 0.3 | 4.37 ± 0.1 |
| **8** | 3.00 ± 0.1 | 5.57 ± 0.3 | 4.77 ± 0.2 |
| **12** | 2.80 ± 0.2 | 5.78 ± 0.2 | 4.85 ± 0.1 |
| | | Spores | |
| **0** | -[4] | - | - |
| **12** | ND | ND | 1.62 ± 0.01 |
| **pH** | 5.94 ± 0.03 | 6.22 ± 0.02 | 6.44 ± 0.03 |

[1]*inoculation counts in the skim milk*
[2]*The mean of duplicate ± standard deviation.*
[3]*ND not detected.*
[4]*no spores were detected initially.*

**Table 3.** Detecting vegetative cells count and spores within biofilm matrix (log CFU/cm$^2$) on SS coupon surface formed.

| Time (hours) | ***Geobacillus stearothermophilus* ATCC® 15952™** | | ***Bacillus licheniformis* ATCC® 6634™** | | ***Bacillus sporothermodurans* DSMZ 10599$^T$** | |
|---|---|---|---|---|---|---|
| | **Vegetative cells** | **Spores** | **Vegetative cells** | **Spores** | **Vegetative cells** | **Spores** |
| 24 | 3.80 ± 0.7[1] | ND[2] | 6.63 ± 0.2 | ND | 5.8 ± 0.4 | 1.0 ± 0.2 |
| 48 | -[3] | - | 7.10 ± 0.2 | ND | 4.9 ± 0.8 | 2.7 ± 0.1 |
| 72 | 4.77 ± 0.4 | 3.10 ± 0.1 | 6.57 ± 0.3 | ND | 6.5 ± 0.2 | 1.7 ± 0.1 |
| 96 | - | - | 6.22 ± 0.5 | ND | 6.5 ± 0.0 | 2.8 ± 0.3 |

***Note:*** *fluctuating the total counts could be a reason of the frequent changing of spent milk with sterilized milk.*

[1] *The mean of duplicate ± standard deviation.*

[2] *ND not detected.*

[3] *G. stearothermophilus samples were pulled after 1, 3, and 6 days.*

**Table 4.** The vegetative cells and spores count in milk medium CFU/ml ($Log_{10}$), and on SS coupons CFU/$cm^2$ ($Log_{10}$).

| **Incubation (Days)** | ***Geobacillus stearothermophilus* ATCC® 15952™** | | ***Bacillus licheniformis ATCC®* 6634™** | | ***Bacillus licheniformis* ATCC® 14580™** | |
|---|---|---|---|---|---|---|
| | Milk | SS coupons | Milk | SS coupons | Milk | SS coupons |
| | | | Vegetative cells | | | |
| 1 | 7.26 ± 0.1[1] | 3.80 ± 0.7 | 9.35 ± 0.6 | 7.54 ± 0.1 | 9.18 ± 0.2 | 9.10 ± 0.1 |
| 3 | 7.65 ± 0.1 | 4.77 ± 0.4 | 8.98 ± 0.2 | 8.55 ± 0.1 | 9.01 ± 0.1 | 9.00 ± 0.4 |
| 6 | 8.29 ± 0.2 | 4.93 ± 0.5 | 9.21 ± 0.1 | 8.34 ± 0.3 | 9.03 ± 0.1 | 8.53 ± 0.2 |
| 7 | 8.58 ± 0.3 | -[3] | - | - | 9.12 ± 0.3 | 8.92 ± 0.1 |
| | | | Spores | | | |
| 1 | 4.26 ± 0.1 | ND[2] | ND | ND | ND | ND |
| 3 | 5.40 ± 0.1 | 3.10 ± 0.2 | ND | ND | ND | ND |
| 6 | 6.05 ± 0.1 | 2.57 ± 0.1 | ND | ND | ND | ND |
| 7 | - | - | - | - | 4.87 ± 0.4 | 2.39± 0.1 |

***Note**: fluctuating the total counts could be a reason of the frequent changing of spent milk with sterilized milk.*

[1]*The mean of duplicate ± standard deviation.*

[2]*ND indicates no detection*

[3]*no samples were taken on these days.*

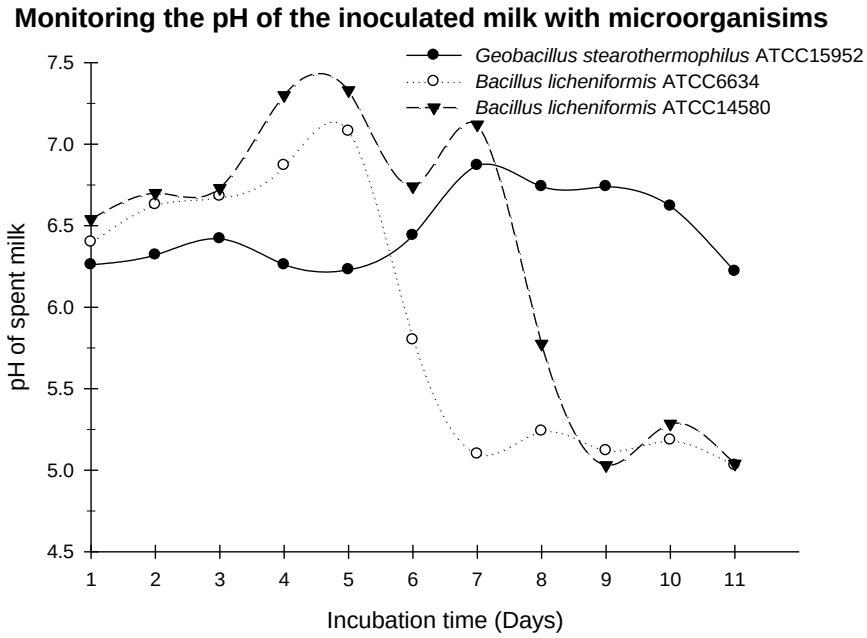



**Figure 1:** pH of the spent milk during the biofilm formation during 11 days of incubation of *Geobacillus stearothermophilus* ATCC® 15952™, *Bacillus licheniformis* ATCC®6634™, and *Bacillus licheniformis* ATCC®14580™.

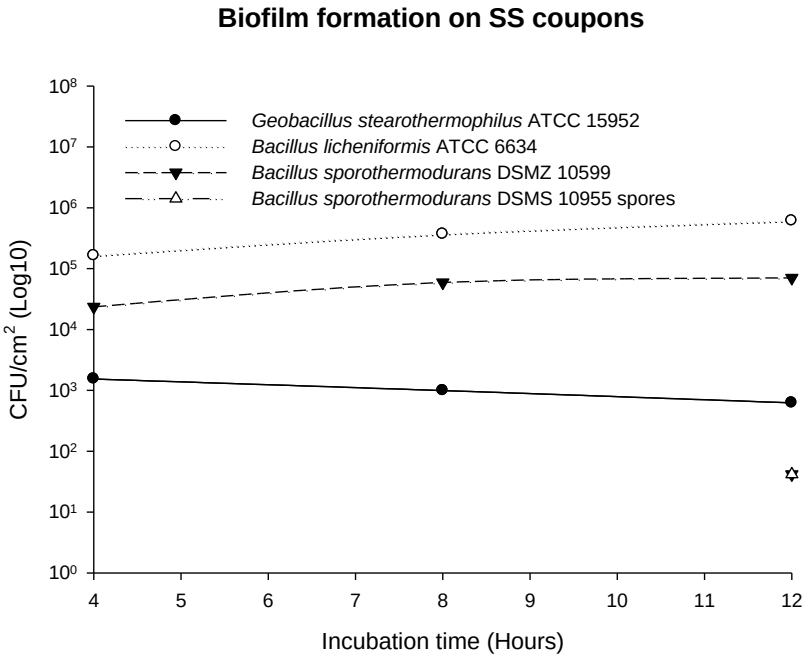



**Figure 2:** Vegetative cell counts and spores of bacterial attachment on stainless coupons during biofilm formation in CFU/cm$^2$.

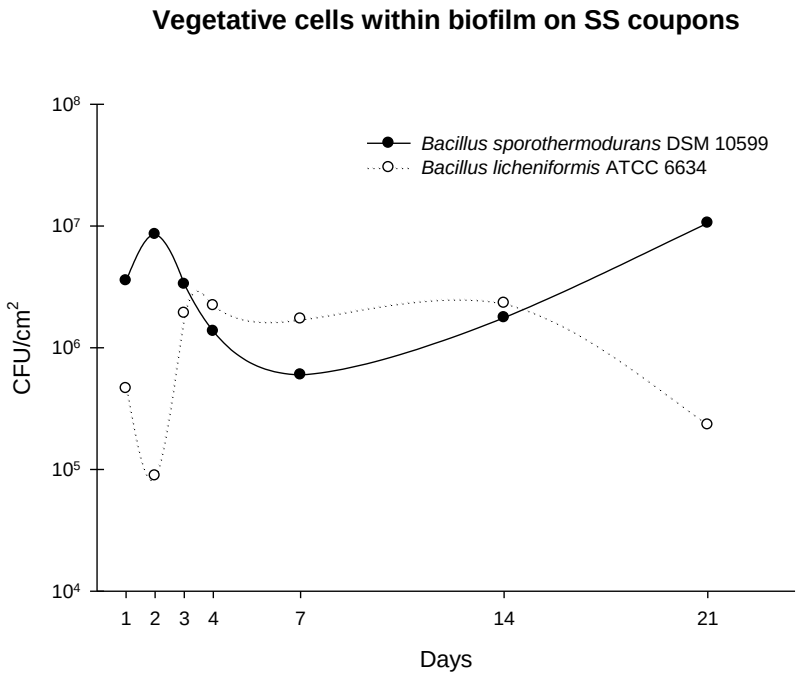
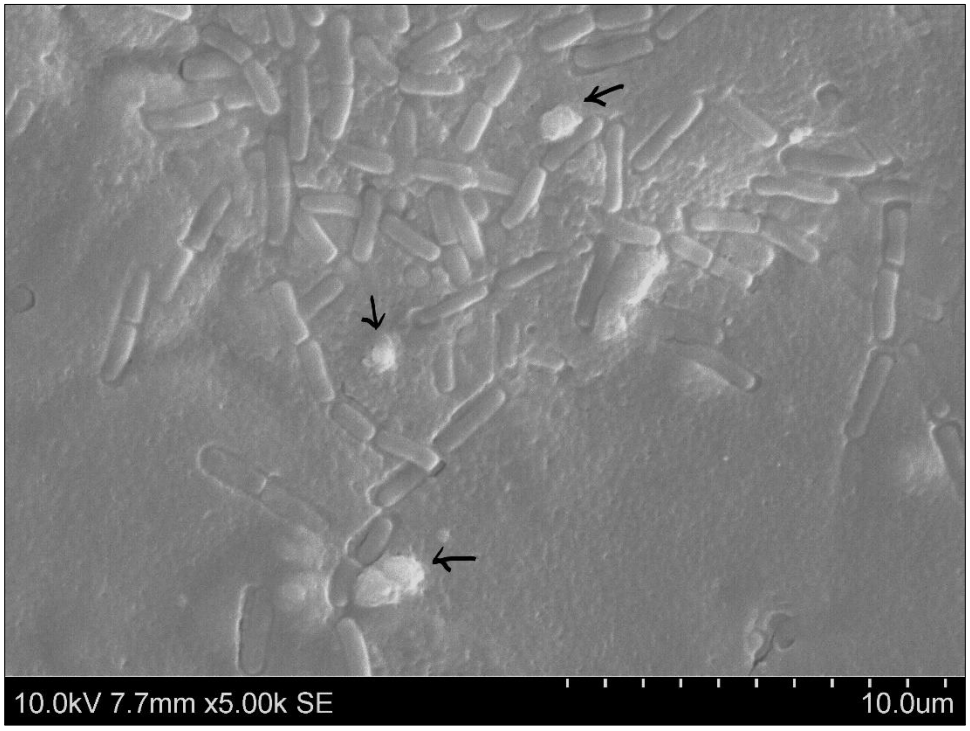



**Figure 3:** Vegetative cell counts of bacterial attachment on stainless steel coupons during biofilm formation under prolonged incubation CFU/cm$^2$.

a)




*b)*

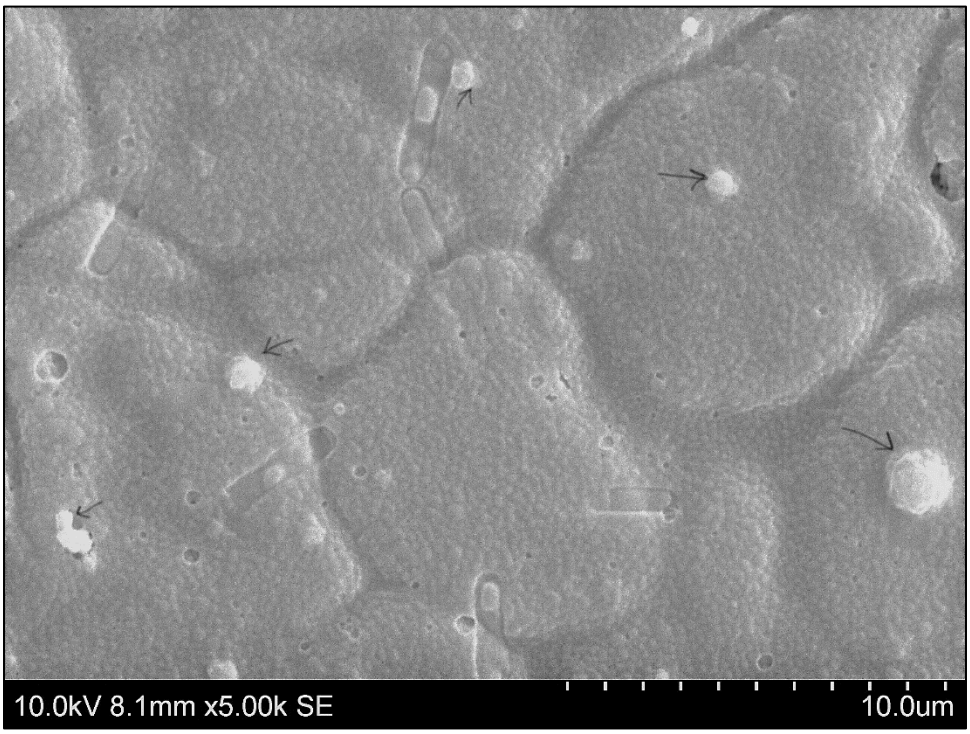
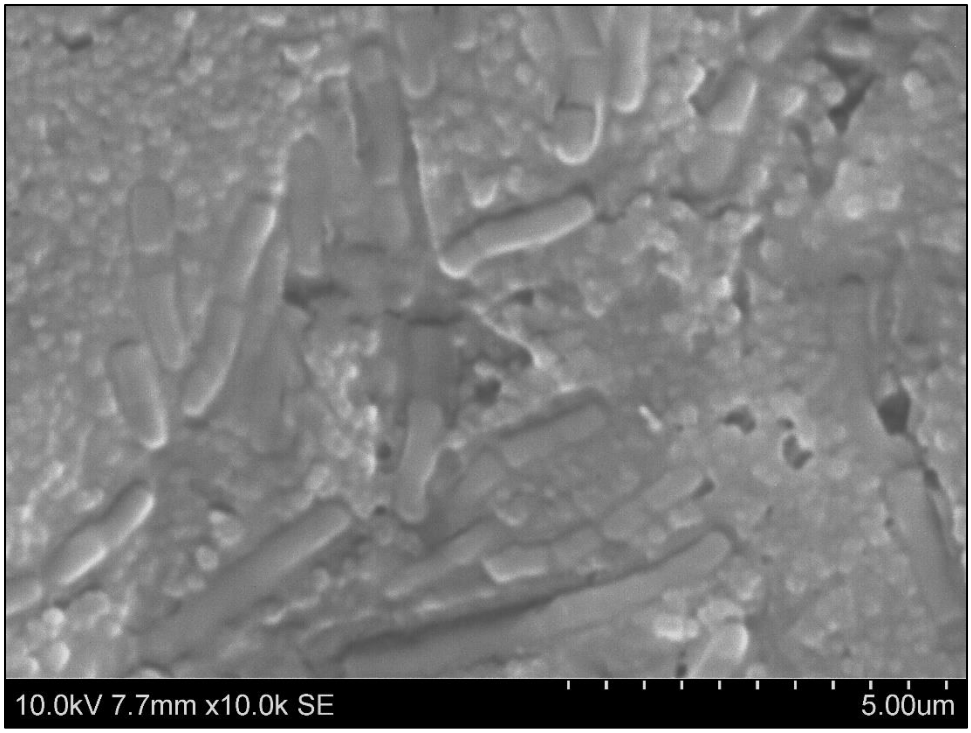
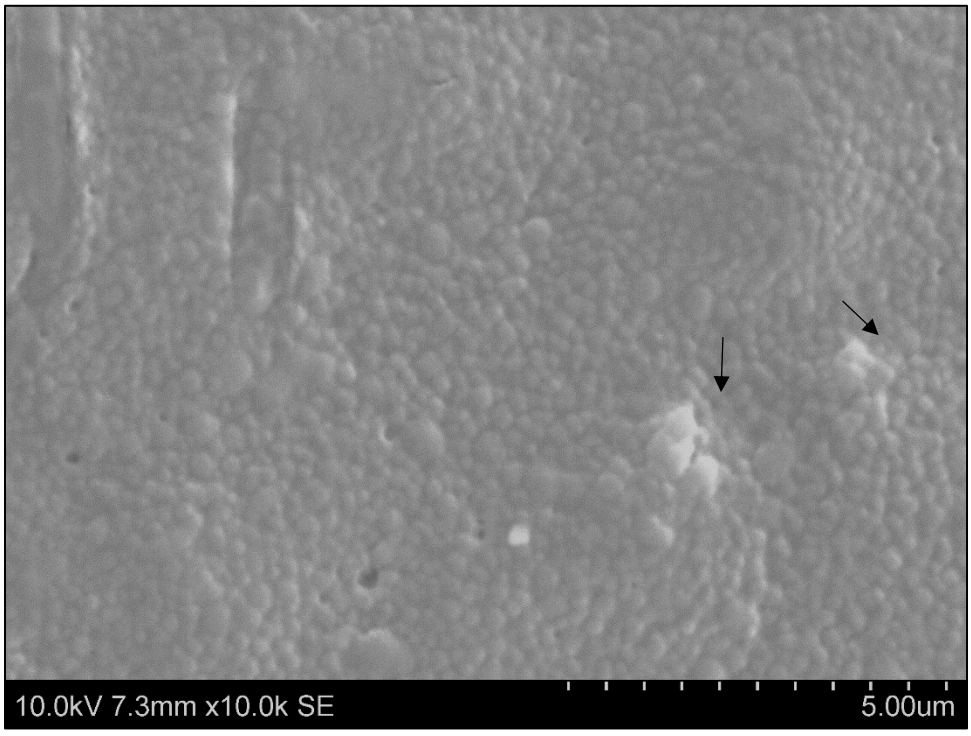



**Figure 4**: Scanning electron micrographs of *Geobacillus stearothermophilus* ATCC®15952™ and its spores within biofilm formed on stainless steel coupons. a) Shows a 7 days old biofilm. b) Shows a 14-21 days old biofilm on SS coupons.

a)




b)



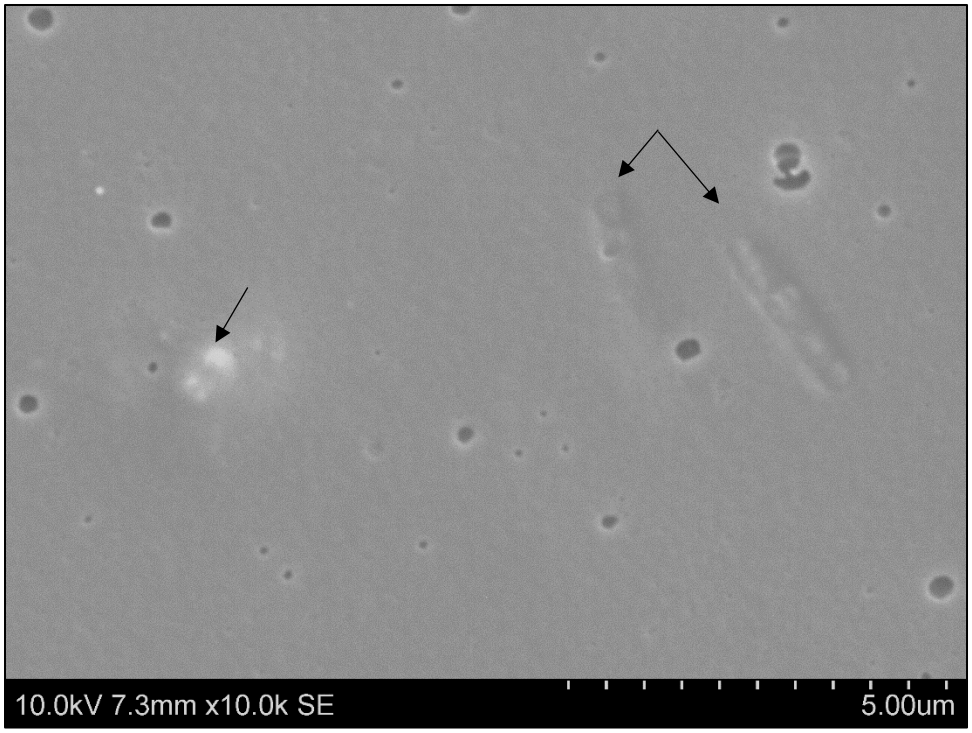
c) 


**Figure 5**: Scanning electron micrographs of *Bacillus licheniformis* ATCC®14580™ and its spores within biofilm matrix on SS coupons a) Shows vegetative cells within 1-day-old biofilm. b) Shows spores within 14-21 days old biofilm. c) Shows embedded vegetative cells and spores within biofilm (21 days old).

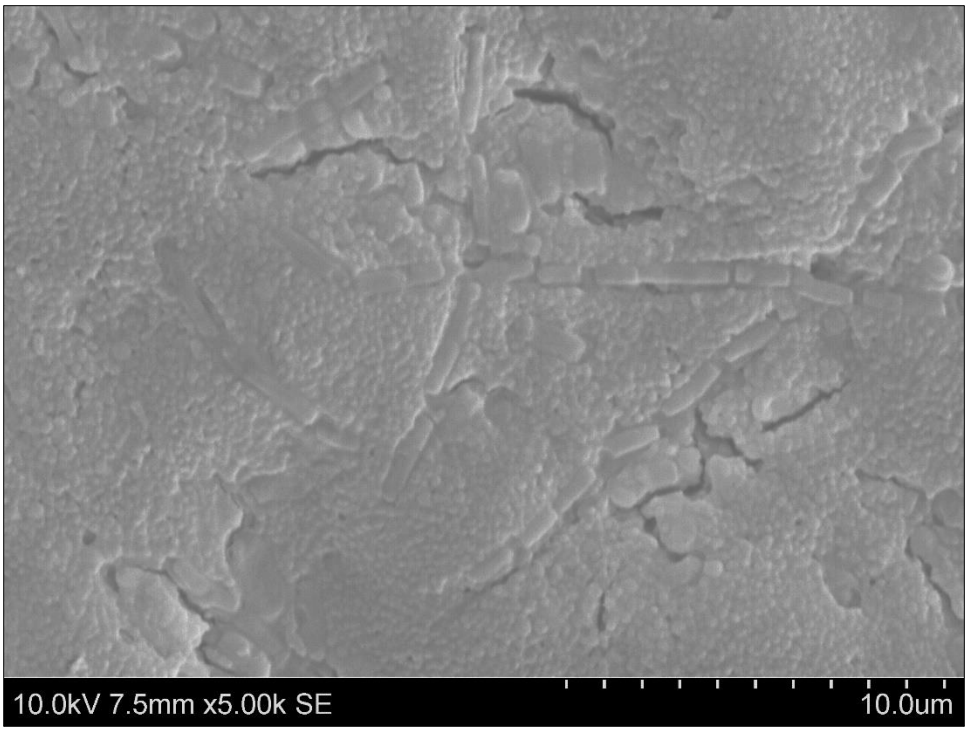
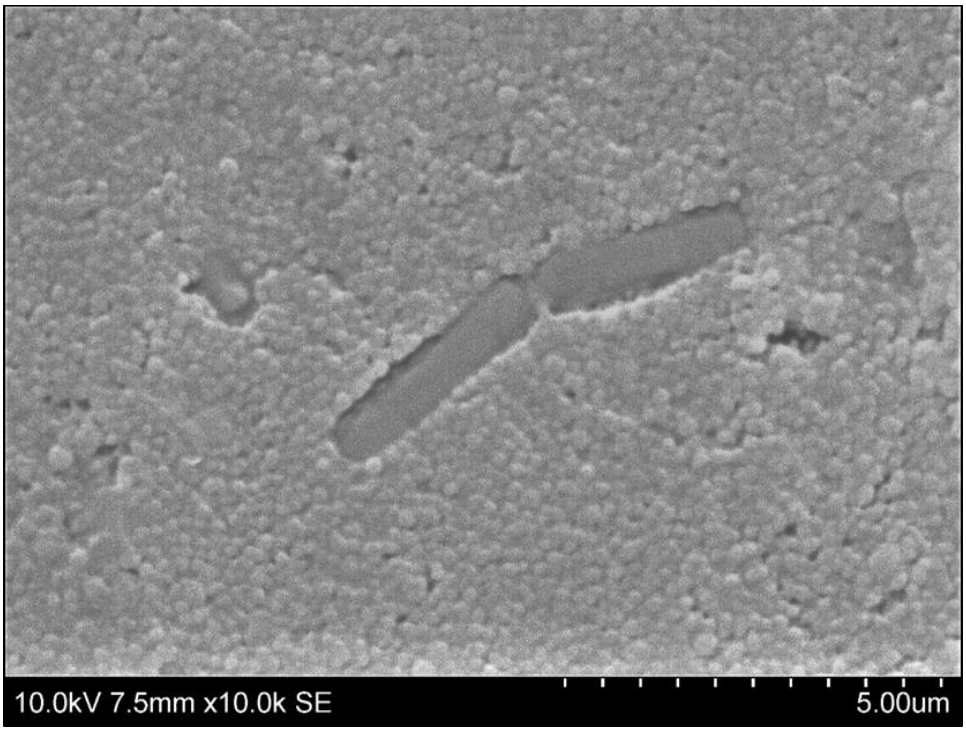



**Figure 6**: Scanning electron micrographs of *Bacillus licheniformis* ATCC®6634™ within biofilm formed on SS coupons but did not form spores within short or long term duration.

a)




b)

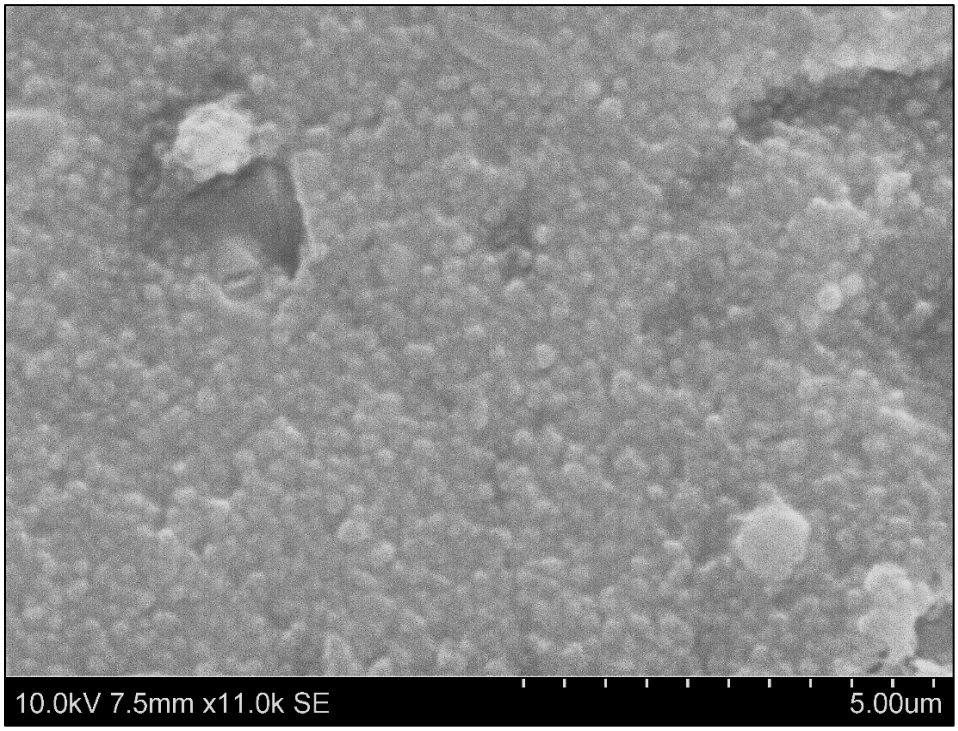



**Figure 7**: Scanning electron micrographs of *Bacillus sporothermodurans* DSMZ 10599$^{T}$ and its spores within biofilm formed on stainless steel coupons. a) Vegetative cells in 12 hours old biofilm. b) Spores in 12 hours old biofilm.

## OVERALL SUMMARY AND CONCLUSIONS

The first study was conducted on commercial cheese samples that have a *P. acidilactici* as a major part of the adjunct culture. The objective of the first study was to understand the effect of the rate of cooling and ripening temperatures on the NSLAB counts and their distribution within the cheese during the ripening process. Also to track the influence of adding *P. acidilactici* on this NSLAB under different controlled ripening temperatures to determine the appropriate ripening condition. The cheese samples ripened for two months had more strain variability and higher counts compared to only cooled cheese or cheese ripened for six months, which indicates higher biological activity that could potentially lead to cheese slits. Predominant isolates remained the same in all samples, which are *Pediococcus acidilactici, Lactobacillus rhamnosus, Lactobacillus paracasei,* and *Streptococcus salivarius* subsp. *thermophilus*, respectively. Their predominance may have led to inhibit the growth of other added cultures such as *Lactobacillus helveticus* and *Lactobacillus casei*. Six months of ripening of cheese samples that were fast cooled to 38°Fand ripened at 50°F lead to control NSLAB counts on cheese, which might help improve cheese quality. In general, if accelerated ripening at 50°F is the goal, our results suggest that the rate of cooling cheese to 38°F or 50°F will not make a difference from the bacterial counts perspective, especially because of the predominance of *Pediococcus acidilactici* as an adjunct. However, bacterial types and their distribution pattern as affected by different rates of cooling might matter the most. Our finding could lead to help standardize the ripening process for semi-hard Italian cheese with the added *Pediococcus acidilactici* as an adjunct culture.

The second study was performed to understand the sporulation within biofilm matrix when formed by common sporeformers in dairy plants in short and long-term incubation. The three strains used in this study were *Bacillus licheniformis* (ATCC®6634™ and ATCC14580), *Geobacillus stearothermophilus* (ATCC® 15952™), and *Bacillus sporothermodurans* (DSMZ 10599$^{T}$). We found changes in the vegetative cells within biofilm matrix in the short-term duration of biofilm formation. In addition, all the species have the ability to form biofilm rapidly. We also observed a variability in species as *Bacillus sporothermodurans* were the only species that formed spores in a short-term (12 hours), but the others did not. *Geobacillus stearothermophilus* formed spores on the stainless steel coupons in 3 days, *Bacillus licheniformis* ATCC®14580™ formed spores after seven days in the milk medium and within biofilm matrix on the stainless steel coupons, while *Bacillus licheniformis* ATCC®6634™ showed no sporulation even after a long-term duration of biofilm formation (21 days). Our hypothesis in this study was proven, which was, if the formed biofilm is kept for longer duration all the organisms will start forming spores at different time intervals based on the species. The outcome of this study will affect the cleaning system that was previously applied to dairy plants. Understanding the species variability in sporulation is a useful tool to eliminate them.