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Foaming properties of pasteurized milk with 1 % fat: could it be affected by lipolysis and proteolysis due to *Pseudomonas* spp. growing in cold-stored milk?

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Food Science – Product Development

Foreword

This study was initially conducted in Spring semester 2020 and completed in Autumn semester of 2020 as my final work in the completion of my master's degree in Food Science, major in Product Development and specialization in Economics and Management, at the Norwegian University of Life Sciences. This thesis is a confidential project and funded by Kavli Trust (Q-meieriene), and was carried out at the Faculty of Chemistry, Biochemistry and Food Science in NMBU Ås.

I would like to take this opportunity to thank all the people whose assistance was a milestone in the completion of this thesis. I wish to express my sincere appreciation to my advisers, Professor Judith Narvhus and Professor Tove Gulbrandsen Devold, for their advice and sharing their expertise in this study. Their persistent help, guidance and encouragement had been monumental in the realization of this thesis.
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Lastly, a heartfelt thank you for the support and the great love of my family and friends, who have kept me going and whose input played a big part in my years of studying in NMBU.

I give the highest praise and deepest gratitude to the Heavenly Father, who has been my rock, strength and provider in this time of my life. Indeed, He is trustworthy and faithful in revealing Himself to those who seek Him (Psalm 9:10). To God be the glory!

Clarideth Cabusao Libut
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Abstract

A velvety, shiny, and compact foam with fine bubbles is desirable in the coffee shop industry to produce coffee drinks like cappuccino or café latte. However, baristas in the cafés are not always able to create this desirable steam-frothed milk foam.

The main objective of this study is to investigate whether the selected strains of *Pseudomonas* spp. showing diverse lipolytic and proteolytic capabilities affect the foaming properties of refrigerated semi-skimmed milk with 1 % fat. This study measured the extent of lipolysis and proteolysis in terms of free fatty acid concentration and casein degradation in pasteurized milk with 1 % fat.

The slight casein degradation in the two milk samples did not show a significant effect on the foaming properties. However, not all the casein degradation in the milk were visible in the electropherograms. Therefore, the effect of bacterial proteases on the foaming properties could not be eliminated. On the other hand, the significant amount of FFA liberated by the *Pseudomonas* strain with the highest lipolytic capability showed a direct correlation with the impaired foaming properties of the milk. Challenges in forming a foam suitable for latte art could be affected by poor microbiological quality. Although psychrotrophic bacteria, which predominate in raw milk, are eliminated during pasteurization, the heat-stable extracellular lipases and proteinases produced by the bacteria, specifically *Pseudomonas* spp., survive the heat-treatment and could remain active in refrigerated milk and continuously degrade the fat and proteins in the milk. It is therefore important to develop a more efficient control measures to limit the contamination of the milk. Since *Pseudomonas* spp. thrive in cold temperatures and lipolysis could occur even at cold storage, good hygiene practices should be improved in every step in the supply chain in order to reduce the contamination.

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1. Introduction

In cafés, milk is used to create steam-frothed foam to produce coffee drinks such as café latte or cappuccino. The milk foam determines the overall characteristic of the coffee with its essential contribution to the body, texture, mouthfeel and the release rate of the coffee aroma (Khezri, Shahriari, & Shahsavani 2017). The steam-frothed foam is usually used in an artisanal manner, for example, as latte art on a coffee. It requires milk to have good foaming properties with at least double the volume of the original milk and have a stable, velvety shiny appearance with fine-textured and densely packed air bubbles, called a "microfoam" (McKenzie & McKenzie, 1995; Coffee Science, 2020; Munchow, Jørgensen, Amigo, Sørensen and Ipsen, 2015). The steam-frothed milk foam is used for latte art immediately after the coffee is made. The coffee is also served immediately, and it is assumed that it is consumed whilst still hot. Thus, the stability of the milk foam should last 10 – 15 min (Huppertz, 2010). However, it has been observed that pasteurized milk used for coffee drinks enigmatically does not consistently produce a good foam. This inconsistency in foaming of milk has resulted in increased interest from the dairy industry to be able to manufacture, and market milk that is well-suited for steam-frothing referred to as "barista milk" (Munchow et al., 2015). It has also stirred the curiosity in investigating the factors affecting the foaming properties of steam-frothed milk foams (Huppertz, 2010).

The factors influencing the foaming properties of milk could be molecular properties and environmental factors. The molecular properties that affect the foaming properties are molecular flexibility, charge density and distribution, and the level of hydrophobicity of the surfactants in the milk. Factors such as temperature, pH, mineral balance, the method of foaming like as cold aeration, steam injection and mechanical agitation, milk protein concentration (Borchedring et al., 2009), and lipids, are environmental factors that affect the

formation and stability of the foams. The influence of lipids on the foaming properties of milk is to impair the foam formation and stability of the milk because lipids coexist with proteins in the interfacial area (Damodaran, 2007). Hydrolytic degradation of triglycerides, (i.e., lipolysis) to monoglycerides, diglycerides and free fatty acids could be the cause of the reduced foaming properties of milk (Deeth & Smith, 1983; Cartier & Chilliard, 1990; Kamath, Wulandewi & Deeth, 2008b; Huppertz, 2010). Lipolysis can either happen spontaneously when the amount of milk lipase is high during late lactation or when bacterial lipases are produced during the growth of bacteria, especially *Pseudomonas* spp. (Deeth, 2006).

Other studies (Andrew, 1983; Corrandini and Innocente, 1994; Buccioni et al., 2013) reported that an increase in proteose-peptone (PP) concentration arising from the degradation of β-casein by plasmin, and during storage as a consequence of the proteolytic activity of endogenous and bacterial enzymes could also impair the foaming properties of milk. A study reported that the proteolytic activity in milk is related to the somatic cell count (SCC), in which the action of PA (plasminogen) - PP becomes higher when the SCC in milk increases (Politis et al., 1989). The foaming properties of milk in the study of Buccioni et al. (2013) showed reducing trend as SCC values increase.

The objective of this study is to investigate whether the liberated free fatty acids and the degradation of proteins by selected strains of *Pseudomonas* spp. during growth in refrigerated milk affect the properties of a steam-frothed milk foam when tested in a pasteurized milk with 1% fat.

2. Background of the study

Milk is a complex liquid food that contains surface-active constituents, e.g., lipids and proteins, that have different structures and molecular composition. The functional properties of milk give the distinct characteristic of a product like texture (appearance), consistency, and mouthfeel, all of which can be qualitatively defined with reference to the molecular properties and mechanisms of each milk constituent. Foaming is an important functional property of milk, particularly for the coffee shop industry wherein milk foam is used in coffee drinks. Knowledge about the properties of each constituent in the milk foam, their mechanisms and coexistence in the foam system would give a better understanding of how to maximize the full potential of the milk to form foams.

2.1. Milk composition

The principal components of milk are water, fat, proteins, lactose, and minerals. Milk also contains trace amounts of other constituents such as vitamins, metal ions and flavor compounds, that also impacts the nutritional, technological and sensorial properties of milk and dairy products (Fox, Uniacke-Lowe, McSweeney, O'Mahony, et al., 2015).
Table 1 shows the main components of milk.

Table 1. **Quantitative composition of cow´s milk in % (Fox et al., 2015).**

Main constituent	%
Water	89
Total solids	12.7
Fat	3.7
Proteins	3.4
Lactose	4.8
Minerals	0.7

Milk fat is a mixture of crystals and liquid fats and is entirely liquid at 40 °C and solid at – 40 °C. The fat globules have an average size of 3 – 4 µm. Milk fat is composed of different fatty-acid esters called triglycerides, which are compounds of alcohol (glycerol) and various

fatty acids. The functional properties of milk containing fat may be influenced by the size and the composition of the dispersed lipid particles (Fox et al., 2015).

Lipids are insoluble in water, and therefore an interfacial tension occurs between oil and water phases when they are dispersed in an aqueous medium. In raw milk, the milk fat globule membrane (MFGM) serves as the surface-active material that covers each fat globule, preventing the coalescence and separation of oil and water in the milk. Milk lipids consist of 97-98 % triglycerides, <0.05 % monoglycerides, <0.5 % diglycerides, <0.05% free fatty acids and <1 % phospholipids (Fox et al., 2015). Phospholipids, monoglycerides, diglycerides, and free-fatty acids belong to the class of polar lipids. Low molecular mass surfactants such as polar lipids could decrease the surface or interfacial tension at a greater extent than high molecular mass surfactants like proteins, due to their differences and orientation and configuration at an interface (Damodaran et al., 2007). Figure 1 presents the orientation of low molecular mass surfactants and high molecular mass surfactants, a fatty acid and a globular protein, respectively, at the liquid interface.

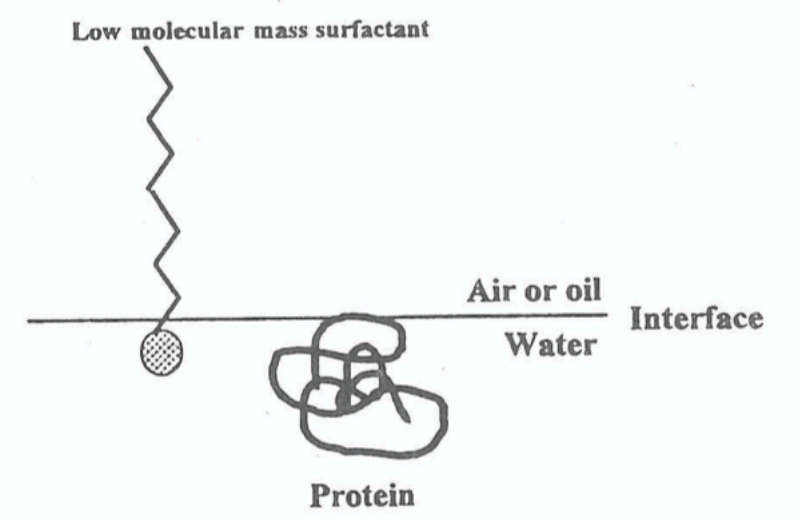


Figure 1. **The orientation of low molecular weight and macromolecular surfactants at liquid interfaces. (Damodaran et al., 2007).**

The entire molecule of a low molecular mass surfactant is adsorbed and oriented in a way that the hydrophilic head group is immersed in the aqueous phase and the lipophilic hydrocarbon

chain is exposed to the nonpolar phase. This division of the entire molecule between the polar and nonpolar phases greatly reduces the interfacial tension (Damodaran et al., 2007).

Proteins constitute approximately 3.5 % of milk and are high-molecular mass surfactants. Their surface hydrophobicity and charge influence the adsorption of proteins at the oil-water or air-water interface. Unlike low molecular mass surfactants, proteins have complex conformational characteristics that do not have a clearly defined hydrophilic head and hydrophobic tails (see Figure 1). Once adsorbed, the proteins unfold and rearrange their three-dimensional structure to expose the hydrophobic parts to the hydrophobic phase. Because of the conformational constraint of the protein, only a portion of the protein molecule lies in the interface and the greatest portion of the protein molecule remains suspended in the aqueous phase in the form of "loops" and "tails". Thus, only a part of the liquid surface or liquid-liquid interface is covered by the protein segments, and the other regions of the surface or interface remain at high free energy levels. For this reason, proteins are generally less surface-active than low-molecular mass surfactants (with energy levels 50 mN/m vs 10-30 mN/m, respectively). But since proteins can form interactions through noncovalent intermolecular interactions and via covalent disulfide cross-linking, the high concentration of protein at the surface leads to the formation of a protein network. Eventually, the proteins form a viscoelastic membrane-like film around oil droplets or air cells. This adsorbed layer serves as a physical barrier to prevent the coalescence of the air bubbles in the foam (Wilde et al., 2004; Damodaran et al., 2007).

Cow's milk constitutes 80% casein and 20% whey proteins. Casein proteins comprise four different types of proteins, namely α_{s1}, α_{s2}, β-casein and κ-casein. Although each type has a distinct primary structure, all of them are hydrophobic and contain many amino acids with nonpolar side chains that are found in clusters along the peptide sequence. They have little secondary structure because they contain a large amount of proline that is difficult to incorporate to the accepted components of the secondary structure of the protein. Because of this, caseins are described as disordered proteins, without specific structure, but flexible. The

flexibility of caseins indicates that in solution, several configurational states could be accessible to the molecules. The application of even small forces could cause the molecules to adopt conformations, like how they are absorbed in the interface and make interactions, and eventually forms the viscoelastic film (Dalgleish, 1997).

Whey proteins are a group of globular proteins consisting of 70 % β-lactoglobulin and α-lactalbumin that are surface-active. The other two major whey proteins are bovine serum albumin (BSA) and the immunoglobulins. The heating procedure applied to whey proteins determines their denaturation in milk. A native whey protein has a definite conformation, which disrupts once the whey protein is exposed to heat above certain critical levels. The disruption of the conformation alters the properties of the whey proteins that influence the functional properties of a dairy product (Cayot and Lorient, 1997).

Lactose is a disaccharide composed of glucose and galactose. Lactose is water soluble and occurs as a molecular solution in milk (Fox et al., 2015).

The minerals in milk occur in solution in milk serum or casein compounds. The essential minerals in milk are calcium, sodium, potassium and magnesium (Fox et al., 2015).

2.2. Foaming properties of milk

Foam is as a two-phase colloidal system in which the gas bubbles are dispersed in a continuous liquid phase. A foam is formed by mechanical stress like mixing, whipping, or shaking, or by injecting air bubbles into the liquid. The foaming properties of the milk used in cafés are assessed by foaming capacity and foaming stability. The foaming capacity refers to the ability to produce a large interfacial area so that a large volume of gas can be incorporated and can be determined as the volume of foam formed from a given quantity of milk. The foaming stability refers to the ability to create a strong and firm interfacial film that can

withstand internal and external forces, that can be determined by the foam´s ability to retain its volume as a function of storage (Damodaran et al., 2007; Huppertz, 2010).

Foam destabilization is influenced by disproportionation, drainage and coalescence that all happen simultaneously and in synergy (Figure 2). Disproportionation is a result of the difference in pressure between the air bubbles due to the Laplace pressure ($\Delta P = \frac{2\gamma}{R}$) in each bubble. The small bubbles have higher gas (small radius R) pressure than large bubbles, so they have the tendency to diffuse through the liquid phase towards large bubbles. Thus, as time increases, the average-sized bubbles become larger, and the large-sized bubbles become much larger. Hence, foams with non-uniform dispersion of air bubbles are susceptible to coarsening that eventually leads to destabilization. Due to gravity (g: acceleration), drainage occur as the water leaks out from the foam based on the difference in density between gas phase (ρ_{gas}) and water phase (ρ_{water}), and the bubbles moves upward. The thin film then becomes thinner, and starts coalescence of the air bubbles (Deng, de Ruiter, Schröen, 2019). Coalescence happens when the film between two air bubbles in the foam ruptures. The driving force of coalescence is the decrease in free energy resulting when a reduction in total surface occurs after a film rupture. The consequence of coalescence in milk foams can cause coarsening of the air: liquid dispersion, and the air bubbles become larger (Walstra, 2003). Figure 2 illustrates these three foam destabilization mechanisms.

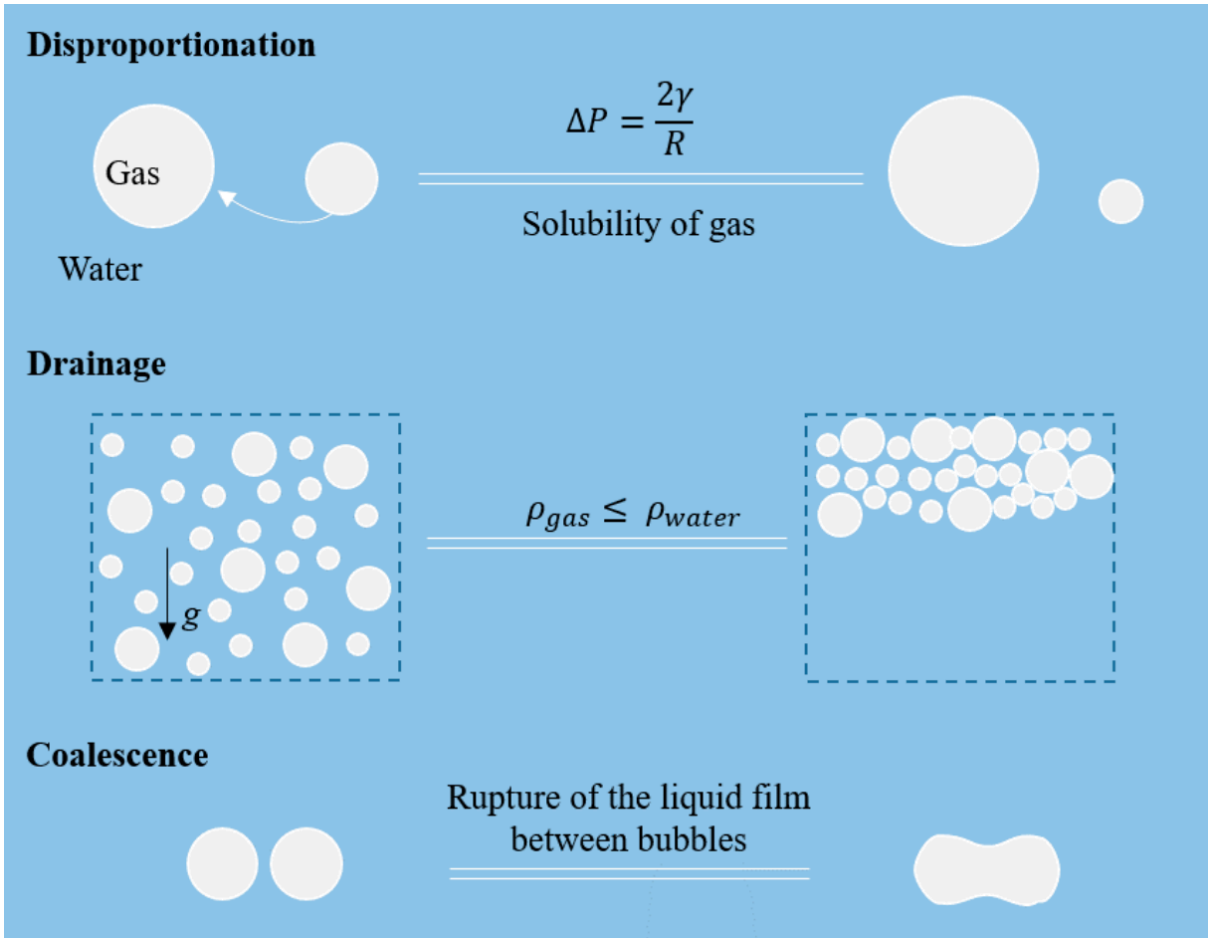
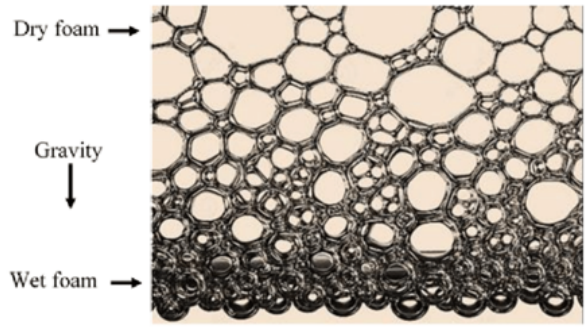
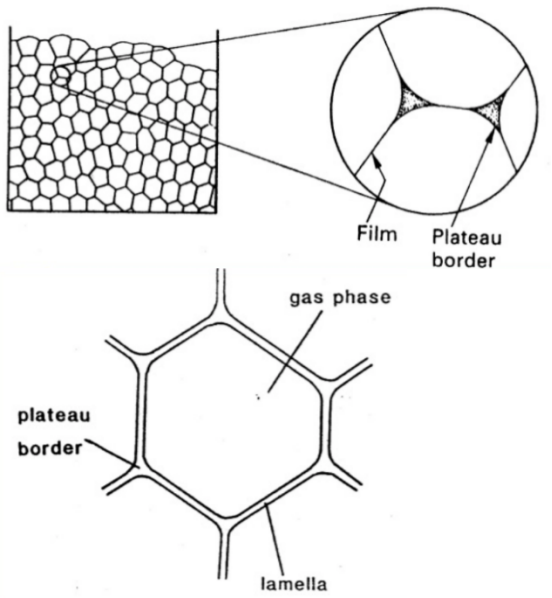


Figure 2. **Foam destabilization mechanisms: disproportionation, drainage and coalescence (Deng et al., 2019)**

Milk foam is classified initially as a spherical foam, containing spherical air bubbles dispersed in a continuous milk phase. In spherical foams or "wet" foams, the liquid drains in a downward movement, at the same time, the continuous phase of the bubbles moves upward, as a result of the large density difference between the air bubbles and the continuous milk phase (Walstra, 2003; Deng et al., 2019). Over time, the spherical bubbles in milk foam turn into polyhedral foams when sufficient drainage has occurred. The spherical bubbles come into contact with each other, and the buoyancy forces cause them to be deformed and become polyhedral bubbles. After attaining polyhedral bubbles, the foam become as "dry" foams. Figure 3 illustrates the foam structures in wet and dry foams and polyhedral foams.



a. **Foam structure in the form of dry and wet and its variation under gravity. (Nour et al., 2018)**



b. **Illustration of polyhedral foam and the lamella and plateau borders in such foams. (Alias, 2013)**

Figure 3a - b. **Structure of foams in the form of dry and wet foams (a), and structure of polyhedral foam (b).**

The network of air bubbles is separated by a thin liquid layer, called lamella, and the corners where more than two bubbles meet are referred to as plateau border.

The air bubbles formed during milk foaming are stabilized by surface-active constituents that are adsorbed at the air and water interface of the bubbles. Milk contains two surfactant components, which are high molecular mass surfactants, e.g., proteins, and low molecular mass surface-active components belonging to lipid class, e.g., polar lipids.

Proteins stabilize foams by forming a viscoelastic film on the surface, while surface-active lipids stabilize foams through the Gibbs-Marangoni mechanism.

The Marangoni effect is the mass transfer on an interface between air: liquid due to a gradient of the surface tension, which occurs in foams under dynamic conditions because of drainage. When the liquid flows through the lamella during drainage, surfactant layers are adsorbed on either side of the film that causes stretching and thinning of the lamella. Once the lamella is stretched, the increase in surface area of the film decreases the surfactant concentration at the

surface and consequently the surface tension increases. Then, the surfactant layers migrate from both sides of the lamella to the opposite direction of the liquid flow. The rapid movement of the surfactant layer reduces the surface concentration gradient. As the surfactant layer moves toward the high-surface tension regions, it carries a volume of underlying liquid with it (water in foams). This liquid restores the presence of fluid between the air bubbles, that consequently restores the thickness of the lamella, hence, preventing coalescence of the air bubbles and retards the rate of drainage (Wilde et al., 2004; Damodaran et al., 2007).

2.3. Competitive destabilization of a foam

Although the individual properties of proteins and polar lipids (e.g., phospholipids and the products of lipolysis) can stabilize foams, the presence of these two types of surfactants together in a foam system produces less-stable foams. The coexistence of milk proteins and polar lipids at the milk foam interface is incompatible as both surfactants compete for the air: liquid interface (Wilde et al., 2004; Huppertz, 2010). Figure 4 illustrates how the competition of the protein and polar lipids at the air: liquid interface is thought to destabilize a lamella that separates the air bubbles in the foam.

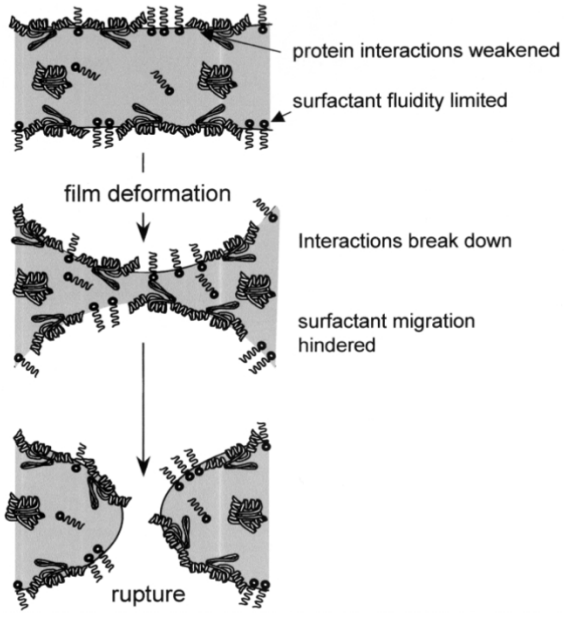


Figure 4. **Competitive destabilization due to the coexistence of proteins and polar lipids at the milk foam interface. (Wilde et al., 2004).**

This incompatibility results in the protein interaction in the surface layer being disrupted, and the rapid migration of the polar lipids towards the area with high-surface tension as required by the Gibbs-

Marangoni mechanism becomes limited. This phenomenon is called competitive destabilization of the foam.

Huppertz (2010) reported that the addition of 1 volume of reconstituted whole milk to 99 volumes of skim milk (i.e., a concentration of ~0.04% fat in the milk) strongly destabilized foam stability. Increasing the volume of whole milk up to ~10% added to skim milk (i.e., a concentration of ~ 0.4% fat in the milk) showed a decrease in foam capacity and foam stability. Huppertz (2010) also exemplified the poor foaming properties of milk due to the presence of phospholipids in the milk foam by comparing the foaming properties of buttermilk (13 % w/w) to the foaming properties of reconstituted skim milk (10 %, w/w) and whole milk (13 %, w/w). The fat globules in buttermilk are generally smaller than those in whole milk (Sodini, Morin, and Jimenez, 2006) and should cause less coalescence of air bubbles in the milk foam based on theory (Prins, 1986). However, the data obtained by Huppertz (2010) showed that the buttermilk had considerably less foam volume compared to the reconstituted skim milk and whole milk, while both the buttermilk and whole milk showed inferior foam stability. Since a considerable proportion of the phospholipids in the buttermilk is present in the serum phase as fragments of the milk fat globule membrane (MFGM), which are surface-active, the phospholipids migrate to the water interface and destabilize the foam. Therefore, this results in the general poor foaming properties of buttermilk (Huppertz, 2010).

Also, low molecular mass surfactants have shown a detrimental effect on the foaming properties of protein-stabilized foams and are often utilized as antifoaming agents (Lee and Tynan, 1988). Since low-molecular mass surfactants are able to reduce the surface tension to lower values than the proteins, relatively small concentrations of surfactant could affect the surface tension of protein-stabilized foams. When the film is deformed, as shown in Figure 4,

the protein-stabilized films and the polar lipid-stabilized films are weakened and no longer strong enough to maintain the integrity of the bubbles. The probability of the rupture of the film (air bubbles in foam) is, therefore, increased (Wilde et al., 2004). The surface layer of the air bubbles in foam that consists of a combination of proteins and polar lipids contains thin and thick regions, wherein the polar lipids saturate the thin areas, and the proteins dominate the thick parts (Wilde et al., 2004).

2.4. Food spoilage microorganisms that have negative impact on milk quality and foaming properties

Members of the genus *Pseudomonas* are common microorganisms in raw milk. They are of major concern for the dairy industry because of their negative effect on the quality of dairy products (Sørhaug & Stepaniak, 1997). Although an effective cold chain is applied on the farm, during transport and in the dairy plant, *Pseudomonas* spp. are able to grow at these low temperatures. Strains of *Pseudomonas* spp. can produce heat-stable proteases (Liu et al., 2007) and lipases (Chen et al., 2003) when the cell density is sufficiently high, that is during the late log or early stationary growth phases. Several studies (Dunstall et al., 2005; Barbano et al., 2007; De Jonge et al., 2011) have reported that the bacterial enzymes produced by *Pseudomonas* spp. remain active after pasteurization at 72 - 75 °C for 15 – 20 s, and even UHT treatment at 130 – 150 °C for 2 – 4 s and could therefore still degrade the proteins and fat in a heat-treated dairy product. Although the bacterial enzymes are heat-stable, most of the proteinases are more unstable at temperatures between 55 – 60 °C than temperatures above 100 °C. This phenomenon is called low-temperature instability. The loss of activity at 55 – 60 °C is said to be the result of enzyme-casein aggregates, and not due to autoproteolysis that occurs in the absence of milk proteins (Sørhaug & Stepaniak, 1997).

Aside from the influence of the milk composition on the foaming properties of milk, the microbiological quality of the raw milk also affects the milk's foaming ability, which may be an effect of lipolysis or proteolysis in milk.

2.5. Lipolysis

Lipolysis is the enzymatic breakdown of triglycerides and the formation of free fatty acids. It is caused either by indigenous lipoprotein lipase (LPL) or by bacterial lipases produced by *Pseudomonas* spp. (Deeth, 2006). The LPL is predominantly associated with the casein micelles and compartmentalized from its substrate (milk fat) by the milk fat globule membrane (MFGM). Slight damage to the MFGM could allow the contact between the LPL and the milk fat, resulting in the hydrolytic degradation of the triglycerides. The LPL becomes active at an optimal temperature of ~37 °C and ~pH 8.5 and can be stimulated by divalent cations like Ca^{2+}. However, due to factors like milk pH, ionic strength, non-optimal temperature, association with the casein micelles, isolation from the substrate by the MFGM, the LPL is unlikely to be fully activated in milk (Fox et al., 2015).

Some free fatty acids and monoglycerides and diglycerides are present in the milk because of incomplete triglyceride synthesis in the mammary gland. Still, the primary cause is lipolysis (Huppertz, 2010). A study (Deeth & Smith, 1983) reported a negative correlation between free fatty acid (FFA) concentration and the foaming properties of milk, wherein FFA concentration as low as 1.5 μequiv mL^{-1} showed a detrimental effect on the foaming properties. Studies have also shown a correlation between a reduction in the surface tension of the milk: air interface and impaired foaming properties of the milk (Buchanan, 1965; Deeth & Smith, 1983 and Kamath et al., 2008b). Foaming of milk either by steam-frothing (Deeth and Smith, 1983; Kamath et al., 2008b) or cold aeration (Kamath et al., 2008b)

showed that a higher concentration of free fatty acids impairs the foaming capacity and foaming stability of milk. The foams formed from both steam-frothing and cold aeration were reported to be coarser and had larger bubbles as FFA concentration increased (Kamath et al., 2008b).

In the review by Huppertz (2010) on the influence of milk composition on foaming properties of milk, he suggested that the rapid coarsening of milk foam is may be due to the polar lipids present in milk. Coarsening of the milk foam happens because of the coalescence of the air bubbles, which could rapidly initiate also because of the competitive destabilization of the foam that happens when the polar lipids compete with the proteins at the air: liquid interface. Huppertz (2010) also mentioned that even if the correlation between the concentration of free fatty acids and the foaming properties of milk has already been established, it would not necessarily mean that free fatty acids are solely responsible for the reduction of foaming properties.

In the study of Buchanan (1965), the addition of varieties of polar lipids to milk showed that the foaming properties of the milk could be dependent on the chain length of the free fatty acids. The results showed that the foaming properties improved with the addition of butyric acid (C4:0). In contrast, the addition of caprylic acid (C8:0) slightly reduced the foaming properties of milk, and the addition of the sodium salt of stearic acid (C18:0) showed a significant reduction in the foaming properties of milk (Buchanan, 1965). This suggests that the foaming properties is reduced as FFA chain length increased.

The lipolytic capability of the bacterial enzymes from *Pseudomonas* ssp. was studied by Tidemann (2016). The growth characteristics and lipolytic capability of eight isolates of *Pseudomonas* ssp. confirmed that in raw milk, *Pseudomonas* continuously grew and

hydrolyzed lipids in refrigerated milk at 4 °C and reached a concentration between 8 to 9 log CFU mL^{-1}. The lipolytic capability was measured by quantification of FFA by Gas Chromatography (GC) using Flame Ionization Detector (FID). It was observed in this study that a high bacterial count does not always correlate with increased lipolytic capability because the *Pseudomonas* strains showed diverse lipolytic capabilities. The results showed that in UHT-treated milk, FFA concentration increased as the incubation temperature and incubation time increased. The highest concentration of the various fatty acids was found after 9 days of storage at 22 °C. It was also concluded in this study that the accumulation of FFA from Day 0 to 4 at low temperature, e.g., 4 °C, occurs if untreated raw milk has been stored beyond 4 days before pasteurization.

2.6. Proteolysis

Plasmin (PL) is an indigenous enzyme in milk, which initially exists in a zymogen form (Plasminogen, PA) and being converted to active PL by the action of tissue-type and urokinase-type activators. Plasmin is associated with casein micelles in milk and degrades β-, α_{s1}- and α_{s2}-caseins to γ-caseins, proteose-peptones and possibly λ-casein. Plasmin contributes to proteolysis during ripening of some cheese varieties depending on cooking temperature and pH during ripening. The enzyme is heat resistant and survives UHT treatment (Bastian and Brown, 1996). The conversion of PA to PL continuously happens, from when the milk is still in the mammary lumen, as well as after milking and during storage of milk. Thus, several factors could influence the concentration of active PL like the stage of lactation, somatic cells content and hygiene of the cows (Mateos et al., 2015).

Proteolysis could also influence the foaming properties of milk as the effect of total proteose peptone content in the milk has been linked to impaired foaming properties. The proteose-peptone (PP) fraction is a mixture of peptides, which are mostly produced by PL, and some are indigenous to milk. The proteose-peptone (PP) fraction represents ~10 % of whey protein

and is composed of 38 components. Several components are derived from the lysis of β-casein by plasmin (PL); and different glycoproteins or hydrophobic constituents (Andrew, 1978; Innocente et al., 1988;). The PP fractions are 5, 8_{slow}, and 8_{fast} (PP5, PP8s and PP8f) and proteose-peptone 3 (PP3). The PP5, PP8f and PP8s are said to have no technological significance, while PP3 has several technological functionalities such as surfactant properties (Fox et al., 2015). An increase in the components (PP5, PP8f and PP8s) as a result of protein degradation by bacterial enzymes during milk storage was reported by Andrew (1983). Slight differences between the breakdown patterns induced by storage and by added plasmin were observed on pasteurized milk in the study.

The relationship between PP concentration in milk and foaming capacity was reported by Innocente et al. (1994). In this study, decreasing milk foaming capacity was observed as PP concentration in milk increased. It was also reported that the emulsion obtained in the presence of PP3 was more stable over time compared to those that contain an unpurified fraction of PP (Innocente et al., 1994). The foaming capacity of milk was also reported to be reduced with increasing somatic cell count (SCC) (Gambini et al., 1995); and an increase in SCC is said to be correlated with increasing plasmin concentration and proteolysis in milk (Kelly and McSweeney, 2003).

The study of Buccioni et al. (2013) mentioned that the proteose peptone could be divided into two groups of proteins. The first group consists of peptides; PP5, PP8f and PP8s originates by lysis of β-Cn. The second group is formed by PP3 (Lactophorin) and several glycoproteins. For this reason, the authors concluded that the impaired foaming properties of milk could be related to high concentrations of PP in which PP3 was not dominant. The concentration of PP fractions was not determined on this study, however, the total PP concentration in the milk sample with poor foaming properties was determined to be 3.37 mg ml^{-1} of milk compared to

PP concentration of 0.11 – 0.75 mg ml^{-1} of the milk sample with good foaming properties. Furthermore, PP3 is not formed after the milk ejection; instead, it is expressed exclusively in mammary tissue of ruminants (Pedersen et al., 2011). PP3 was also reported to be a suitable substrate for PL, which reduces the lactophorin to a shorter fragment (residue 54 – 135) that showed poor emulsifying properties (Sørensen and Pedersen, 1993).

Proteases from *Pseudomonas* are predominantly active towards the casein fraction, producing a grey color of the milk, bitter off-flavors, gelation of UHT treated milk and decreased yield of soft cheeses. The bacterial enzymes destabilize the casein micelles through hydrolysis of caseins that results in the formation of a gel structure or coagulation of heat-treated milk during storage. The coagulation in UHT-treated milk starts with an interaction between whey proteins (β-lactoglobulin) and casein (κ-casein) during heat treatment. Then, the bacterial proteinases hydrolyze the β-lactoglobulin - κ-casein interaction (βκ-complex) and then protein networks are formed into a gel. The proteases are mostly metalloproteases, which are rich in alanine and glycine residues and poor in cysteine and methionine residues. Calcium is needed for the activity and stability of these proteases, which prefers to hydrolyze κ-casein and β-casein, thereafter α_{s1} casein in milk proteins. Caseins are more prone to proteolysis of either endogenous proteinases or bacterial proteinases because of their open and flexible structures (Samaržija et al. 2012; Mateos et al., 2015).

The proteolytic capabilities of enzymes from seven isolates of *Pseudomonas* ssp. were studied by Bækkelund (2016) in parallel with the study of lipolytic capabilities of the same strains by Tidemann (2016). The proteolytic capabilities of the *Pseudomonas* strains were measured in terms of casein degradation in the milk by quantifying the free amino acids using High-Performance Liquid Chromatography (HPLC) and determining protein degradation and

observing peptide formation by Capillary Electrophoresis (CE). The study showed that there was a considerable increase in free amino acids as incubation temperature and incubation time (storage) increased. CE analysis also suggested a complete degradation of the κ-casein, and a reduction of the contents of β-casein A1 and A2.

2.7. Objective of the study

It is of great interest for the dairy industry to identify the cause of impaired foaming properties of milk, specifically for the pasteurized milk with 1 % fat being the preferred milk used to create low-fat steam-frothed milk foam. Therefore, this study aims to investigate whether the liberated free fatty acids and casein degradation by selected *Pseudomonas* strains affect the foaming properties of pasteurized milk with 1 % fat. The results of this study could provide more concrete information that could lead to solutions in producing milk with consistent good foaming properties and suitable for steam-frothed milk.

3. Materials and Methods

Overview

Raw milk (40 L) was collected from the University farm, Ås Gård, on three occasions (09.03.20, 30.04.20, and 10.06.20) immediately after milking. Five cows from the herd were randomly selected. This milk was mechanically drawn from the cow's udder and was collected directly to a sterile milk churn, and then delivered to the pilot plant at NMBU. It was desirable to use freshly drawn milk to avoid any unknown historical factors such as the growth of diverse bacteria during cold storage. The raw milk was treated by separation (Milky FJ130, Bob White Systems, Vermont, USA), standardization to 1 % fat (MilkoScan FTIR, Hilleroed, Denmark), homogenization (Rannie MODEL LAB 4580/71, Copenhagen, Denmark) and pasteurization using equipment in the NMBU pilot plant.

Figure 5 shows the experimental design of this study.

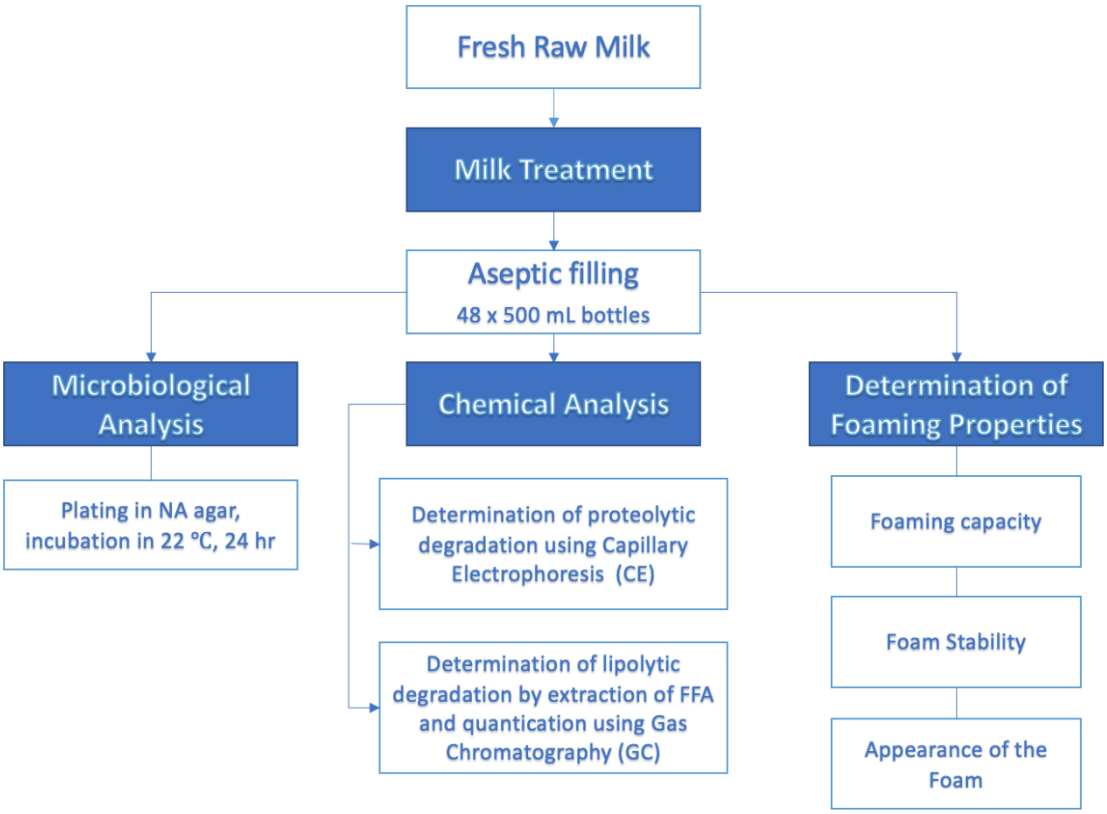


Figure 5. **Experimental design**

The experiment included the measuring of the growth of bacteria in the milk (microbiological analysis), determination of FFA content in the milk by FTIR and quantification by GC; and determination of proteolytic degradation by CE (chemical analyses); and determination of the foaming properties of the milk samples. The treated milk was divided for these analyses as shown in Figure 6.

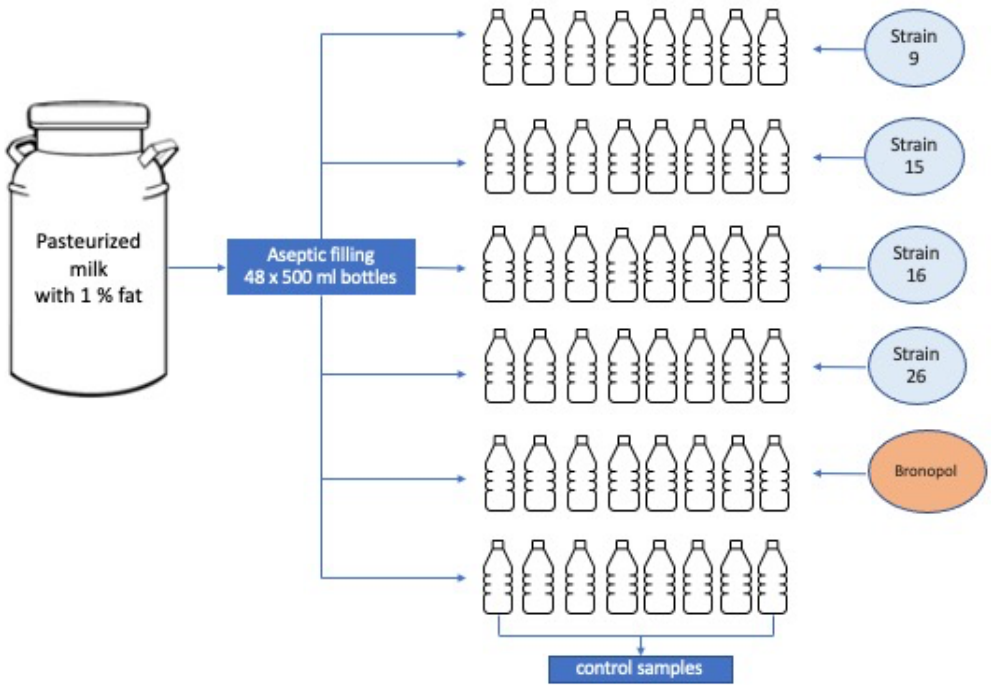


Figure 6. **Schematic diagram of the division of the treated milk.**

The treated milk was divided into 48 x 500 mL sterile bottles. The 48 bottles made up the 6 samples that were used in order to study the growth of the selected *Pseudomonas* strains and their effect on the foaming properties of milk from Day 0 – 7 (total of 8 days). Of these, 4 x 8 bottles were inoculated with four *Pseudomonas* strains (9, 15, 16 and 26). Further, 8 bottles were not inoculated and used as control samples, and 8 bottles were added Bronopol to hinder any bacterial growth. All the samples were stored at 4 °C.

Effect of COVID-19 pandemic on the study

This experiment was repeated three times. However, due to the COVID-19 pandemic, the microbiological analysis and the assessment of foaming properties of the milk were performed in two different locations (NMBU Ås and a private residence in Bærum). The first trial was interrupted in Day 3 at the start of the lockdown. The milk samples were then transported in a thermal box with ice, from the cooling room in the laboratory at NMBU Ås, to a refrigerator in Bærum. The microbiological analysis and the determination of the foaming properties were therefore carried out in Bærum from Day 4 until Day 7 of Trial 1.

In an attempt to continue the experiment during the lockdown period, the treated milk samples for Trial 2 were inoculated and then transported from NMBU Ås to Bærum. The milk treatment was carried out in the pilot plant in NMBU Ås, and then the samples were stored in a refrigerator in Bærum at 4 °C. The microbiological analysis and the determination of the foaming properties in the second trial were also performed in Bærum.

All the tests during the third experimental trial were eventually completed in NMBU Ås.

The growth of *Pseudomonas* in the milk and the foaming properties were measured daily from Day 0 – 7.

For the chemical analyses, the milk samples from Trial 1 and 3 were taken as samples. For each milk sample, 2 x 50 mL was set aside daily and stored at -20 ºC until the chemical analyses. The uninoculated standard milk from Day 0 (M0) and all the 6 milk samples from Day 7 were analyzed further to determine the lipolytic and proteolytic degradation in the milk. The samples from Day 7 consisted of the milk inoculated with *Pseudomonas* strains (9, 15, 16 and 26); plus, the two controls, uninoculated standard milk (M), and the milk sample with Bronopol (MB). Lipolysis was determined by extraction of free fatty acids and quantification by Gas Chromatography. The degradation of proteins, a result of proteolytic action, were analyzed using Capillary Electrophoresis.

3.1. Pilot Study

A standardized method in assessing the foaming properties of milk was developed during the pilot study. The pilot study also included selecting strains of *Pseudomonas* from the previous studies performed by Tidemann (2016) and Bækkelund (2016) that exhibited diverse lipolytic and proteolytic properties. A procedure for creating the steam-frothed foam was also developed in cooperation with Kavli, in an attempt to mimic the process being performed by café staff (also called "baristas"). The assessment of the foaming properties of milk was determined according to the desired foaming properties of a steam-frothed milk foam used in cafés.

3.1.1. Selection of the *Pseudomonas* strains

The pilot study included the selection of the *Pseudomonas* strains from the study of Tidemann (2016) and Bækkelund (2016) based on the degree of their lipolytic and proteolytic capabilities. The free amino acids and free fatty acids produced by the 8 isolates of *Pseudomonas* after 9 days incubation at incubation temperatures 4, 8 and 22 ºC were compared to each other. The *Pseudomonas* strains were then selected based on having the highest and moderate lipolytic and proteolytic capabilities.

3.1.2. Foaming properties of different types of milk

The foaming properties of pasteurized milk (store-bought) with different fat content and UHT semi-skimmed milk (delivered by Tine) were studied to develop a standardized method for assessing the foaming properties. The foaming properties of the different milk types were also considered to determine the fat content that should be used for the experiment.

The milk samples used in the pilot study were UHT semi-skimmed milk with 1.2 % fat, pasteurized semi-skimmed milk with 1 % fat, pasteurized full-fat milk with 3.5 % fat,

pasteurized skimmed milk with 0.1 % fat. A coffee machine (Nespresso Creatista Plus, Nestle Nespresso, Lausanne, Switzerland) was used to make a standardized steam-frothed foam, mimicking the procedure performed by a barista. The test was done in two parallels for 2 days.

3.1.2.1. Foaming capacity

150 mL of each milk sample was used to create a steam-frothed foam using the Nespresso Creatista Plus coffee machine. The volume of the foam (mL) was measured after it had been poured into a 500 mL measuring cylinder (BRAND Silberbrand ETERNA 500:10 mL, Boro 3.3, low-form, H: 250 mm, Duran®, Germany) and referred to as time 0.

Three drops of methylene blue dye were added in the measuring cylinder before the steam-frothed milk foam was added in order to facilitate visualization of the division between the foam and the liquid phase. The methylene blue was prepared by slowly dissolving 1.5 g to 100 mL 95 % ethyl alcohol (Sigma-Aldrich, Missouri, USA). Then, 30 ml of the saturated alcoholic solution of methylene blue was added to 100 mL distilled water and 0.1 mL of 10 % potassium hydroxide (Sigma-Aldrich, Missouri, USA).

The stainless jug containing the milk and steam-frothed foam was slightly pounded on a hard surface and then swirled two times to disperse the bubbles. The volume of the foam (mL) created from each milk sample at time 0 was compared to determine the foaming capacity of each milk sample.

3.1.2.2. Foam stability

A maximum time of 30 minutes was set as a standard time in measuring the foam stability of each milk sample. The changes in the volume of the foam (mL) from 0 to 30 min whilst standing at room temperature were observed. The foam stability of the milk was assessed by

measuring the volume of the foam (mL) at 0, 15 and 30 min to determine which of the milk samples was able to produce a steam-frothed foam that could last up to 30 min.

3.1.2.3. Appearance of the foam

The appearance of the steam-frothed milk foam produced by each milk sample was observed at 0, 15 and 30 min. An attempt to measure the bubble size under a microscope (Wild M8, Wild Heerbrug, Switzerland) using 40x magnification was made. A teaspoon-full of foam was placed on a glass slide to see an image of the bubbles under a microscope and then photographed by a phone camera (iPhone 7plus, Apple Inc., California, USA).

The changes in the appearance of the foam in the measuring cylinder were also observed by eye from 0 to 30 min. Photos of the top view of the foam while inside the measuring cylinder were also taken using the phone camera. The appearance of the foam was assessed using the parameters from Kavli (personal communication, November 18, 2019). Figure 7 shows how the size of the bubbles was described and evaluated.

Figure 7. **Parameter in assessing the appearance of the steam-frothed milk foam (**Kavli, personal communication, November 18, 2019).

The foam was defined as compact and having fine bubbles when it looked smooth and had a velvety appearance. Small bubbles on the foam were mostly visible on the outer side of the foam than in the center, but the foam itself was still thick and compact. Large bubbles were visible when the foam was not thick enough and not compact.

3.2. Main Experiment

3.2.1. Milk Treatment

3.2.1.1. Preparation of the pasteurized standard milk with 1 % fat

The raw milk was first heated to 55 °C in a warm bath to reduce the viscosity of the cream and increase the separation efficiency (Bylund, 1995). To standardize the milk with 1 % fat, the skim milk was separated from the cream using a cream separator (Milky FJ130, Bob White Systems, Vermont, USA). Afterwards, the milk was manually standardized to 1.0 % fat, and the fat concentration was determined using the FTIR analyzer (MilkoScan ™ FT1, Hilleroed, Denmark) in NMBU pilot plant. The standardized milk was then homogenized (Rannie MODEL LAB 4580/71, Copenhagen, Denmark) at 60 °C using 180 bar, to finely distribute the fat globules in the milk (Bylund, 1995). Then, the milk was pasteurized by placing the milk churn in a water bath and heated the milk to 65 °C for 30 minutes. The milk was then cooled down to 16 °C before filling into 48 sterile bottles, containing approximately 500 mL per bottle.

3.2.1.2. Inoculation of the milk

A 0.5 mL aliquot of each frozen culture (strains 9, 15, 16 and 26) was inoculated into each of 8 x 500 mL milk, gently mixed and then stored (incubated) at 4 °C. For each strain, a milk sample was prepared for Day 0 – 7, giving a total of 8 bottles per culture.

Two control sequences were prepared. 13 tablets of Bronopol (Broad Spectrum Microtabs II, Weber Scientific, Hamilton, NJ, USA) was added to about 500 mL standardized milk, giving a total of 8 bottles for samples to be used from Day 0 – 7. This control served as a negative control without bacterial growth (MB). The other control was uninoculated pasteurized milk (M).

3.2.2. Microbiological analysis

3.2.2.1. Preparation of the agar

Nutrient Broth (NB, Oxoid, Hampshire, England) and Nutrient Agar (NA, Oxoid, Hampshire, England) were used as growth media for the selected *Pseudomonas* strains. Nutrient Broth (NB) was prepared by dissolving 8 g in 1 L of distilled water, while Nutrient Agar (NA) was prepared by dissolving 24 g in 1 L distilled water. These mixtures were placed in a water bath and mixed carefully until all the particles were completely dissolved. The NB was cooled down before dispensing (5 mL) in 10 mL test tubes. The NB and NA were then autoclaved at 121 °C for 15 minutes.

The NA was cooled down in a 48 °C before pouring into the petri dishes. The NB tubes and NA plates were stored at 4 °C.

3.2.2.2. Cultivation of *Pseudomonas* strains

The effect of the growth of *Pseudomonas* on the foaming properties of milk was investigated in this thesis. Four isolates showing diverse proteolytic and lipolytic properties from those used in the previous studies performed by Tidemann (2016) and Bækkelund (2016) were selected and used to investigate the effect on the foaming properties. These *Pseudomonas* strains were identified to be as members of the *Pseudomonas fluorescens* group and Strains 9, 15, 16 and 26 were selected.

These strains had been stored frozen at -20 °C. These were then thawed in lukewarm water and sub-cultured to NB broth, and were incubated at 22 °C for 24 hours. The purity of the selected *Pseudomonas* strains was checked by plating a loop-full of the subculture to a NA plate and incubating the plates aerobically at 22 °C for 24 hours. Serial dilutions of an overnight culture of each strain in NB were made, and the dilutions 10^{-5}, 10^{-6}, and 10^{-7} were plated on NA to ascertain the cell density of each strain following overnight incubation.

Once the purity of the *Pseudomonas* was confirmed and the bacterial growth in 24 hours had been determined, the *Pseudomonas* strains 9, 15, 16 and 26 in NB broth were centrifuged using 8,000 rpm for 10 min at 4 °C in order to obtain the *Pseudomonas* in the form of a solid residue (pellet).

Table 2 shows the cell densities of *Pseudomonas* strains 9, 15, 16 and 26 after overnight growth in NB; the amount of the NB broth containing each culture and the Ringers solution and dilutions used to resuspend each culture in UHT milk.

Table 2. **The growth of *Pseudomonas* strains 9, 15, 16 and 26 at 22 °C for 24 hours; the amount of NB broth containing each culture, and the Ringers solution and dilutions used to resuspend each culture in UHT milk.**

Culture	Growth in 22 °C, 24 hr (log CFU mL^{-1})	NB broth (mL)	Ringers solution (mL)	Suspended culture in Ringers solution (mL)	UHT milk (mL)
Strain 9	7.86	20	20	3	30
Strain 15	8.09	20	20	3	30
Strain 16	7.55	30	30	30	30
Strain 26	7.07	30	30	30	30

In order to start the experiment at log 3 and use 0.5 mL inoculate, 20 mL of the NB broth from Strains 9 and 15 were suspended to 20 mL of Ringer's solution. Then a 3 mL from each of the suspended Ringer´s solution was transferred to the 30 mL UHT milk. The Strains 16 and 26 used 30 mL of the NB broth culture to be suspended in 30 mL Ringers solution, and the pellet from the suspended strains was resuspended in 30 mL UHT milk. This procedure gave 4 x 30 mL of milk, each containing one of the strains of *Pseudomonas*. The milk solution containing the suspended *Pseudomonas* strains was divided into 5 x 6 mL tubes. Of these, 1 tube was used per experimental trial, and 1 tube was kept as a reserve. The tubes were kept frozen at -20 °C until used for the experiment.

3.2.2.3. Determination of the growth of the selected *Pseudomonas* strains in the pasteurized milk with 1 % fat

Plate counting on NA using a spread plate technique was done for each milk sample to determine the growth of each strain during the whole incubation period of 7 days at 4 °C. The plates were incubated at 22 °C for 24 hours. On Day 0, the dilution factors used for the milk samples containing Strains 9, 15, 16 and 26 were -3 and -4. For Days 1 – 7, the dilution factors of -4, 5, 6 and 7 were used. For the standardized milk (M) and the standardized milk with Bronopol (MB), the dilution factors used were -1 and -2 from Day 0 – 7.

3.2.3. Chemical analyses

3.2.3.1. Determination of lipolytic degradation by extraction of free fatty acids and quantification using Gas Chromatography – Mass Spectrometry

To investigate whether lipolysis by the *Pseudomonas* strains affected the foaming properties of milk, the free fatty acids were extracted using a modified Folch method (Folch et al., 1957) as described by Inglingstad et al. (2017) and quantified using Gas Chromatography-Mass Spectrometry (GC-MS). The lipid extraction was carried out by using organic solvents to extract the lipids from a complex mixture of water-soluble organic compounds.

3.2.3.1.1. Lipid extraction

The frozen milk samples were thawed for 15 min in lukewarm water and then mixed in a vortex mixer. For each milk sample, 1 g was accurately weighed and transferred to a glass tube, following the addition of 10 mL MeOH and 10 mL diethyl ether: hexane (1:1, v/v). Methanol was used to disrupt the hydrogen bonding or electrostatic forces between the polar lipids and the protein. The ethyl ether was used to extract most non-polar components of the lipids (Work et al., 1972).

Thereafter, 50 µL distilled water was added to organic solvents to achieve a phase separation, that consists of the organic phase with lipids and water phase with components soluble in water (R. Inglingstad, personal communication, October 14, 2020).

The following internal standard solutions were also added to the milk sample solution: 100 µL C11:0 FFA IS (undecanoic acid, 5 mg/mL in diethyl ether: hexane, Larodan, Malmø, Sweden) and 200 µL C19:0 FFA IS (trinonadecanoic acid, 5 mg/mL in diethyl ether: hexane, Larodan, Malmø, Sweden). Each of the milk sample solutions was then mixed in a vortex, and then gently mixed in an orbital shaker (PSU-20i, Biosan, Riga, Latvia) in a horizontal position for 5 min at 37 °C at 150 rpm. Afterwards, the milk sample solution was centrifuged for 3 min at 2000 x g. The supernatant, which is the organic phase containing lipids, was transferred to a glass tube. These steps were repeated for subsequent extraction of the lipids in the milk sample.

The rest of the supernatant was removed through evaporation using N_2 at 40 °C in a fume cupboard. 1000 µL of Chloroform (Sigma-Aldrich, Missouri, USA) was then added by rinsing the tube with the first 500 µL of the chloroform and then transferring it to a GC vial, following another tube-rinsing with 500 µL chloroform. The samples in GC-vials were stored at -20 °C until the Solid-phase Extraction (SPE).

3.2.3.1.2. Solid-phase extraction

The Solid Phase Extraction (SPE) method was used to separate the lipids into polar and neutral fractions. The SPE was performed using an automated system (GX-275 ASPEC, Gilson, Wisconsin, USA). The aminopropyl mini-columns (Bond Elut Straight Barrel NH2, 500 Mg, 3 mL, Agilent Technologies, USA) were activated with 7 mL heptane (Sigma-Aldrich, Missouri, USA), and were added to the solution with the extracted lipids. The neutral lipids were eluted first, with 5 mL chloroform (Sigma-Aldrich, Missouri, USA). Then

the free fatty acids were eluted with 5 mL of 2% acetic acid in diethyl ether (Sigma-Aldrich, Missouri, USA). The solution with the free fatty acids was then transferred into a glass tube, and the solvents were set to evaporate under N_2 gas at 40 °C.

3.2.3.1.3. Formation of Fatty Acid Methyl Esters (FAME)

The methylation of free fatty acids was performed by adding 1 mL of 10 % Boron trifluoride-methanol solution (BF_3MeOH) (Sigma-Aldrich, Missouri, USA) to the glass tubes containing the free fatty acids from SPE. The solution with free fatty acids was placed in a water bath for 5 minutes at 70 °C. After cooling, 1 mL of heptane (Sigma-Aldrich, Missouri, USA) was added to the solution with free fatty acids to form two phases (organic and aqueous). The upper phase (organic) in all the samples was transferred to GC tubes and stored at 4 °C until the GC analysis.

3.2.3.1.4. GC Analysis

The isolated FAME was identified and quantified using Gas Chromatography (TRACE 1310, Thermo Fisher Scientific, Massachusetts, USA) and Mass Spectrometry (Thermo ISQ™ QD, Thermo Fisher Scientific, Massachusetts, USA). For each sample, 1 µL was injected into the GC machine using an autosampler (Thermo AI/AS, Thermo Fisher Scientific, Massachusetts, USA), with an injector temperature of 250 ºC and split flow of 10. The free fatty acids were separated using a 60 m capillary column (Rtx®-2330, Restek, Pennsylvania, USA), with a diameter of 0.25 mm and 0.20 µm film thickness. The GC oven temperature program is listed in Table 3.

Table 3. **Oven temperature program for the GC.**

Oven Temperature Program	
Initial Temperature (°C)	50
Hold Time (min)	5
Temperature 2 (°C)	140
Hold Time (min)	30
Rate (°C/min)	100
Temperature 3 (°C)	145
Hold Time (min)	30
Rate (°C/min)	10
Temperature 4 (°C)	175
Hold Time (min)	20
Rate (°C/min)	3
Temperature 5 (°C)	260
Hold Time (min)	10
Rate (°C/min)	50

The MS was running in full scan mode (50-600 m/z) with 0.2 sec scan time and 70 eV and generated a quantification of the individual free fatty acids present in the milk samples. The software that was used to control the GC/MS system was Chromeleon 7.8 CDS (Thermo Scientific, Massachusetts, USA).

3.2.3.2. Determination of proteolytic degradation of caseins using Capillary Electrophoresis

To investigate whether proteolysis by the *Pseudomonas* strains in milk affected the foaming properties of the standardized milk, the peptide formation and protein degradation was analyzed using Capillary Electrophoresis (CE) with UV detection. The CE is a technique that separates protein molecules in an electric field according to size and charge. It is performed in a small glass tube, called capillary, which is filled with an electrolyte solution. The analytes migrate through the electrolyte solution under the influence of an electric field and are separated as a result of their differences in electrophoretic mobility. This mobility varies with charge, solvent viscosity and size (Kemp, 1998).

The sample buffer and the run buffer were prepared as described by Bækkelund (2016), wherein 39.3 mg of dithiothreitol (DTT, Sigma-Aldrich, St. Louis, MO, USA) were added

per 5 mL of the sample buffer. The sample buffer (pH 8.670.1) consisted of 167 mM hydroxymethyl-aminomethane (TRIS; Sigma, St. Louis, MO, USA), 42mM 3-morpholino-propane sulphonic acid (MOPS; Sigma), 67 mM ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA; Merck, Darmstadt, Germany), 17 mM D,L-dithiothreitol (DTT; Sigma), 6M urea (Merck) and 0.05% (w/w) methyl hydroxyethylcellulose 30000 (MHEC; Serva, Heidelberg, Germany). The run buffer (pH 3.070.1) consisted of 0.19 M citric acid (Merck), 20 mM sodium citrate (Merck), 6 M urea and 0.05% (w/w) MHEC (Heck et al., 2008).

The milk samples were thawed in lukewarm water for 15 min, and then 270 mL of each sample was mixed with 1230 ml of the sample buffer in an Eppendorf tube. The Eppendorf tubes were rapidly shaken using a mini-shaker (Gene 2) and then incubated on a rotating PSU-20i orbital shaker (Biosan, Riga, Latvia) at room temperature for 1 hour. Afterwards, the fat on the samples was separated by centrifuge using an Eppendorf 5415D Microcentrifuge (Eppendorf, Hamburg, Germany) at 5000 x g for 5 minutes. Then, the sample material was carefully aspirated using a 2.5 mL sterile syringe with a 0.8 x 40 mm disposable needle (Becton Dickinson SA, Madrid, Spain). Careful aspiration was done to avoid the sediment at the top and bottom of the tube that consists of proteins including fat. The sample material was then filtered with a 0.45 mm PES filter (VWR, Pennsylvania, USA) into new Eppendorf tubes. 50 mL of the sample material was transferred to CE sample tubes for CE analysis. The rest of the sample material in the Eppendorf tubes was kept frozen at -20 °C for backup.

An Agilent capillary electrophoresis system using Agilent ChemStation software and HPCE standard capillary tube (G1600-61211) with 50 mm inner diameter and 56 cm length was used for the analysis (Agilent Technologies, Germany). Protein separation was carried out according to the methodologies of Heck et al. (2008) and Mestawet et al. (2014), wherein the

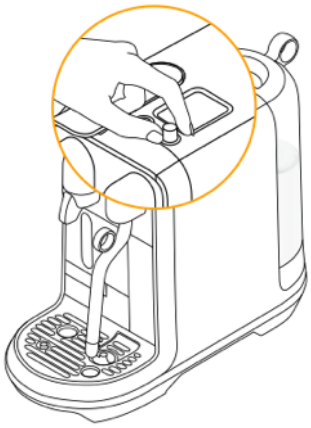
samples were run in sequences of 14 samples that took 72 minutes for each run. The capillary was rinsed with 0.1 M NaOH for 5 minutes before the first run and after every fourth run, followed by 20 minutes rinsing with the run buffer. Before each run, the capillary was rinsed with the "run buffer" for 5 minutes. Then, a fresh sample buffer was used after each run. The sample material was injected at the anode at a pressure of 34.5 mbar for 20 sec, and the UV detection of the peptides and proteins was performed at 214 nm (Mestawet et al., 2014).

3.2.4. Determination of the foaming properties of the pasteurized milk with 1 % fat

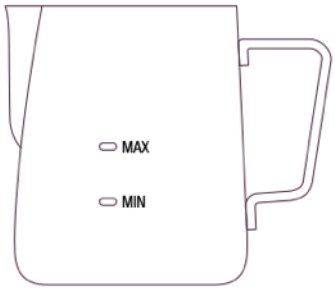
The foaming properties of the milk samples were tested daily from Day 0 – 7 using the standard method that had been developed in the pilot study. The Nespresso Creatista Plus (Nestle Nespresso, Lausanne, Switzerland) has a fully automated steam wand and an advanced micro milk foam technology integrated into the coffee machine that enabled a standard method in creating a steam-frothed foam. The steam-frothed foam was done by injecting steam into 150 mL of the milk sample that had been poured into the stainless jug provided with the coffee machine. The coffee machine was set to the "Milk" setting to create the steam-frothing, and the temperature was set to 60 °C. The coffee machine used a maximum pressure of 19 bar to inject the steam. The steam wand was placed in the center of the stainless jug containing the milk sample. Care was taken that the jug was in contact with the temperature sensor located in the drip tray, in order to have the correct and stable temperature treatment of the milk. Figures 8 shows the steps in operating the Nespresso Creatista Plus machine.

Step 1. Fill in the water tank with fresh potable water.

Step 2. Make sure the cup support is locked to accommodate the jug.

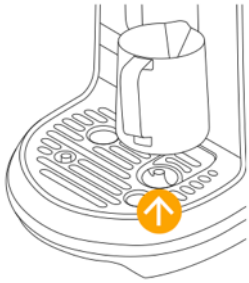


Step 3. Rotate the select dial to select the “Milk”.



Step 4. Fill the milk jug with 150 mL of the cold milk sample (4 °C).

Step 5. Lift the steam wand & position in the center of the milk jug, and press START to begin milk frothing.



Step 5. Ensure the milk jug is in contact with the temperature sensor.

Figure 8. **Steps in creating a steam injected milk foam using Nespresso Creatista Plus (Nespresso Creatista Plus, 2020).**

The automatic foaming sequence took approximately 50 sec. Thereafter, the jug was tapped on a hard surface to disperse the larger bubbles in the foam, and then gently swirled two times to make the top of the milk foam shiny. The tapping and swirling were done consistently for all milk samples.

3.2.4.1. Foaming capacity

To determine the foaming capacity of each milk sample, the volume (mL) of foam produced after automatic steam injection was measured in the following way. First, three drops of methylene blue were added into the bottom of a 500 ml measuring cylinder (BRAND Silberbrand ETERNA 500:10 mL, Boro 3.3, low-form, H: 250 mm, Duran®, Germany). The

purpose of this was to stain the milk phase (and not foam phase) and therefore make it easier to see the division between the liquid phase and the foam. The milk and the steam-frothed foam were quickly poured into the measuring cylinder in a tilted position to prevent the milk from plunging onto the surface. This technique avoided disruption of the bubbles and adding more mechanical stress to the milk. The volume (mL) of the foam was read as soon as the measuring cylinder had been placed on a flat surface in a horizontal position, and while pouring the rest of the foam from the stainless jug to the measuring cylinder. The foam volume was read right away because the volume of the liquid phase (milk) can rapidly increase in 30 seconds. This procedure was carried out with two parallels on each sample. Figure 9 shows the procedure used to create and measure the volume (ml) of milk foam.

Step 1. Add three drops of methylene in the measuring cylinder.


Step 2. Steam injection of the foam using the Nespresso Creatista Plus machine.


Step 3. Pound the jug on a hard surface, and then gently swirl the jug 2x to disperse the largest bubbles.


Step 4. Quickly pour the milk foam into the stained measuring cylinder in a tilted position, and then measure the volume (mL) of the foam.

Figure 9. **Procedure in creating and measuring the foam volume (ml).**

The test was done daily on all the milk samples of standardized milk inoculated with Strains 9, 15, 16, 26; in addition, the controls of uninoculated standardized milk and standardized milk with Bronopol were foamed.

3.2.4.2. Foam stability

The foam stability was measured daily by observing the changes of the foam volume (mL) during standing at room temperature. The foam volume of the milk samples was read after 0, 5, 15 and 30 minutes. This test was done in two parallels on all the milk samples. The observed changes in the volume (ml) of foam over time and the differences in the volume of foam between the milk samples with the selected *Pseudomonas* strains 9, 15, 16 and 26, and the two control samples (uninoculated standardized milk and the standardized milk with Bronopol) were analyzed. When the foams were uneven, the foam volume was measured visually as average.

3.2.4.3. Appearance of the foam

To determine if the selected *Pseudomonas* strains have an effect on the size of the bubbles of the milk foam, the appearance of the foam was observed visually from Day 0 – 7. The top view of the foam in the measuring cylinder was also photographed using a phone camera. The size of the bubbles was assessed according to the parameter from Kavli (personal communication, November 18, 2019) used in the pilot study. The parameter illustrated how a compact, fine bubbles; small bubbles and large bubbles should appear. The foam appearance on all the milk samples and the changes in the size of the bubbles over time from 0, 5, 15 and 30 minutes were observed and compared to each other.

3.2.5. Statistical Analysis

The data on this study were analyzed using RStudio Open-Source Package (Version 1.3.1073, 2009-2020, Boston, MA, USA). The differences were considered significant when $p \leq 0.05$.

3.2.5.1. Analysis of Variance

The following linear model was used to determine the significant differences in the results of the analyses, namely growth of the *Pseudomonas* strains in the milk, the content of FFA by quantification using GC, and the foaming capacity and stability of milk.

$$y_{ij} = \mu + R_i + e_{ij}$$

Where y_{ij} is the observation; μ is the overall mean; R_i is the foaming attitude; j is the no. of variables; i is the number of samples (6 samples: uninoculated standardized milk, standardized milk with Bronopol, standardized milk with *Pseudomonas* strains 9, 15, 16 and 26), e_{ij} is the residual error.

3.2.5.2. Tukey test

Tukey test was performed to compare and determine if there are significant differences in the content of FFA between the milk samples. The following test statistic was used:

$$q_s = \frac{Y_{\max} - Y_{min}}{SE}$$

Where Y_{max} and Y_{min} are the larger and smaller means of the two groups being compared in the test. *SE* is the standard error of the entire data.

4. Results

The effect of the selected *Pseudomonas* strains on the foaming properties of the pasteurized milk with 1 % fat was determined. The standard method in determining the foaming properties of milk, which was developed during the pilot study, was applied to the actual experiment.

The growth of the *Pseudomonas* strains in the milk samples was monitored daily. The lipolytic and proteolytic degradation by the selected *Pseudomonas* strains were also determined.

Statistical analysis was done on each test to determine significant differences in the results. The second trial in the experiment showed deviating results; therefore, this study is only based on the first and third trial.

4.1. Pilot study

The pilot study aimed to develop a standardized method in creating a steam-frothed foam and assessing the foaming properties of milk used in the coffee shops.

The foaming properties of the milk samples (UHT semi-skimmed milk with 1.2 % fat, pasteurized semi-skimmed milk with 1.2 % fat, pasteurized full-fat milk with 3.5 % fat, pasteurized skimmed milk with 0.1 % fat) were assessed. The foaming capacity was assessed by measuring the volume of the steam-frothed milk foam after it had been poured into the measuring cylinder (0 min). The foam stability was assessed by measuring the foam volume (mL) above the liquid phase during standing in room temperature from 0, 15 and 30 min.

4.1.1. Foaming capacity

The foaming capacity of the milk samples was measured at 0 min. Figure 10 shows the average results of trials performed on two days.

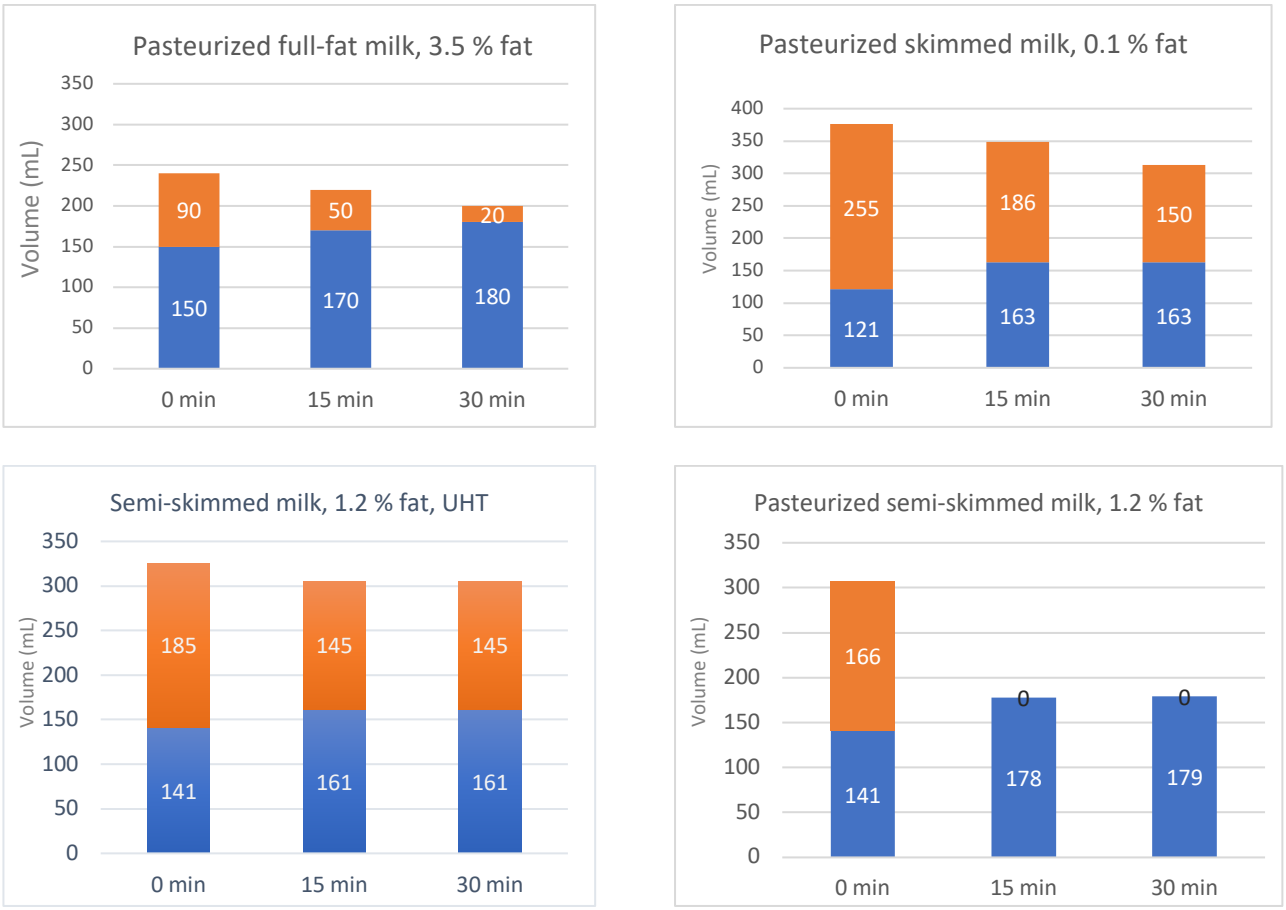


Figure 10. **Foaming properties of the UHT semi-skimmed milk (1.2 % fat), pasteurized full-fat milk (3.5% fat), pasteurized semi-skimmed milk (1.2 % fat) and pasteurized skimmed milk (0.1 % fat). (blue: liquid; orange: foam) Average of 2 days trial.**

It was observed on this pilot study that the milk with higher fat content produced less foam compared to the milk with less fat content. The pasteurized skimmed milk had the highest foam volume at 0 min, which was 255 ml, while the pasteurized full-fat milk (3.5 % fat) had the lowest foam volume that was 90 ml.

The UHT semi-skimmed milk (1.2 % fat) produced more foam at 0 min than the pasteurized semi-skimmed milk.

The milk (liquid phase) on all the milk samples did not increase by more than 50 mL of the original milk volume.

4.1.2. Foam stability

As shown on Figure 11, the foam stability of all the milk samples in the pilot study showed decreasing foam volume (mL) as time increased from 0 min to 30 min, except for the UHT semi-skimmed milk (1.2 % fat) that remained stable between15 min and 30 min. The pasteurized semi-skimmed milk (1.2 % fat) showed impaired foam stability as the foam collapsed after standing in room temperature for 15 min.

4.1.3. Appearance of the milk foam

The appearance of the milk foam varied between the milk samples. The steam-frothed foam from the pasteurized skimmed milk with 0.1 % fat and UHT semi-skimmed milk with 1.2 % fat was compact and had fine, small bubbles at 0 min. The milk foam from both pasteurized skimmed milk and UHT semi-skimmed milk became dry, and the bubbles also increased in size as time increased from 0 to 30 min. The pasteurized full-fat milk with 3.5 % fat was observed to have small bubbles but less compact foam. The pasteurized semi-skimmed milk with 1.2 % fat had considerably larger bubbles, and the foam was not compact at all.
The steam-frothed foam made from these milk samples were also observed using a microscope (Wild M8, Wild Heerbrugg, Switzerland). Figure 11 shows a photo image of the foam from the pasteurized skimmed milk at 0 min, taken using a microscope with 40x magnification.

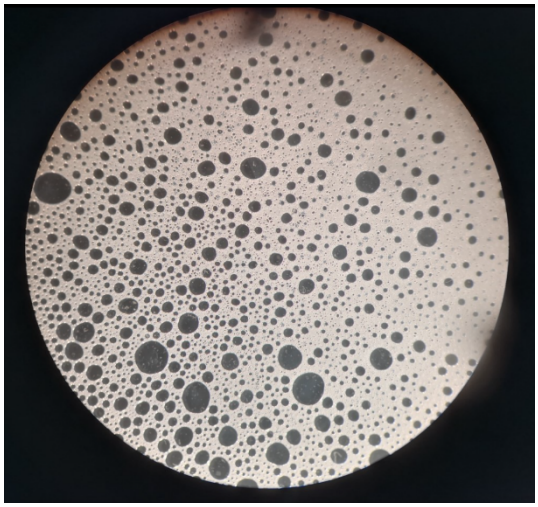


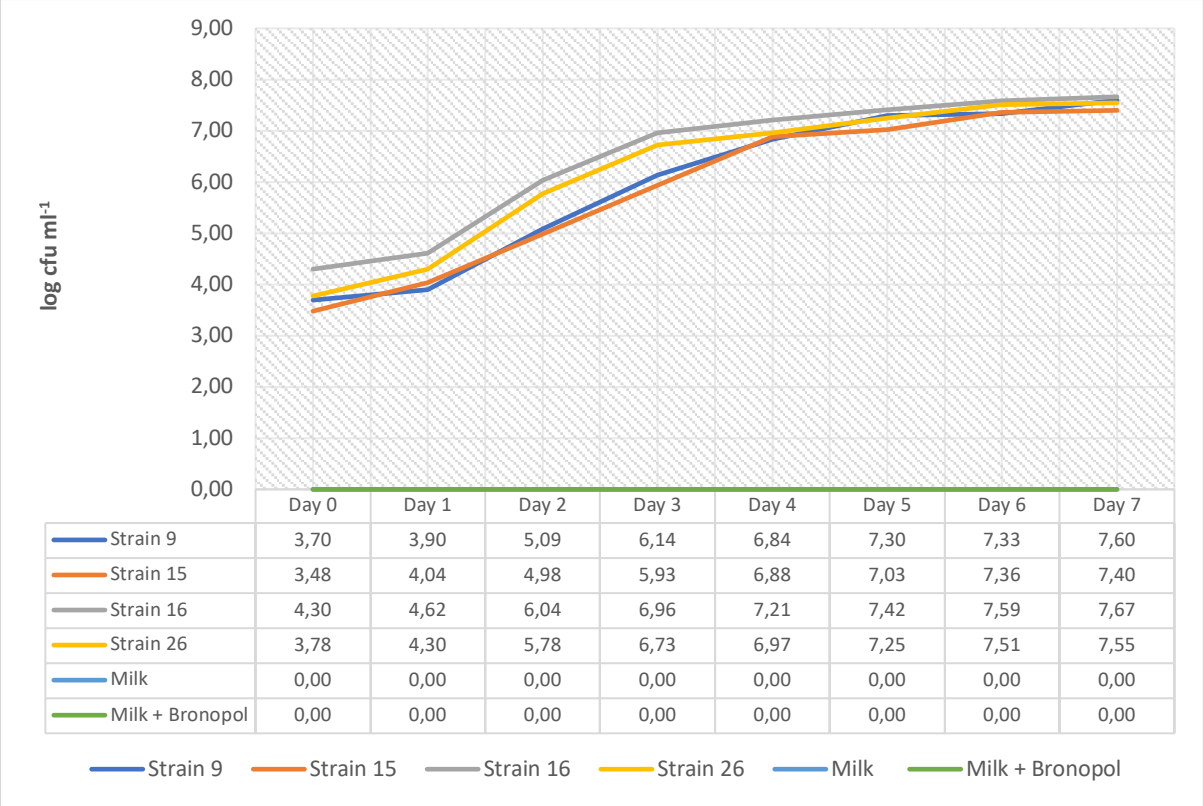
Figure 11. **Photo of the foam made from pasteurized skimmed milk with 0.1 % fat using 40x magnification.**

The foam appeared to have different bubble sizes, from small to big sizes. The distribution of the bubbles on the liquid phase was also uneven.

4.2. Main Experiment

4.2.1. Growth of the selected *Pseudomonas* strains in the pasteurized milk with 1 % fat

The growth of bacteria at 4 ^{o}C in all the milk samples was measured by plating on Nutrient agar and incubated at 22 ºC for 24 hours. The added isolates of *Pseudomonas* showed an increase in growth from Day 0 until Day 7, as shown in Figure 12.



	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Strain 9	3,70	3,90	5,09	6,14	6,84	7,30	7,33	7,60
Strain 15	3,48	4,04	4,98	5,93	6,88	7,03	7,36	7,40
Strain 16	4,30	4,62	6,04	6,96	7,21	7,42	7,59	7,67
Strain 26	3,78	4,30	5,78	6,73	6,97	7,25	7,51	7,55
Milk	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Milk + Bronopol	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00

Figure 12. **Growth of *Pseudomonas* strains in standardized milk with 1 % fat (Average of Trial 1 and 3).**

All the *Pseudomonas* strains showed growth in pasteurized milk and reached nearly 7 log CFU mL^{-1} by Day 4. Strain 16 showed the highest cell number from the start of the incubation until Day 7 among the *Pseudomonas* strains in this study. The growth of Strain 9 and 15 started lower and grew more slowly, but also ended up at about 7.5 log CFU mL^{-1} by Day 7. The controls used (Milk and Milk + Bronopol) did not show any growth during the incubation period.

The result on the statistical analysis done for the growth of the selected *Pseudomonas* strains showed no significant difference between the cell densities of Strains 9, 15, 16 and 26 from Day 0 – 7, ($p \geq 0.05$).

4.2.2. Chemical Analysis

4.2.2.1. Content of the free fatty acids in the milk samples as analyzed by Gas Chromatography - Mass Spectrometry (GC-MS)

The lipolytic degradation by the selected *Pseudomonas* strains was determined by quantifying the various free fatty acids in the milk samples using GC-MS. The milk samples on Day 7 were compared with the uninoculated milk sample from Day 0 (M0) to analyze the extent of lipolysis by the selected *Pseudomonas* strains. The results from the GC analysis are illustrated in Figure 13. The results show the average concentration (mg/g^{-1}) of the free fatty acids in the milk samples from Trial 1 and 3, that were all stored at 4 °C and inoculated with the selected *Pseudomonas* strains (9, 15, 16 and 26). The two control samples, namely uninoculated standardized milk (M), and standardized milk with Bronopol (MB), were also analyzed. The concentrations below the detection limit were recorded as 0.

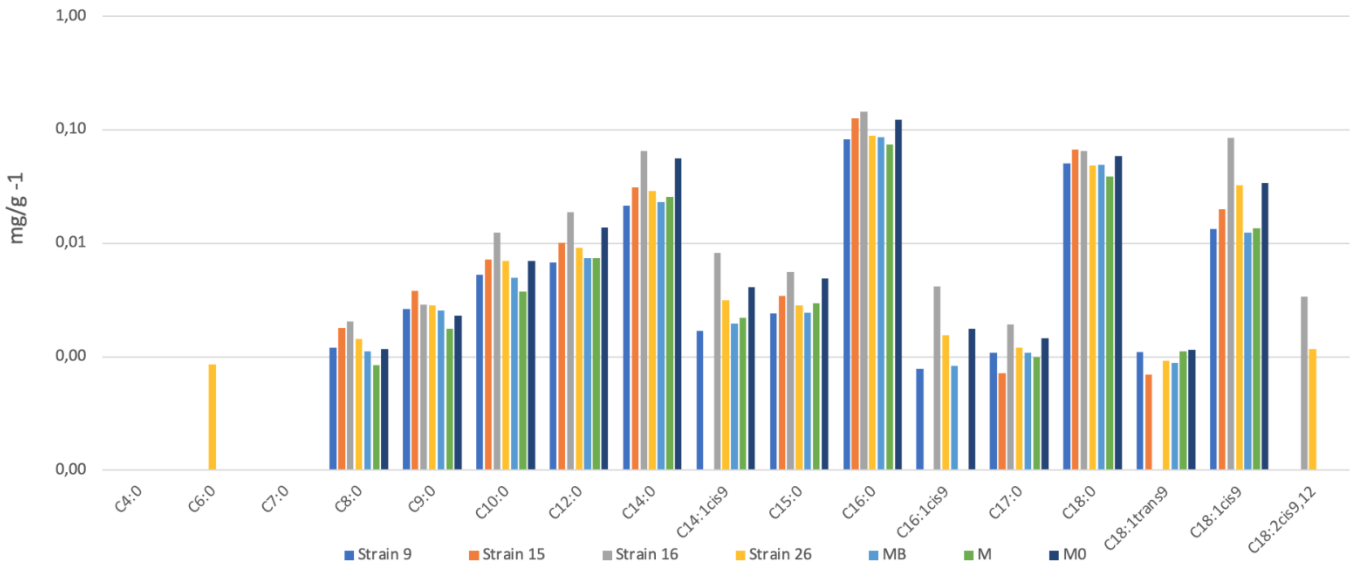


Figure 13. **Free fatty acids in pasteurized milk at Day 0 (M0); pasteurized milk incubated at 4 °C and inoculated with selected *Pseudomonas* strains (9, 15, 16 and 26) and with Bronopol (MB), and without inoculation and Bronopol (M) from Day 7. (Average of Trial 1 and 3)**

The results show that the pasteurized milk inoculated with Strain 16 showed a higher amount of free fatty acids after Day 7 when compared to the milk sample from Day 0 (M0). The results also indicated that Strain 16 could liberate more free fatty acids (C16:0 and C18:1cis9)

compared to the rest of the *Pseudomonas* strains (9, 15 and 26) and the control samples pasteurized uninoculated milk (M) and pasteurized milk with Bronopol (MB).

The statistical analysis on Table 4 shows the results using Analysis of Variance (ANOVA) and the control milk sample from Day 0 (M0) as a reference.

Table 4. **Analysis of variance for the free fatty acids in pasteurized milk determined by GC-MS. (Average of Trial 1 and 3).**

Observations	p value
C4:0	0.1775
C6:0	0.1853
C7:0	0.1775
C8:0	0.2811
C9:0	0.4130
C12:0	0.4626
C14:0	8.19e-08
C14:1cis9	0.455
C15:0	0.5131
C16:0	<2e-16
C16:1cis9	0.2747
C17:0	0.2667
C18:0	2.70e-15
C18:1trans9	0.2366
C18:1cis9	9.53e-06
C18:2cis9,12	0.2220
M	0.0141
MB	0.0385
Strain 9	0.0320
Strain 15	0.4925
Strain 16	0.0474
Strain 26	0.1502

When compared to sample M0, the concentrations of the free fatty acids C14:0, C16:0, C18:0 and C18:1cis9 are significantly different ($p \leq 0.05$). The results also showed that milk samples M, MB, and the milk samples inoculated with Strain 9 and 16 are significantly different from M0 ($p \leq 0.05$). The % FFA in the milk samples is presented on Figure 14 and shows the distribution of C14:0, C16:0, C18:0 and C18:1cis9 in each milk sample.

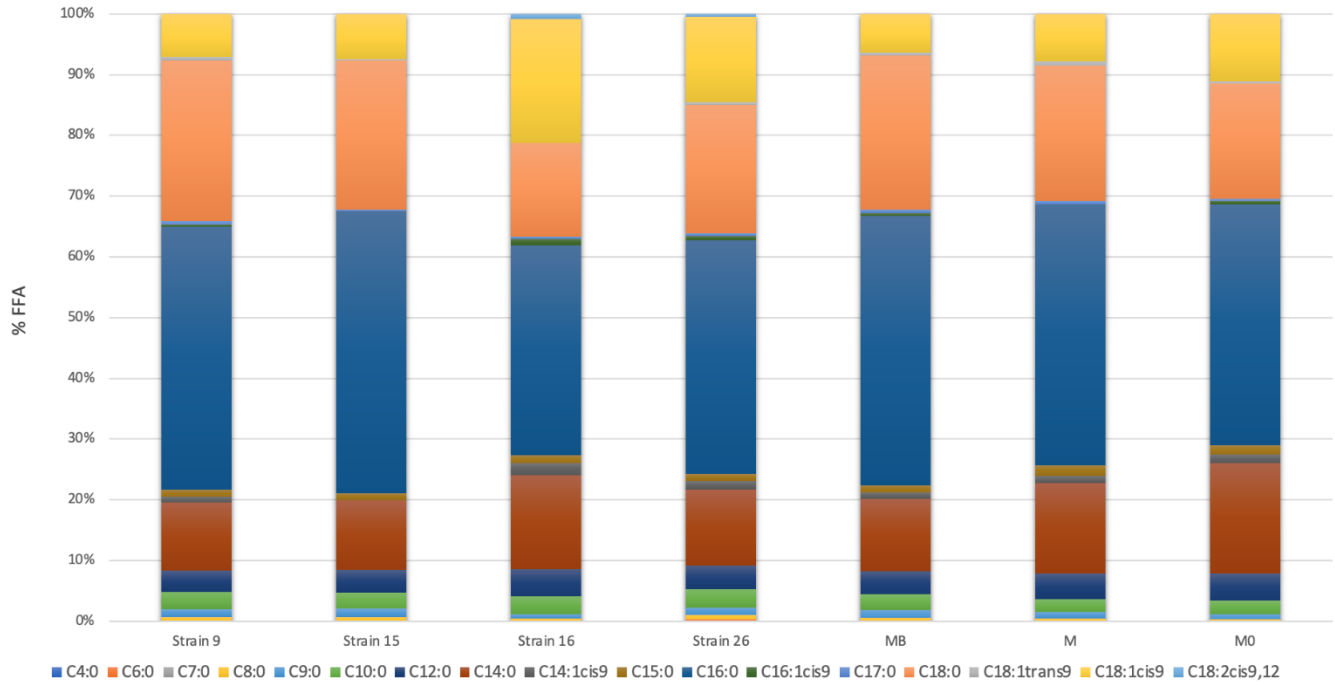


Figure 14. **Distribution of FFA in the milk samples.**

The distribution of FFAs in the milk samples shows that the C18:1cis9 is higher in the milk sample inoculated with Strain 16 compared to M0 and the rest of the milk samples. The proportion of C14:0 is also higher in the milk sample inoculated with Strain 16 than the rest of the inoculated milk samples, but 5 % lower compared to M0.

A post hoc analysis using Tukey test was done to determine if there are similarities or significant differences between the content of free fatty acids in the sample groups.

Table 5 shows the results.

Table 5. **Tukey test showing the difference in means, confidence levels and the adjusted p-values for all the possible pairs of milk samples.**

Sample groups	Difference in means	Lower CI	Upper CI	Adjusted p-value
M - M0	-0.0080	-0.0177	0.0016	0.1709
MB - M0	-0.0067	-0.0164	0.0029	0.3621
Strain 15 – M0	-0.0022	-0.0119	0.0075	0.9929
Strain 16 – M0	0.0065	-0.0032	0.0161	0.4161
Strain 26 – M0	-0.0047	-0.0143	0.0050	0.7729
Strain 9 – M0	-0.0070	-0.0167	0.0027	0.3183
MB – M	0.0013	-0.0083	0.0110	0,9910
Strain 15 – M	0.0058	-0.0039	0.0155	0.5440
Strain 16 – M	0.0145	0.0048	0.0242	0.0004
Strain 26 – M	0.0034	-0.0063	0.0130	0.9408
Strain 9 – M	0.0010	-0.0086	0.0107	0.9910
Strain 15 – MB	0.0045	-0.0051	0.0142	0.7956
Strain 16 – MB	0.0132	0.0035	0.0229	0.0016
Strain 26 – MB	0.0021	-0.0076	0.0118	0.9945
Strain 9 – MB	-0.0002	-0.0099	0.0094	1.0000
Strain 16 – Strain 15	0.0087	-0.0010	0.0183	0.1100
Strain 26 – Strain 15	-0.0024	-0.0121	0.0072	0.9880
Strain 9 – Strain 15	-0.0048	-0.0144	0.0049	0.7520
Strain 26 – Strain 16	-0.0111	-0.0208	-0.0014	0.0138
Strain 9 – Strain 16	-0.0134	-0.0231	-0.0037	0.0012
Strain 9 – Strain 26	-0.0023	-0.0120	0.0073	0.9907

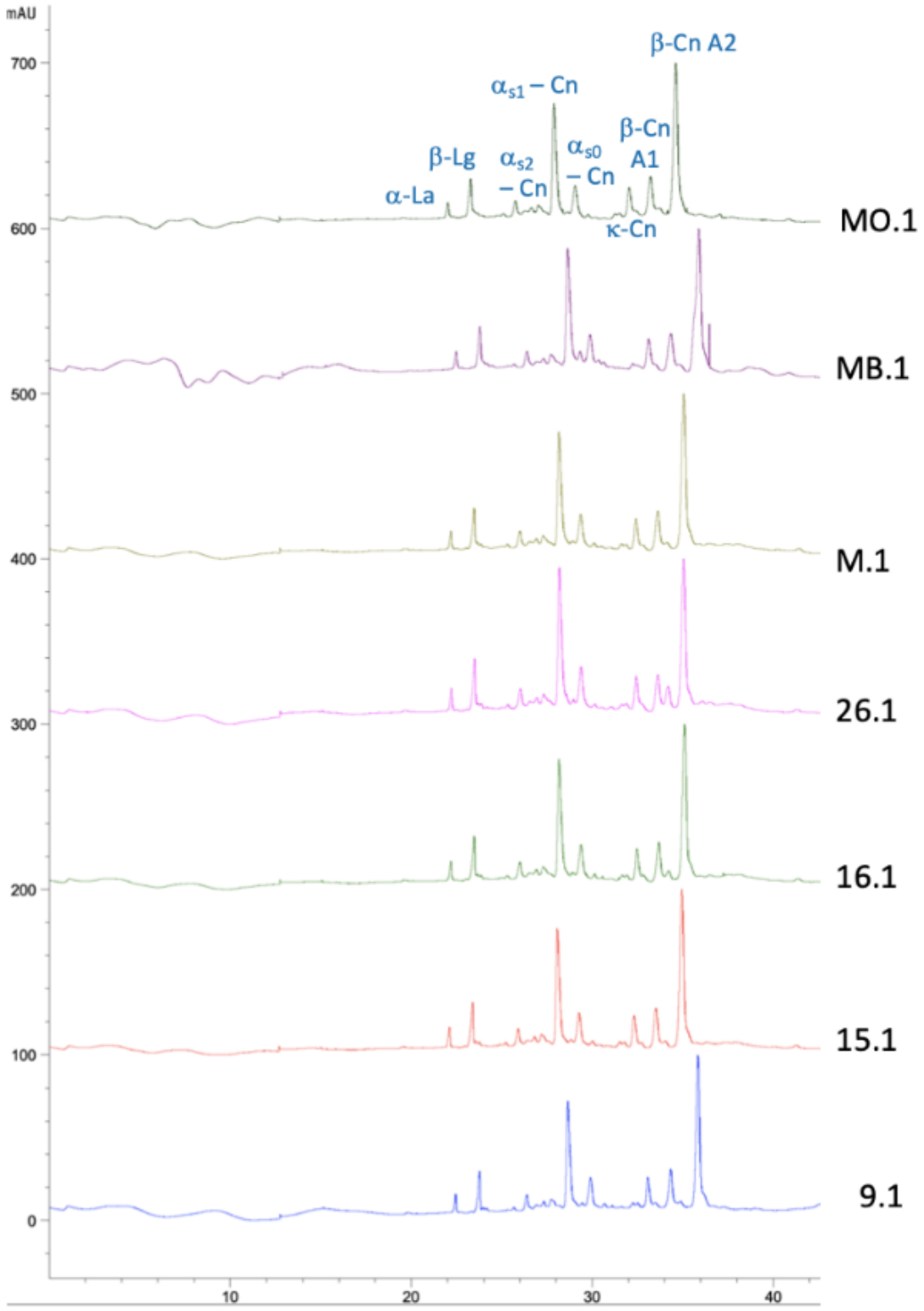
The confidence levels and p-values show that the only significant between group difference is for the content of free fatty acids in the milk samples “Strain 16 and M”, “Strain 16 and MB”, “Strain 26 and Strain 16” and “Strain 9 and Strain 16”. These sample groups were the only ones that do not have “0” in the lower and upper confidence interval and with adjusted p-value ≤ 0.05.

The result indicated that only the content of free fatty acids in the milk inoculated with *Pseudomonas* Strain 16 is significantly different from the control samples “M” (uninoculated standardized milk) and “MB” (standardized milk with Bronopol), adjusted p-value ≤ 0.05.

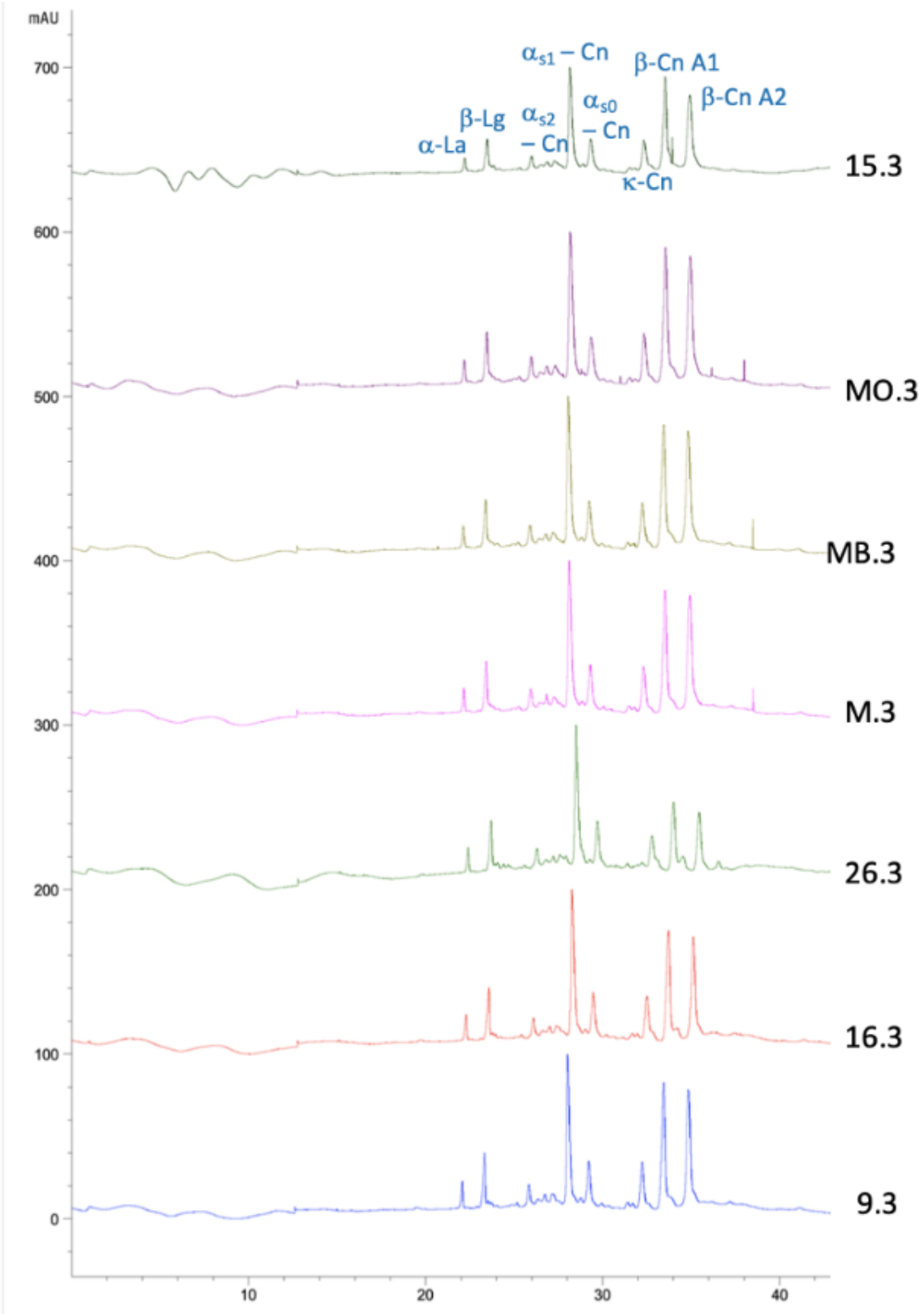
4.2.2.2. Protein degradation by the selected *Pseudomonas* strains

Capillary Electrophoresis was used to determine the peptide formation and protein degradation caused by the selected Pseudomonas strains. Figure 15a-b illustrates the capillary electropherograms from the milk samples in experiments Trial 1 and 3. The sample M0 was

used as a comparison in each figure on both trials, as it was the milk sample on Day 0 that was not inoculated and did not contain Bronopol.



a. **Capillary electropherogram of milk samples from Trial 1.**



b. **Capillary electropherogram of milk samples from Trial 3.**

Figure 15a-b. **Capillary electropherogram of the pasteurized milk samples incubated at 4 °C and inoculated with *Pseudomonas* strains (9, 15, 16 and 26), uninoculated pasteurized milk (M), and the pasteurized milk with Bronopol (MB) from Day 7, and the uninoculated pasteurized milk from Day 0 (M0).**

There was no visible protein degradation in the milk samples from Trial 1 when compared to the control sample M0.1 (uninoculated pasteurized milk from Day 0). A small amount of peptide formation can be observed on milk sample 26.1 between retention time 32 – 34 minutes. The amount of proteins β-Cn A1 and β-Cn A2 are different from Trial 3, as the peak of β-Cn A1 is lower on Trial 1 than in Trial 3; and the peak of β-Cn A2 is higher on Trial 1 compared to Trial 3.

In Trial 3, a slight degradation of α_{s2}, α_{s1}, κ-Cn, and β-Cn was observed on milk sample 15.3. A small peak indicating peptide formation was also observed on milk sample 15.3 at retention time around 34 minutes. The lower peaks of κ-Cn, β-Cn A1 and β-Cn A2 were observed on milk sample 26.3, that also indicated slight degradation of κ-and β-Cn.

4.2.3. Foaming Properties of the milk samples

The foaming properties of the standardized milk inoculated with *Pseudomonas* Strains 9, 15, 16 and 26; and the two controls Milk, and the Milk with Bronopol, were measured daily with two parallels. The foam volume was measured from the time that the foam was formed after steam injection until after 30 min standing at room temperature from Day 0 - 7. These results are shown in Figures 17 - 18. All the milk samples showed reduced foaming properties as incubation time increased.

4.2.3.1. Foaming Capacity

The foaming capacity was determined by measuring the foam volume (ml) above the liquid phase at 0 min. Figures 16 - 17 show the average results of Trial 1 and 3 for foam volume of all the milk samples from Day 0 – 7.

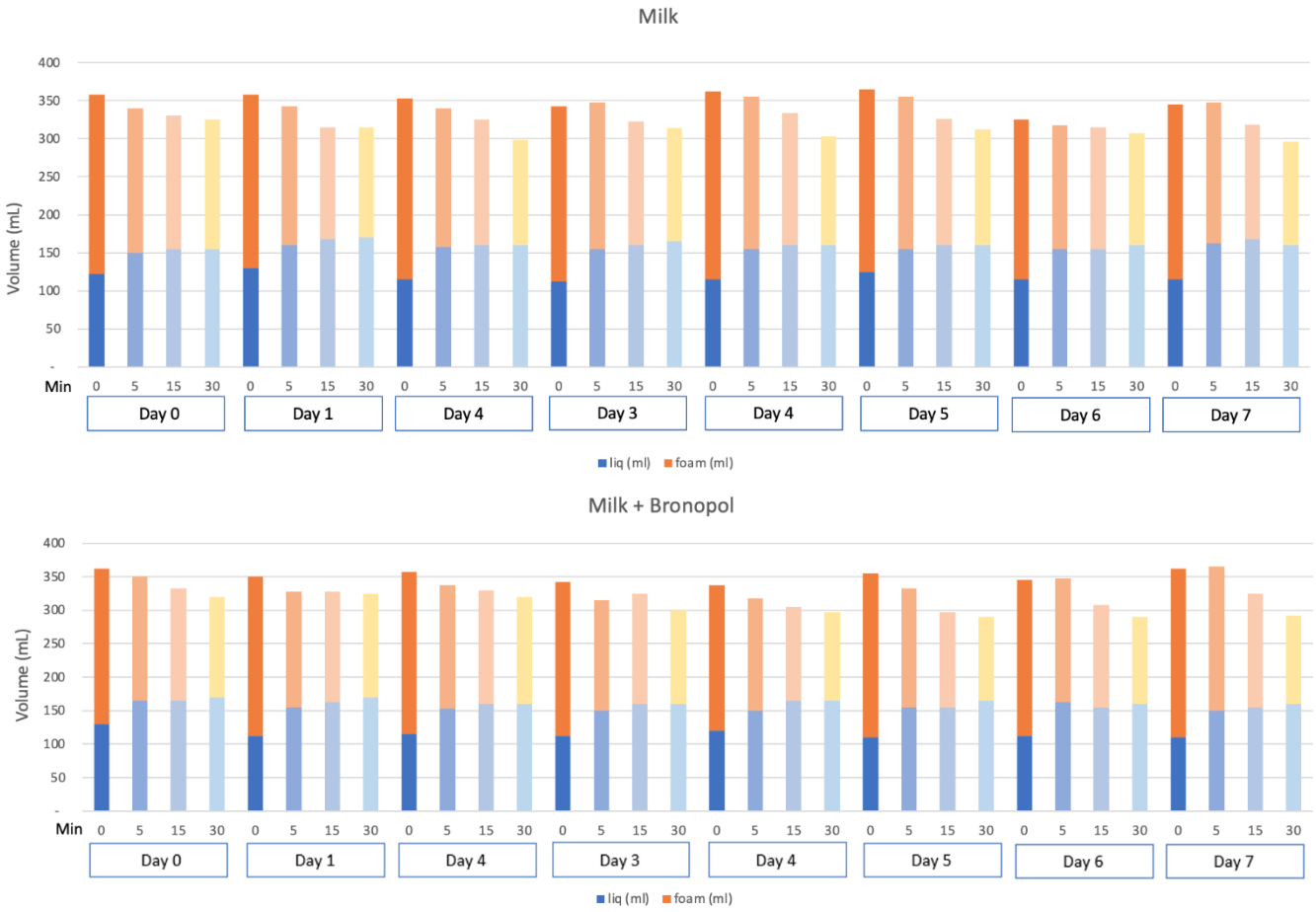


Figure 16. **Liquid and foam volume (ml) of the control samples: uninoculated standardized milk and the standardized milk with Bronopol. Average of Trial 1 and 3.**

The foaming capacity of the two control samples (uninoculated standardized milk and the standardized milk with Bronopol) were similar and consistent from Day 0 – 7. The foam volume (mL) of the controls samples at 0 min was above 200 mL from Day 0 until Day 7. The liquid phase at 0 min was also consistent at <130 mL from Day 0 – 7.

Figure 17 shows the liquid and foam volume (mL) of the milk samples inoculated with *Pseudomonas* strains 9, 15, 16 and 26.

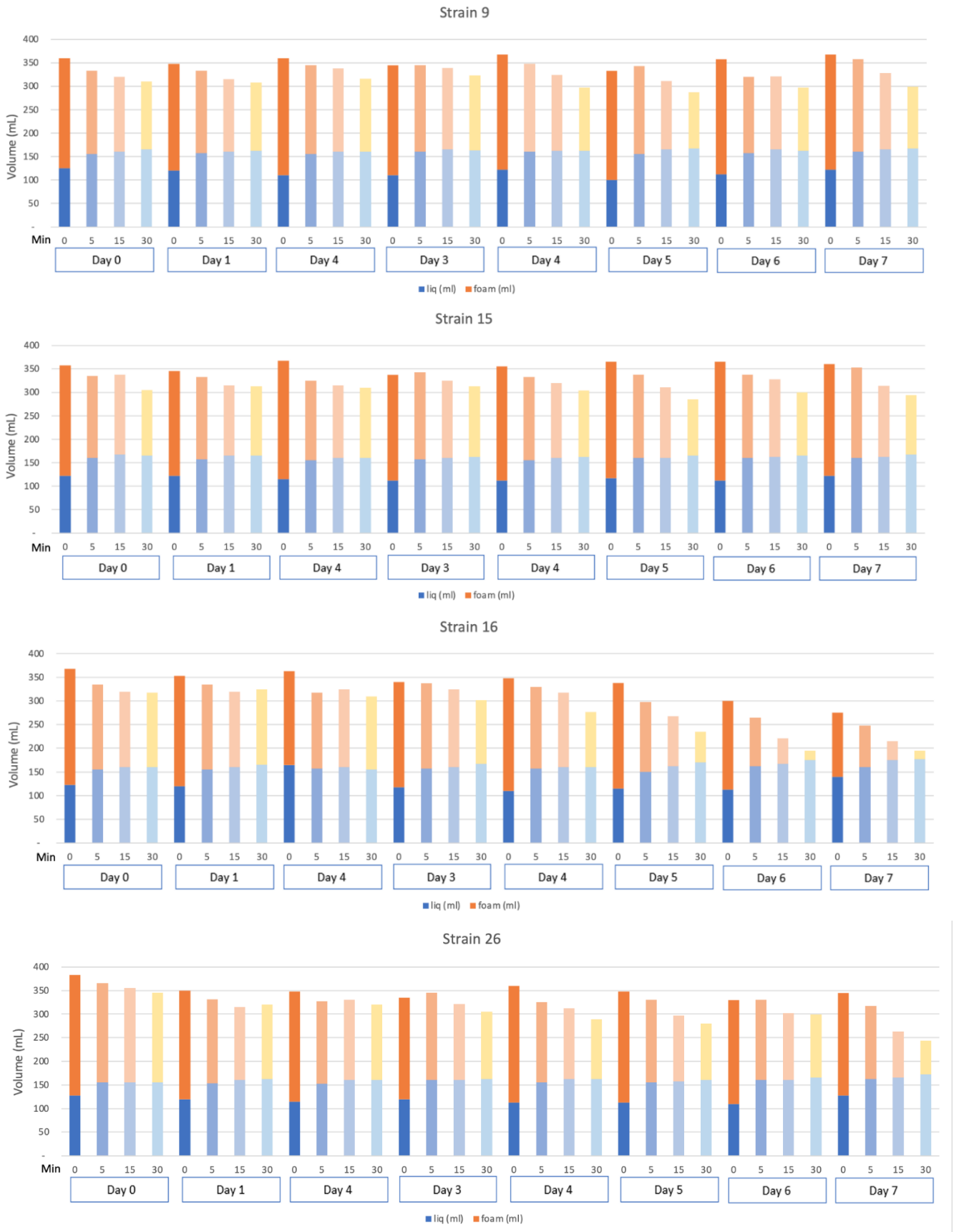


Figure 17. **Liquid and foam volume (mL) of the milk samples inoculated with *Pseudomonas* strains 9, 15, 16 and 26. Average of Trial 1 and 3.**

The foam volume of all the milk samples at 0 min was consistently above 200 mL from Day 0 – 7, except for the milk sample Strain 16. The foam volume of Strain 16 at 0 min started decreasing after 5 days of storage and was reduced to 135 mL by Day 7. Considerable increase in the volume (ml) of the liquid phase was also observed on Strain 16 by Day 7. Statistical analysis using Analysis of Variance (ANOVA) was done to determine whether there is a significant difference in the foaming capacity between the milk samples. The result is shown in Table 6.

Table 6. **Analysis of variance on the foam volume at 0 min of the standardized milk inoculated with *Pseudomonas* strains 9, 15, 16 and 26; and the standardized milk with Bronopol (Average Trial 1 and 3).**

Milk sample	p-value
Standardized milk with Bronopol (MB)	0.6532
Standardized milk inoculated with Strain 9	0.4278
Standardized milk inoculated with Strain 15	0.4278
Standardized milk inoculated with Strain 16	0.0176
Standardized milk inoculated with Strain 26	0.9448

The results showed that the foam volume of Strain 16 at 0 min is significantly ($p \leq 0.05$) different from the control sample MB and the milk samples inoculated with strains 9, 15 and 26. The effect of incubation (storage) time on each milk sample was also analyzed to determine whether the foaming capacity of each milk sample differs daily. Table 7 shows the results.

Table 7. **Statistical analysis for the foaming capacity of the standardized milk inoculated with *Pseudomonas* strains 9, 15, 16 and 26; and the two control samples (uninoculated standardized milk, and standardized milk with Bronopol) from Day 0 – 7. (Average Trial 1 and 3).**

Milk sample	p-value
Uninoculated standardized milk (M)	0.5386
Standardized milk with Bronopol (MB)	0.4703
Standardized milk inoculated with Strain 9	0.2930
Standardized milk inoculated with Strain 15	0.2888
Standardized milk inoculated with Strain 16	0.0388
Standardized milk inoculated with Strain 26	0.1515

The statistical analysis showed that storage time has an effect on the foaming capacity of the milk inoculated with Strain 16 ($p \leq 0.05$). The result means that the reduced foaming capacity from Day 0 – 7 is significant ($p = 0.0388$).

4.2.3.2. Foam Stability

The foam stability of the milk samples was determined by measuring the foam volume (mL) after standing from 0 – 30 min. As shown in Figures 18 and 19, the foam volume of all the 6 milk samples decreased as standing time increased.

The time difference between 0 – 5 min, 0 – 15 min and 0 – 30 min on each milk sample was analyzed using a linear model to determine the significant difference in the foam volume (ml) from 0 – 30 min. The results are shown in Table 8.

Table 8. **Statistical analysis for the foam volume (mL) of the standardized milk inoculated with *Pseudomonas* strains 9, 15, 16 and 26; and the control samples: uninoculated standardized milk and the standardized milk with Bronopol (Average Trial 1 and 3).**

Milk sample	0 – 5 min p-value	0 – 15 min p-value	0 – 30 min p-value
Uninoculated standardized milk (M)	0.7681	0.9527	0.6764
Standardized milk with Bronopol (MB)	0.3431	0.0909	0.0103
Standardized milk inoculated with Strain 9	0.8032	0.0947	0.0241
Standardized milk inoculated with Strain 15	0.9285	0.1967	0.0727
Standardized milk inoculated with Strain 16	0.4569	0.1209	0.0395
Standardized milk inoculated with Strain 26	0.3999	0.0274	0.0178

The decrease in foam volume (mL) from 0 to 30 min was significant in the milk samples MB, standardized milk inoculated with *Pseudomonas* Strains 9, 16 and 26.

The foam volume of MB, Strain 9 was significantly reduced by Day 7, while the foam volume of Strain 16 and 26 was reduced considerably by Day 3 and 4, respectively. A significant ($p \leq 0.05$) decrease in foam volume after standing 15 min was also observed on Strain 26 by Day 7.

The foam volume (mL) of MB and Strain 9 was significantly reduced to <133 ml after standing 30 min by Day 7. The foam volume of Strain 16 was reduced to 134 mL after standing 30 min by Day 3 and went further down to 18 mL on Day 7. The foam volume of Strain 26 was reduced to 126 mL by Day 4 and was also reduced to 71 ml on Day 7.

The difference in the foam stability between the milk samples was determined by multivariate Analysis of Variance (ANOVA) using foam volumes after 0, 5, 15 and 30 min. The results are shown on Table 9.

Table 9. **Multivariate ANOVA of the foam stability of the standardized milk inoculated with *Pseudomonas* strains 9, 15, 16 and 26; and the standardized milk with Bronopol using foam volumes after 0, 5, 15 and 30 min. (Average Trial 1 and 3).**

Milk sample	0 min p-value	5 min p-value	15 min p-value	30 min p-value
Standardized milk with Bronopol (MB)	0.6532	0.5783	0.7828	0.6434
Standardized milk inoculated with Strain 9	0.4278	0.6705	0.9331	0.6507
Standardized milk inoculated with Strain 15	0.4278	0.3964	0.7462	0.5579
Standardized milk inoculated with Strain 16	0.0176	0.0005	0.0071	0.0056
Standardized milk inoculated with Strain 26	0.9448	0.3228	0.4166	0.5108

The statistical analysis showed that the milk sample inoculated with Strain 16 is significantly ($p \leq 0.05$) different among the milk samples in terms of foam stability at all time points. The impaired foam stability of Strain 16 started at Day 3, and a further significant decrease in foam stability was observed from Day 5 to Day 7.

4.2.2.1. Appearance of the milk foam

The changes in the foam of all the milk samples from 0 – 30 min were observed by the naked eye. The size of the bubbles was described using the parameters from Kavli (personal communication, November 18, 2019) that were used during the pilot study. The appearance of the foam made from the milk samples from Trial 1 is presented in Figures 18 – 22 and 24. The foam appearance of the control sample "M" and Strain 16 was compared on Trial 3 and shown in Figure 23.

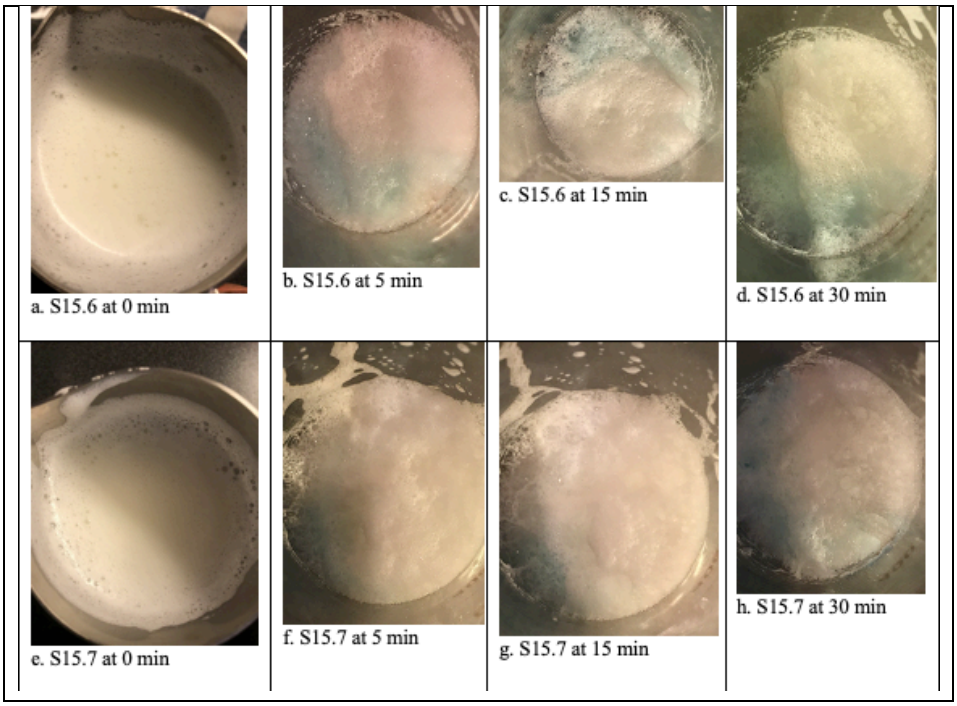
Figures 18a – x. **The foam appearance of uninoculated standardized milk (M) from 0 – 30 min at Day 2 – 7 (Trial 1).**

Figures 19a – l. **The foam appearance of the standardized milk with Bronopol (MB) from 0 – 30 min at Day 3, 6 & 7 (Trial 1).**

The foam of the uninoculated standardized milk (M) and the standardized milk with Bronopol (MB) right after steam injection (0 min) appeared to have small spherical bubbles and was compact. The spherical bubbles of these two control samples were observed to increase in size as standing time increased to 30 min. After standing for 5 min on Day 4, a portion of the foam of sample M started to collapse. On Day 5, the foam started forming larger bubbles after 5 min. The foam bubbles also began to form irregular sizes after 5 min on Day 7 and also became dry as time increased. The sample MB, on the other hand, started having dry foam with irregular bubble sizes on Day 6 after standing 15 min.

Figures 20a – t. **The foam appearance of the standardized milk inoculated with *Pseudomonas* strain 9 from 0 – 30 min at Day 0, 4 & 5 (Trial 1).**

The foam appearance of the standardized milk inoculated with *Pseudomonas* strain 9 (S9) at 0 min was also observed to have small and spherical bubbles right after steam injection. However, the foam was not as compact as the foam of the uninoculated standardized milk (M). The bubbles were also larger from Day 4 at 5 min and had irregular shapes and sizes after standing for 30 min. A portion of the foam started to collapse at 15 min on Day 6, and large irregular bubbles were seen after 30 min. Larger and irregular bubbles began forming at 15 min on Day 7.

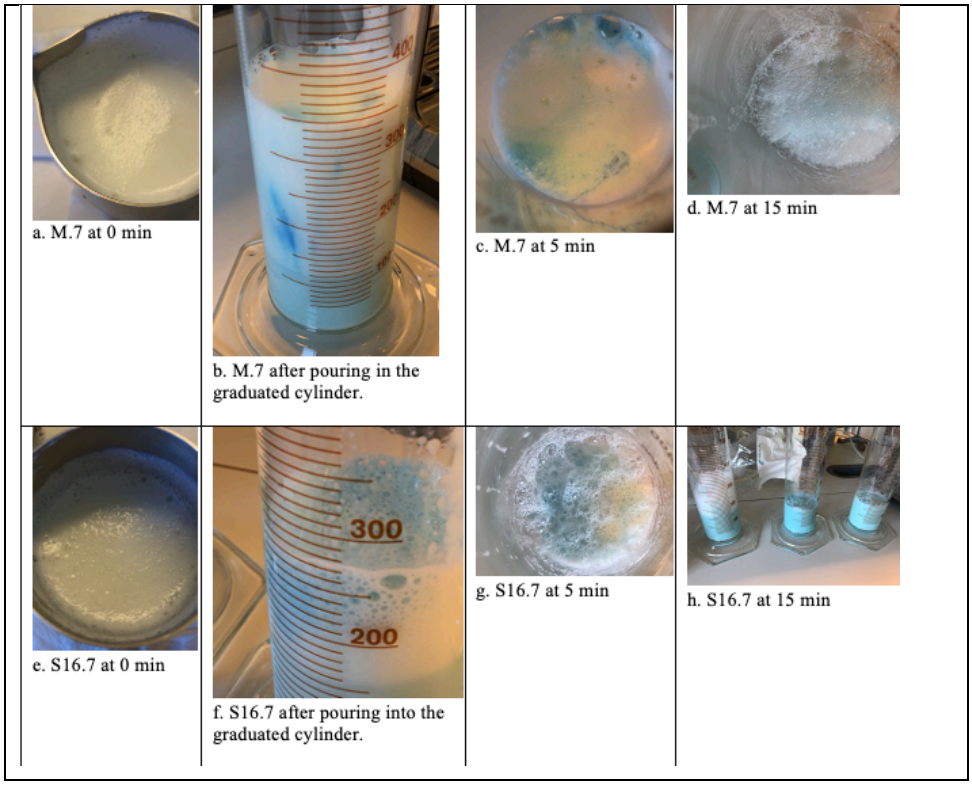


Figures 21a – h. **The foam appearance of standardized milk inoculated with *Pseudomonas* strain 15 from 0 – 30 min at Day 6-7 (Trial 1).**

The foam appearance of the standardized milk inoculated with *Pseudomonas* strain 15 (S15) at 0 min on Day 6 and 7 was less compact and had few larger bubbles. Irregular bubble sizes were apparent after standing 5 min on Day 7.

Figures 22a – l. **The foam appearance of standardized milk inoculated with *Pseudomonas* strain16 from 0 – 30 min at Day 5 – 7 (Trial 1).**

The foam of the standardized milk inoculated with *Pseudomonas* strain 16 (S16) was less compact and had larger bubbles compared to the rest of the milk samples. The shape and sizes of the bubbles were irregular from Day 5 at 15 min. The foam was thinner on Day 6, and a portion of the foam was almost reaching the liquid phase of the milk at 15 min, which collapsed after 30 min. On Day 7, the foam was much thinner and already had large and irregular bubble shapes at 5 min. The foam was also reaching the liquid phase of the milk after standing from 15 min.



Figures 23a – h- **The foam appearance of uninoculated standardized milk (M) and standardized milk inoculated with *Pseudomonas* strain 16 from 0 – 15 min at Day 7 (Trial 3).**

On Trial 3, a considerable difference on the foam appearance of sample S16 from sample M was observed. Figure 23 shows that the foam of S16 on Day 7 at 0 min was almost liquid and had larger bubbles compared to the foam of sample M, which still had a smooth appearance. After 5 min, the foam of sample M had a few large spherical bubbles, while sample S16 already had irregular bubble shape and size. The foam of sample M had larger bubbles after standing for15 min, while the foam of sample S16 had collapsed entirely at that time.

Figure 24. **The foam appearance of standardized milk inoculated with *Pseudomonas* strain 26 from 0 – 30 min at Day 0, 4 & 5 (Trial 1).**

The foam of the standardized milk inoculated with *Pseudomonas* strain 26 (S26) had larger bubbles as time increased from 0 - 30 min. The foam appeared to become drier on Day 4 after standing for 30 min. Varying bubble size and shape started forming from 15 min on Day 6. On Day 7, the foam appearance of S26 at 0 min was less compact and had larger bubbles compared to Day 6.

4.2.3.4. Summary of the results on the foaming properties of the milk samples

The foam volume of all the milk samples decreased from 0 to 30 min. It was also observed that the small spherical bubbles of the milk foam in all milk samples were transformed to larger spherical bubbles, then to irregular bubble shapes as time increased, from 0 min to 30 min.

The most significant decrease in foaming capacity and impaired foam stability was observed in milk samples inoculated with Strain 16 after 5 – 7 of storage. Using the foam volumes after 0, 5, 15 and 30 minutes, the statistical analysis showed that Strain 16 is significantly ($p < 0.05$) different from Strain 9, 15, 26 and MB at all time points.

The foam of the standardized milk inoculated with *Pseudomonas* strains 9, 15, 16 and 26 increased in bubble size as storage time increased from Day 0 – 7 compared to the control samples M and MB. Larger and irregular bubble shapes were also formed as standing time increased from 0 to 30 min.

Among all the milk foams, only the foam formed from the standardized milk inoculated with Strain 16 was considerably less compact and had larger bubbles by Day 5. The foam of Strain 16 was the only foam that did not become dry over time as the foam was thinner and had collapsed sooner (after 15 min on Day 7 during Trial 3) compared to the other milk samples.

5. Discussion

Since some studies report that protein and fat degradation by the bacterial enzymes affect the foaming properties of milk, it is of interest to study whether the growth in milk of selected strains of *Pseudomonas* spp. influence the foaming properties of milk. The genus *Pseudomonas* are the psychrotrophic bacteria which tend to dominate in raw milk and produce of heat-stable lipases and proteases that degrade the quality of milk (De Jonghe et al., 2011; Mateos et al., 2014). This study aims to acquire more specific information on the effects of these enzymes in order to develop more efficient control measures to maximize the functional properties of milk, specifically the foaming properties.

The foaming properties of pasteurized milk inoculated with four selected strains of *Pseudomonas* spp. were investigated in this thesis. In order to relate the results to the experiences of baristas in the cafés, an automated steam-frothing machine was used in this thesis. The parameters used to assess the foaming properties were also adapted from how the milk foam is being assessed in the cafés. The growth of the *Pseudomonas* strains in the milk was measured, as well as lipolytic and proteolytic degradation. The enzymatic degradation was examined by chemical analysis performed on GC and CE.

5.1. Pilot study

Standard method in assessing the foaming properties of milk was developed in the pilot study.

5.1.1. Foaming properties of the different types of milk used in the pilot study

The foaming properties of milk are influenced by many factors, including fat content and milk treatment. The temperature condition used in the manufacture, like UHT treatment, strengthens the foam stability and hinders the partial coalescence of the milk fat globules

(Kamath, Huppertz, Houlihan and Deeth, 2008a). The effect of fat content and processing condition (e.g., temperature) was investigated in the pilot study by comparing the foaming properties of the different types of milk. Milk foams were formed from different types of milk at 65 °C using a coffee making machine with an automated frothing steam wand. The foaming capacity and foam stability were measured, and the appearance of the milk foams was compared to each other.

The primary purpose of the pilot study was to identify and develop a standard method in creating a steam-frothed milk foam and in assessing the foaming properties of milk that is relatable to the experience of the baristas and the parameters used in the cafés.

The milk samples that were used in the pilot study came from different sources (e.g., different milk, different batches, different processing conditions). Thus, the milk samples could have several unknown factors that affect the foaming properties. The microbiological quality of these milk samples was also not determined. Therefore, the discussion is solely based on the characteristics of the foam constituents (i.e., milk protein and milk fat).

The foaming capacity was assessed by measuring the foam volume of the milk samples immediately after the milk and the foam had been poured into the measuring cylinder after steam injection, referred to as 0 min. The milk sample that produced the highest foam volume at 0 min was designated as having the highest foaming capacity. The results from the pilot study showed that the foaming capacity of pasteurized skimmed milk was higher than the foaming capacity of pasteurized full-fat milk, pasteurized semi-skimmed milk; and UHT-treated semi-skimmed milk.

Skim milk is known to produce copious amounts and stable foam. In the pilot study, the pasteurized skimmed milk had the greatest amount of foam at 0 min compared to the rest of the milk samples, and the foam volume was 70 % more than the original volume of the

milk. In the foam system formed by skimmed milk, proteins dominate the foaming attitude of the product; thus, it is classified as protein-stabilized foam. Milk proteins are surface-active and can reduce interfacial tension. The air: liquid interface in such foams was reported to have the presence of, and enriched with, casein micelles (Brooker, 1985; Borcherding et al., 2008).

The foam volumes at 0 min of pasteurized full-fat milk and pasteurized semi-skimmed milk were about half of the volume of the foam formed from the pasteurized skimmed milk. The reduced foaming capacity of the pasteurized full-fat and semi-skimmed milk could be due to competitive destabilization. The combination of low-molecular mass surfactants such as polar lipids and high molecular mass surfactants such as protein has a destabilizing effect on the foaming properties of milk. As surface-active lipids in the milk compete with proteins at the air: liquid interface, the protein interactions are disrupted, and the migration of the surface-active lipids via the Gibbs-Marangoni mechanism is hindered, resulting to reduced foaming capacity of the pasteurized full fat and semi-skimmed milk (Wilde et al., 2004).

The UHT-treated semi-skimmed milk had greater foam volume at 0 min than the pasteurized full-fat and semi-skimmed milk. The greater foaming capacity of the UHT-treated semi-skimmed milk could be attributed to the heat-induced association of whey proteins with the casein that are present on the surface of homogenized milk fat globules (Huppertz & Kelly, 2006; Kamath et al., 2008a).

Drainage has great relevance to steam-frothed milk foam and is an important factor in foam stability. The liquid flow through the lamella describes foam drainage. This liquid flow is driven by both gravity and capillarity (Hutzler, Weaire, Saugey, Cox and Peron, 2005). Capillarity is the ability of the liquid to flow against gravity in a narrow space such as the lamella (Davidson Institute, 2012). The drainage continues until the correct capillary

suction has a larger curvature of the capillary (lamella), which happens when the surface tension is higher (Prins, 1986; Walstra, 2003; Huppertz, 2010).

The foam stability in this pilot study was assessed by measuring the foam volume of the milk samples after 0, 15, and 30 min. The foams produced in pasteurized skimmed milk and UHT-treated semi-skimmed milk were more stable compared to that in pasteurized full-fat milk and pasteurized semi-skimmed milk.

The volume of the liquid phase in the foamed pasteurized skimmed milk increased from 121 mL to 163 mL after 15 min, and remained steady even after 30 min, while the foam volume decreased from 186 mL after standing 15 min in room temperature and slightly decreased to 150 mL after 30 min. Skim milk is a protein-stabilized foam, and therefore, the protein interactions form a viscoelastic film on the surface that stabilizes the foam system (Damodaran et al., 2007). Increase in the volume of the liquid could be due to the coalescence of the air bubbles, which typically occurs to minimize the surface area that was enlarged when the foam was formed (Walstra, 2003). The stability of the volume of the liquid and slight decrease in the foam volume over time could be due to less rupture of the air bubbles, which suggests that the bubbles around the lamella of this foam were very small since the lamella was “well-defined” or not stretched (Kamath et al., 2008a). Thus, the reduction in the foam volume and the increase in the liquid volume is primarily caused by drainage and that the foam is becoming drier.

With regards to the UHT-treated milk, the volume of the liquid phase increased from 141 mL to 161 mL after 15 min and remained the same after 30 min. The foam volume also remained steady at 145 mL even after standing 30 min. The foam stability of the UHT-treated semi-skimmed milk is more likely due to the heat-induced association of the whey proteins with caseins on the surface of the milk fat globules. This association makes the milk fat

globules less susceptible to the re-formation of fat crystals during cooling of the foams (Kamath et al., 2008a).

The foam stability of the pasteurized full-fat milk and semi-skimmed milk was reduced as standing time at room temperature increased. The foam volume of the pasteurized full-fat milk decreased from 123 mL to 36 mL after 30 min, whereas the foam volume of pasteurized semi-skimmed milk had collapsed after 15 min. In addition to the competitive destabilization, the influence of temperature on the milk fat also impairs the foaming stability of milk. Although the foams from the pasteurized full-fat and semi-skimmed milk were steam-frothed at 65 °C, in which the milk fat is in liquid form, re-crystallization of globular milk fat could occur during standing in room temperature. Since the pasteurized full-fat milk had higher fat content than the pasteurized semi-skimmed milk, it has a higher concentration of fat globules that are readily adsorbed at the interface that makes the foam more stable (Walstra, 1996; Kamath et al., 2008a).

The rapid increase in the liquid phase from the time that the milk and foam had been poured on the measuring cylinder up until 5 min could be attributed to the coalescence of the air bubbles that occurs normally to minimize the surface area, which had been enlarged during the foam formation (Walstra, 2003).

5.1.2. Appearance of the foam

The appearance of the foam was assessed from 0 min, and after standing 15 and 30 min in room temperature. The foam appearance of the milk samples was compared to each other and was described according to the parameters from Kavli (personal communication, November 18, 2019). The fine bubbles and compact foam of the pasteurized skimmed milk can be attributed to the protein-enriched interface that stabilized the foam. Over time, from 0 – 30 min, the appearance of the foam became coarse and dry, which was probably due to drainage

and coalescence. When the spherical bubbles came into contact with each other and gravity allowed them to be deformed into polyhedral bubbles, the top layer of the foam appears to be dry and coarse (Huppertz, 2010).

In the case of the foam appearance of the pasteurized full-fat milk and pasteurized semi-skimmed milk, the presence of fat in the foam system could have led to competitive destabilization. Thus, the surface tension is higher, which resulted in a larger radius of curvature of the capillary. Consequently, the plateau border becomes thicker, and the foam of the pasteurized full-fat milk and pasteurized semi-skimmed milk appeared to be wetter and less compact. Due to the competitive destabilization, the coalescence of the air bubbles may not have been prevented, and thus larger bubbles were formed over time (Prins, 1986; Walstra, 2003; Huppertz, 2010).

An attempt to take a microscopic image was made on all the foams. However, it was difficult to standardize the amount of foam on each slide, and so they were not the same, and also the foams started draining on the slide. The air or liquid was also assumed to have been evaporated; thus, the photo did not capture the real appearance of the foams in the measuring cylinder. The image of the foam at 0 min formed from the pasteurized skimmed milk did not represent the fine bubbles and compact foam as described. The foam was moving on the microscope slide, and the bubbles seemed disrupted.

5.1.3. Discussion summary and conclusion of the pilot study

Among the different types of milk that were used in the pilot study of the foaming properties of milk, pasteurized semi-skimmed milk with 1 % fat was used as the milk sample for the actual experiment. This type of milk had the least foaming capacity and most unstable foam compared to the pasteurized skimmed milk, UHT-treated semi-skimmed milk and pasteurized full-fat milk. The pasteurized semi-skimmed milk has lower fat content,

which means it has fewer and smaller fat globules in the milk that can be readily adsorbed at the interface of the foam system. Thus, the foam formed from the pasteurized semi-skimmed milk was less stable compared to the pasteurized full-fat milk. According to Kavli, the pasteurized semi-skimmed milk with 1 % fat is also the most problematic milk in terms of having consistent good foaming properties (A.B. Hauge, personal communication, February 3, 2020). Therefore, this type of milk was chosen to be studied in the main experiment.

A rapid increase of the liquid phase in all the milk samples from 0 min to 5 min was observed during the pilot study. Therefore, the foam volume measurement was determined to include 5 min in the measurement readings.

The image of the foam taken using a microscope did not capture the same foam appearance that was in the measuring cylinder. Therefore, taking a photo of the foam using a microscope was not taken further to the actual experiment.

5.2. Main Experiment

The growth of the selected *Pseudomonas* strains in the milk stored at 4 °C was measured for 7 days in the main experiment. The extent of lipolytic and proteolytic capabilities of the selected *Pseudomonas* strains were measured in terms of FFA content and casein degradation. The effect of the liberated FFA and casein degradation on the foaming properties of the milk were investigated by assessing the foaming capacity, foaming stability and foam appearance of each milk sample.

The action of the selected *Pseudomonas* strains that were inoculated to the milk following the milk treatment could explain the significant observations on this study. An overview of what could have happened in the milk with poor foaming properties in real-life situations as compared to the experimental set-up of this thesis is presented in Table 10.

The comparison shows the milk processing from the farm up to the storage of the treated milk from "real-life" situation, the experimental set-up, and the previous studies of Tidemann (2016) and Bækkeleund (2016).

Table 10. **Milk treatment and processing times of the milk samples from the experimental set-up of this thesis and the previous studies of Tidemann (2016) and Bækkelund (2016) and in real-life situation.**

Milk processing	Real-life	Tidemann & Bækkelund	Experimental set-up
Milk collection			
From farm to silo tanks	About 3 days	About 3 days	Same day (<1 hr)
Heat treatment			
From silo tanks to heat treatment	About 2 - 3 days	About 2 - 3 days	Same day (<1 hr)
Total no. of days before the heat treatment	5 – 6 days	5 – 6 days	0 day
Type of heat-treatment	Pasteurized	UHT	Pasteurized
% fat	1 %	3.5 %	1 %
Filling / Packing	Same day	Same day	Same day (<1 hr)
Presence of *Pseudomonas* spp.	Unknown	Inoculated 3 log CFU ml^{-1}	Inoculated 3 log CFU ml^{-1}
Storage	4 °C	4, 8, 22 °C	4 °C
From production until before using the milk	5 + max 11 days	5 + 9 days	0 + 7 days

The raw milk in real-life and from the previous studies of Tidemann (2016) and Bækkelund (2016) could have been stored for 5 – 6 days before the heat treatment. In contrast, the raw milk from this experiment was heat-treated at the same day of milking and then inoculated with selected strains of *Pseudomonas*.

5.2.1. Growth of the selected *Pseudomonas* strains in milk

The raw milk that was used in the experiment was freshly drawn from the cow´s udder and was pasteurized on the same day of milking and was then bottled aseptically. Freshly drawn milk was used to make sure that there were no unknown factors, such as the growth of diverse bacteria during storage that could influence the foaming properties of milk.

In this study, the selected *Pseudomonas* strains grew in refrigerated milk at 4 °C, which confirmed that *Pseudomonas* has the ability to grow and thrive at temperatures below 7 °C (Adams & Moss, 2008). The selected *Pseudomonas* strains showed slow growth at the beginning of the log phase, as expected, but reached 7 log CFU ml^{-1} by Day 7. The results obtained in this thesis used approximately the same starting concentration of the strains and had the same cell numbers at Day 0 as Tidemann (2016) and Bækkeleund (2016). However, the growth of the *Pseudomonas* strains in this experiment grew more slowly and attained lower cell numbers. Aside from the heat treatment used and the number of days in the growth study, another difference between these studies was the number of days that the raw milk had been stored before the heat treatment. Factors between the differences in the heat treatment and storage times that could have influenced the growth of the *Pseudomonas* strains were not, and could not be, determined. Reasons for the difference in the growth of the selected *Pseudomonas* strains between these experiments are therefore unknown.

Pseudomonas spp. are considered the most predominant species in milk and are important biofilm-makers and producers of thermo-resistant enzymes. A study showed that *Pseudomonas* spp. from environmental origin could grow in refrigerated raw milk up to 6 log CFU ml^{-1} in 96 hours (Capodifoglio et al., 2016). Since raw milk is always not processed in the dairy within 4 days of milking, heat treatment such as thermisation (heating around 65 °C for 15 sec) is often used in Norway, especially when the raw milk is stored over by the weekend. Such treatment allows the milk to be stored for another 3 – 4 days at <7 °C without significantly increasing the growth of the bacteria, provided that psychrotrophic bacteria do not recontaminate the milk. Thermisation is thought to kill almost all psychrotrophic bacteria

because of inhibition of further growth of bacteria and serves as a thermal shock for the remaining bacteria (Champagne et al., 1994).

In addition to the prolonged storage of raw milk on the farm and at the dairy plant, pasteurized milk can also be more prone to recontamination since it is not aseptically packaged, unlike UHT processed milk. Recontamination by *Pseudomonas* spp. following pasteurization was reported to be the main cause of pasteurized milk deterioration at temperatures below 7 °C (Sørhaug and Stephaniak, 1997). The growth of the selected *Pseudomonas* strains in the pasteurized milk at 4 °C helps to consider applying more efficient hygienic control measures from the farm to the dairy plant and consider which type of milk is most practical to use in the cafés.

5.2.2. Chemical Analysis

5.2.2.1. Content of the free fatty acids in the milk samples by Gas Chromatography

Cooling the raw milk to low temperatures like 4 °C without acceptable hygiene practices prior to heat treatment of the milk would allow *Pseudomonas* spp. to grow and to produce heat resistant lipases. Lipase production occurs to compensate for the reduced transportation and diffusion of the nutrients to the bacterial cells (Buck et al., 1986). Furthermore, lecithinase and phospholipases C and A are lipases from psychrotrophic bacteria that can disrupt the native membrane structure of fat globules by hydrolyzing phospholipids, and as a consequence the milk triglycerides, which become available to the native milk lipase, LPL (Shah, 1994; Deeth, 1997). The activation of LPL by certain apolipoproteins is enhanced in the presence of phospholipids, such as phosphatidylcholine (Blaton et al., 1974). Phosphatidylcholine itself is hydrolyzed by LPL that acts as phospholipase A_1 in the presence of serum cofactors (Stock and Galton, 1980) producing lysophosphatidylcholine and free

fatty acids. Lysophospholipids are said to be agents that can disrupt the MFGM and could therefore encourage lipolysis of milk fat in its native form (Deeth, 1997). According to Deeth (1997), a similar scenario could also be caused by phospholipase A from *P. fluorescens.* This suggest that the production of lipase by *Pseudomonas* spp., even in low temperatures, could initiate fat degradation in raw milk, especially those that have prolonged storage time.

The extent of lipolysis in this study was measured by the concentration of FFA. The amount of free fatty acids that had been liberated after 7 days of storage in 4 °C by the selected *Pseudomonas* strains were quantified using a GC analysis.

Statistical analysis of the results showed that the content of FFA in the milk sample inoculated with Strain 16 was significantly higher compared to the FFA concentration of the uninoculated pasteurized milk from Day 0 (M0). On the other hand, the milk inoculated with Strain 9 and the other two control milk samples from Day 7 (uninoculated pasteurized milk (M) and pasteurized milk with Bronopol (MB)), had lower FFA concentration compared to M0. Significant amounts of intermediate- (C14:0 and C16:0) and long-chain (C18:0 and C18:1cis9) FFA were also detected ($p \leq 0.05$). Strain 16 liberated the highest amount of these free fatty acids, while these FFAs were lower in the milk inoculated by Strain 9, and in the control samples M and MB. The *Pseudomonas* strain 26 followed Strain 16 in liberating the most amounts of long-chain FFAs C18:0 and C18:1cis9. In the Tukey test, it also shows that the differences in the FFA concentration of Strain 16 with that of Strain 9, 15, 26, M and MB were significant ($p \leq 0.05$).

This same trend was observed in the previous study by Tidemann (2016), wherein Strain 16 showed the greatest lipolytic capability among the selected *Pseudomonas* strains. The FFA concentrations in the milk samples in this current thesis, however, were lower compared to the ones reported by Tidemann (2016). The lower FFA concentration is probably due to the

difference in fat concentration and the lower growth of bacteria. The study of Tidemann (2016) used UHT treated milk with 3.5 % fat and had cell density close to 8 log CFU mL^{-1} by the end of the log phase. The free fatty acids of milk are distributed in the fat and the fat globule membrane fraction, hence, milk with higher fat content potentially contains a greater source of FFA (Kintner and Day, 1965). In addition, the lipolytic capability could also be affected by the growth rate of the bacterial strains and also that strains were incubated for two more days in Tidemann's work (2016). This suggests that the FFA concentration could possibly be higher if the milk had been stored longer in low temperature. Thus, prolonged storage of raw milk in the farm and before the heat treatment in the dairy could expose the raw milk to higher risk of lipolysis (Shah, 1994; Deeth, 1997).

5.2.2.2. Protein degradation by the selected *Pseudomonas* strains

Bacterial proteases such as the ones produced by *Pseudomonas* spp. are highly active proteases that predominantly affect κ-Cn and β-Cn. The proteases from *Pseudomonas* spp. are produced mainly at the end of the log phase and are said to have the capability to hydrolyze all available casein to soluble peptides during growth in milk at 4 °C (Sørhaug and Stephaniak, 1997). This, however, was not observed based on the electropherograms in this thesis when the proteolytic degradation by the enzymes produced by the selected *Pseudomonas* strains was investigated. The electropherogram of Trial 1 did not show protein degradation aside from the small peak on Strain 26, which came after β-Cn A1 between retention time 32 - 34 min. Protein degradation can, however, be visually observed on the electropherograms from Trial 3 for Strain 26, wherein the peaks of κ-Cn, β-Cn A1 and A2 were reduced compared to the control samples (M.3 and MB.3; and the uninoculated standardized milk from Day 0 (M0)). Slight protein degradation was also observed from Strain 15 from Trial 3, where a reduction of β-Cn A2 and A1, following the formation a

small peptide fraction at retention time 34 min was seen; as well as a slight reduction of κ-Cn, and α_{s2} -Cn, α_{s1}-Cn? These results are also different from the study of Bækkelund (2016) in which β-Cn A2 and A1 were reduced and κ-Cn was degraded entirely by Strains 9, 15, 16 and 26 after 9 days storage at 4 °C. The study of Bækkelund (2016) mentioned that it was not possible to visually observe the protein degradation of the selected *Pseudomonas* strains after 4 days of storage in 4 °C. It was only after 9 days of storage that the proteolytic degradation was seen clearly in electropherograms when the cell density of the strains had almost reached 8 log CFU ml^{-1}. The two days difference between the storage time of this thesis and the study of Bækkelund (2016) could have made a difference in the results. Furthermore, the growth of the selected *Pseudomonas* strains in the previous study was much higher, wherein the strains were able to reach the end of log phase by Day 2 at cell density between 6 - 8 log CFU-ml^{-1}, compared to the growth of the strains in this thesis that were only between 6 – 7 log CFU mL^{-1} at the end of the log phase at Day 3. It might be that the connection between the production of extracellular enzymes and the cell density that occurs at the end of the log phase and the start of the stationary phase could only be right and applicable for cultures that have already reached 8 log CFU ml^{-1} (McKellar, 1989; Dunstall et al., 2005). According to studies, the formation of proteases from *Pseudomonas* spp. in milk is said to start during late log phase and at the early stationary growth phase of the bacteria at cell counts of 7 – 8 log CFU ml^{-1} and increases with increasing storage time (McKellar, 1989; Guinot-Thomas et al., 1995; Matselis & Roussis, 1998; Stevenson Rowe, Wisdom & Kilpatrick, 2003). This suggests that the presence of heat-stable proteases from *Pseudomonas* spp. in milk can be related to prolonged milk storage even at low temperatures.

The electropherograms shown in this thesis indicated a difference in the protein (casein) composition, specifically beta-Cn A1 and A2 when the height of the peaks in Trial 1 and

3 was compared. Although the raw milk used in both trials came from one farm (Ås farm), different cows in the herd might have obviously been selected for the milking. Thus, the individual differences between the cows could have affected the casein composition of b-Cn A1 and A2 as an effect of genetic variation between the cows.

5.2.3. Foaming properties of the milk samples

The assessment of the foaming properties was carried out to investigate the effect of the liberated FFA and casein degradation by the selected *Pseudomonas* strains in the refrigerated pasteurized milk with 1 % fat.

The foam volume (ml) that is above the liquid phase at 0 min determined the foaming capacity of the milk samples in this thesis. The results showed that there were no significant differences between the foaming capacity of the milk samples, and the foaming capacity of each milk sample from Day 1 - 7, except for the milk sample with Strain 16 ($p \leq 0.05$) that showed reduced foaming capacity by Day 5. Based on this trend, the significant decrease in the foaming capacity shown by Strain 16 as storage time increased could be related to the high FFA content of that milk sample. Significant amounts of intermediate- (C14:0 and C16:0) and long-chain (C18:0 and C18:1cis9) were detected in this milk after 7 days of storage at 4 °C. The presence of FFA and proteins in the milk probably reduced the foamability of the milk as these two surfactants compete at the air: liquid interface. FFA are surface-active low molecular mass surfactants that greatly reduce the surface tension, more than the proteins. When low molecular mass surfactant molecules such as FFA are present in the foam system together with high molecular mass surfactants like proteins, the stretchability of FFA at the interface becomes limited, at the same time, the protein interactions at the interface becomes disrupted (Wilde et al., 2004). Since FFAs are low molecular mass surfactants, they do not have the cohesive and viscoelastic properties that are required to withstand the internal pressure of the air bubbles. Thus, the bubbles rapidly expand and then rupture during the steam injection (Damodaran et al., 2007). This same observation was also reported when FFA concentration showed a direct correlation with

the impaired foaming properties of milk (Buchanan, 1965; Deeth & Smith, 1983; Kamath et al. 2008b).

Furthermore, the long-chain FFAs liberated by Strain 16 could have possibly contributed to the reduced foaming capacity of the milk. Long-chain FFAs are said to be highly surface-active, and this was reported to decrease the surface tension of milk (Buchanan, 1965; Sherbon, 1988). Specifically, the presence of long-chain (C18:0) FFA considerably reduced the foaming properties of milk in the study of Buchanan (1965). Their solubility can explain the higher surface activity of the long-chain FFA, wherein the hydrophobic part (C-chain) of the molecule is larger relative to the hydrophilic part (carboxyl group); thus, it is more attached to the lipophilic environment than in the water in a foam system. This makes the surface activity higher since it would take larger free energy to remove the FFA molecule from the interface (Walstra, 2003).

With regards to the foam stability, the foam volume (ml) of the milk samples decreased as standing time in room temperature increased. A significant difference was observed in the foam volume of the control milk sample with Bronopol (MB), and the milk samples inoculated with Strain 9, 15, 16 and 26 after standing 30 min in room temperature.

The foam volume of MB after 30 min (133 ml) was similar to that of the other control milk sample (uninoculated pasteurized milk), which was 136 ml. The significant difference is due to the voluminous foam of MB at 0 min, which was 253 mL that was reduced to 133 mL after standing 30 min. The significant reduction could be due to the measurement reading that was challenging when dry foam layers were uneven, and the foam volumes were measured as average visually.

Milk samples inoculated with Strains 9 and 15 showed a significant difference in foam stability only after standing 30 min, which could be attributed to the effect of drainage in the foam. The slight protein degradation in the milk sample inoculated with Strain 26 could

probably have affected the decrease in foam stability of the milk sample, in which it was observed to significantly decrease after standing 15 min in room temperature on Day 7. These differences did not show any significance when the foam stability of all the milk samples was compared to each other. Among the milk samples, it was the milk inoculated with Strain 16 that showed a significant difference at all time points. The considerable amounts of intermediate- (C14:0, C16:0) and long-chain (C18:0, C18:1cis9) FFAs liberated by Strain 16 must be related to the impaired foaming stability of the milk, which had already started decreasing by Day 3. This same trend was also observed when the role of lipase and lipolysis in frothing of milk (Buchanan, 1965) and the relationship of surface tension, free fatty acids and foaming properties (Kamath et al., 2008b) were studied. The presence of surface-active FFAs decreases the surface tension of milk; therefore, they compete with the proteins for the interfacial area. Surface-active material needs to be absorbed into the air: liquid interface of the air bubbles to stabilize the air incorporated in the milk foam. The pasteurized milk with 1 % fat contains two surfactants, which are the proteins and the FFA. Both surfactants can stabilize the foam. The proteins stabilize the foam by forming a viscoelastic film on the surface through protein-protein interactions. The FFAs stabilize the foam through Gibbs-Marangoni mechanism that requires a rapid absorption of the FFAs from both sides of the lamella to the areas in the adsorbed film with low surfactant concentration to maintain a rapid equilibrium of concentration gradients. So, when both two surfactants are present in the interface, their mechanisms become limited and interrupted, leading to poor foam stability. The adsorption of FFAs leads to disruption of protein-protein interactions; thus, proteins are unable to form a viscoelastic film around the air bubbles that results to unstable foam (Damodaran, 2007; Wilde et al., 2014).

5.2.3.1. Appearance of Foam

As storage time increased, the foams of all the milk samples appeared to have smaller bubbles but were still compact, compared to the early days of the experiment (Day 0 – 4). All the foams, except for the one formed from the milk inoculated with Strain 16, became dry as standing time increased from 0 – 30 min. The foam formed from the milk inoculated with Strain 16 was rather coarse and less compact at 0 min and had larger bubbles and also became wet as standing time increased. The formation of large bubbles in the milk inoculated with Strain 16 could be related to the FFA concentration in the milk, which was significantly higher compared to the FFA content of the rest of the milk samples. As a low molecular mass surfactant, FFA is capable of displacing proteins from the interface that disrupts the formation of viscoelastic film by the protein interactions, thus, it weakens the elastic film around the air bubbles. When the film is weakened, the probability of coalescence of the bubbles is increased, leading to the formation of larger bubbles (Wilde, et al., 2004; Walstra, 2003).

Since FFAs have higher surface activity than proteins, they dominate the air: liquid interface (Mackie & Wilde, 2005). Thus, the foam formed from the milk with Strain 16, which has a higher FFA concentration, had larger bubbles and is less compact, compared to the rest of the foams formed from the milk samples with lower FFA content. The same observations were also reported by Kamath et al. (2008b) wherein the foams formed from the milk with higher FFA concentration were coarser. This also explains why the appearance of the foam formed from the milk with Strain 16 were coarser compared to the rest of the samples. The coalescence of the bubbles can also result to the drainage of the liquid from the film (Huppertz, 2010, Deng et al., 2019), which explains the wet appearance of the foam formed from the milk with Strain 16. When all the foams from all the milk samples became dry over time from 0 – 30 min, the foam from the sample with Strain 16 became wet, and eventually

collapsed after standing 30 min. In addition, the foam consisting of both proteins and surface-active FFAs are said to be elastic and more fluid, which could mean that it is less viscoelastic compared to the foams that are more saturated with proteins (Wilde, et al., 2004).

The test results in the foaming properties of milk suggest that prolonged storage time before the heat treatment of the milk allows the growth of the *Pseudomonas* spp. and the production of heat-stable enzymes that could affect the foaming properties of milk, particularly lipase. On this study, the impaired foaming properties of the milk was observed at cell density 7 log CFU ml^{-1} of Strain 16 that liberated the most FFAs (0.42 mg/g^{-1}).

6. Conclusion

Since not all the casein degradation can be visible in electropherogram, the effect of proteolysis on the foaming properties of milk cannot be eliminated.

Nevertheless, the results of this thesis showed that the long-chain FFAs had an effect on the foaming properties of milk. A relationship between the significant decrease in foaming capacity and foaming stability with increasing concentration of long-chain FFA was observed. Furthermore, the difference in foam appearance was noticeable on the foam formed from the milk with higher FFA concentration. The higher concentration of the long-chain FFAs on this thesis was liberated by the *Pseudomonas* strain with high lipolytic capability that was also described and studied by Tidemann (2016).

Based on these results, it can be concluded that lipolysis has a definite and significant effect on the foaming properties of milk.

7. Recommendations

To confirm the differences in the foam appearance, it is recommended to take the foam image directly from the measuring cylinder containing the foam to get "real-time" pictures of the foam. Measuring the diameter of the bubbles and the distribution of these air bubbles in the interface is also recommended to confirm whether the presence of fat affects the foam appearance and air bubble distribution in the milk foam.
Measuring the surface tension in studying the foaming properties of milk is also recommended to confirm the effect of lipolysis on the surface activity of the FFAs and the foaming properties.

To maximize the functional properties of milk, it is recommended to initiate more efficient control measures to limit the milk from being contaminated with bacteria that produce enzymes with high hydrolytic activity. An effort to improve good hygiene practices in every step in the supply chain is recommended to reduce the contamination. Heat-processing in the farm such as thermisation, could also be an option to treat milk that cannot be collected immediately, especially during weekends. This is to hinder the growth of psychrotrophic bacteria and thus, to limit the presence of quality-degrading enzymes. Since *Pseudomonas* spp. produce both lipases and proteases; high cell density does not always mean that the degradation is caused by lipolytic or proteolytic enzymes. Thus, it would also be beneficial to have a quality measurement that could indicate the extent of lipolysis or proteolysis, e.g., FTIR for FFA concentration. This would enable the sorting of milk, based on the content of heat-resistant enzymes.

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Appendix

Appendix 1. **Liquid and foam volume (mL) of the different types of milk that were used in the pilot study. (Results of two trials)**

Table 1. Liquid and foam volume (mL) of the pasteurized full-fat milk (3.5 % fat), pasteurized semi-skimmed milk (1.2%), UHT semi-skimmed milk (1.2 % fat) and pasteurized skimmed milk (0.1 % fat).

19.02.20	Liq	Foam	Liq	Foam	Liq	Foam
Parallel 1	0 min		15 min		30 min	
Pasteurized full-fat, 3.5% fat	140	150	160	60	120	40
Pasteurized semi-skimmed, 1.2% fat	140	160	180	0	180	0
UHT semi-skimmed, 1.2% fat	140	200	160	170	160	170
Pasteurized skimmed milk, 0.1% fat	110	270	160	200	160	180
19.02.20	Liq	Foam	Liq	Foam	Liq	Foam
Parallel 2	0 min		15 min		30 min	
Pasteurized full-fat, 3.5% fat	145	150	165	65	125	60
Pasteurized semi-skimmed, 1.2% fat	145	165	180	0	175	0
UHT semi-skimmed, 1.2% fat	145	200	160	175	160	170
Pasteurized skimmed milk, 0.1% fat	115	270	165	190	165	185
20.02.20	Liq	Foam	Liq	Foam	Liq	Foam
Parallel 1	0 min		15 min		30 min	
Pasteurized full-fat, 3.5% fat	150	90	170	50	180	20
Pasteurized semi-skimmed, 1.2% fat	140	170	175	0	180	0
UHT semi-skimmed, 1.2% fat	140	170	160	120	160	120
Pasteurized skimmed milk, 0.1% fat	130	240	160	180	160	120
20.02.20	Liq	Foam	Liq	Foam	Liq	Foam
Parallel 2	0 min		15 min		30 min	
Pasteurized full-fat, 3.5% fat	150	100	175	55	180	25
Pasteurized semi-skimmed, 1.2% fat	140	170	175	0	180	0
UHT semi-skimmed, 1.2% fat	140	170	165	115	165	120
Pasteurized skimmed milk, 0.1% fat	130	240	165	175	165	115
	Liq	Foam	Liq	Foam	Liq	Foam
Ave	0 min		15 min		30 min	
Pasteurized full-fat, 3.5% fat	146	123	168	58	151	36
Pasteurized semi-skimmed, 1.2% fat	141	166	178	0	179	0
UHT semi-skimmed, 1.2% fat	141	185	161	145	161	145
Pasteurized skimmed milk, 0.1% fat	121	255	163	186	163	150

Appendix 2. **Microbiological analysis**

Appendix 2.1. **Growth of bacteria in milk from Trial 1, 2 and 3; and the average results of Trial 1 and 3.**

Table 2. Growth of bacteria in milk from Trial 1.

Sample	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
	log cfu mL^{-1}	log cfu mL^{-1}	log cfu mL^{-1}	log cfu mL^{-1}	log cfu mL^{-1}	log cfu mL^{-1}	log cfu mL^{-1}	log cfu mL^{-1}
Strain 9	4.28	4.32	5.70	6.28	7.30	7.45	7.48	7.90
Strain 15	3.78	4.08	4.70	5.60	7.08	7.20	7.34	7.48
Strain 16	4.79	5.28	6.93	7.18	7.32	7.38	7.54	7.65
Strain 26	4.32	4.99	6.40	7.11	7.18	7.26	7.30	7.40
M	0	0	0	0	0	0	0	0
MB	0	0	0	0	0	0	0	0

Table 3. Growth of bacteria in milk from Trial 2.

Sample	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
	log cfu mL^{-1}	log cfu mL^{-1}	log cfu mL^{-1}	log cfu mL^{-1}	log cfu mL^{-1}	log cfu mL^{-1}	log cfu mL^{-1}	log cfu mL^{-1}
Strain 9	4.06	4.98	5.38	6.18	5.53	6.45	6.68	6.08
Strain 15	3.61	4.52	4.85	5.52	4.78	6.15	5.41	6.08
Strain 16	4.06	4.82	4.95	6.26	5.52	6.54	5.68	5.94
Strain 26	3.75	4.83	5.26	4.95	5.11	6.15	5.32	5.18
M	0	0	0	2.53	0	0	4.38	0
MB	0	0	1	1	0	0	4.54	0

Table 4. Growth of bacteria in milk from Trial 3.

Sample	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
	log cfu mL^{-1}	log cfu mL^{-1}	log cfu mL^{-1}	log cfu mL^{-1}	log cfu mL^{-1}	log cfu mL^{-1}	log cfu mL^{-1}	log cfu mL^{-1}
Strain 9	3,11	3,48	4,48	6	6,38	7,15	7,18	7,3
Strain 15	3,18	4	5,26	6,26	6,68	6,85	7,38	7,32
Strain 16	3,81	3,95	5,15	6,73	7,1	7,45	7,63	7,68
Strain 26	3,23	3,6	5,15	6,34	6,75	7,23	7,72	7,69
Milk	0	0	0	0	0	0	0	0
MB	0	0	0	0	0	0	0	0

Table 5. Growth of bacteria in milk. Average results of Trial 1 and 3.

Sample	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
	log cfu mL^{-1}	log cfu mL^{-1}	log cfu mL^{-1}	log cfu mL^{-1}	log cfu mL^{-1}	log cfu mL^{-1}	log cfu mL^{-1}	log cfu mL^{-1}
Strain 9	3.695	3.9	5.09	6.14	6.84	7.3	7.33	7.6
Strain 15	3.48	4.04	4.98	5.93	6.88	7.025	7.36	7.4
Strain 16	4.3	4.615	6.04	6.955	7.21	7.415	7.585	7.665
Strain 26	3.775	4.295	5.775	6.725	6.965	7.245	7.51	7.545
M	0	0	0	0	0	0	0	0
MB	0	0	0	0	0	0	0	0

Appendix 2.2. **Statistical analysis for the growth of the selected *Pseudomonas* strains in milk.**

```
> melk2$day2 <- melk2$Day^2
> model2 <- lm(`log cfu mL` ~ Day+day2, melk2)
> summary(model2)

Call:
lm(formula = `log cfu mL` ~ Day + day2, data = melk2)

Residuals:
     Min       1Q   Median       3Q      Max
-0.72594 -0.16661  0.00344  0.14714  0.74781

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  3.55219    0.14737  24.105  < 2e-16 ***
Day          1.15705    0.09835  11.765 1.46e-12 ***
day2        -0.08330    0.01351  -6.167 1.01e-06 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 0.3502 on 29 degrees of freedom
Multiple R-squared:  0.944,     Adjusted R-squared:  0.9402
F-statistic: 244.6 on 2 and 29 DF,  p-value: < 2.2e-16
```

Figure 1. **Linear model showing no significant difference in the growth of the *Pseudomonas* Strains 9, 15, 16 and 26 in milk stored at 4 °C.**

Appendix 3. **FFA quantification by GC-MS.**

Table 6. FFA in the milk samples quantified by GC (Trial 1).

FFA	9.1	15.1	16.1	26.1	MB.1	M.1	M0.1
C4:0	0,00	0,00	n.a.	n.a.	n.a.	n.a.	n.a.
C6:0	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C7:0	0,00	0,00	n.a.	n.a.	n.a.	n.a.	n.a.
C8:0	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C9:0	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C10:0	0,00	0,01	0,01	0,01	0,01	0,00	0,01
C12:0	0,01	0,01	0,01	0,01	0,01	0,01	0,01
C14:0	0,02	0,03	0,05	0,02	0,03	0,02	0,02
C14:1cis9	0,00	0,00	0,01	0,00	0,00	0,00	0,00
C15:0	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C16:0	0,07	0,13	0,13	0,08	0,12	0,06	0,09
C16:1cis9	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C17:0	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C18:0	0,05	0,06	0,06	0,04	0,06	0,03	0,05
C18:1trans9	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C18:1cis9	0,01	0,02	0,07	0,03	0,02	0,01	0,02
C18:2cis9,12	0,00	0,00	0,00	0,00	n.a.	n.a.	n.a.

Table 7. FFA in the milk samples quantified by GC (Trial 3).

FFA	9.3	15.3	16.3	26.3	MB.3	M.3	M0.3
C4:0	0,00	0,00	n.a.	n.a.	n.a.	n.a.	n.a.
C6:0	0,00	0,00	n.a.	0,00	n.a.	n.a.	n.a.
C7:0	0,00	0,00	n.a.	n.a.	n.a.	n.a.	0,00
C8:0	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C9:0	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C10:0	0,01	0,01	0,02	0,01	0,00	0,00	0,01
C12:0	0,01	0,01	0,02	0,01	0,01	0,01	0,02
C14:0	0,02	0,03	0,08	0,03	0,01	0,03	0,09
C14:1cis9	0,00	0,00	0,01	0,00	0,00	0,00	0,01
C15:0	0,00	0,00	0,01	0,00	0,00	0,00	0,01
C16:0	0,09	0,12	0,16	0,10	0,06	0,09	0,15
C16:1cis9	0,00	n.a.	0,00	0,00	0,00	n.a.	0,00
C17:0	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C18:0	0,05	0,07	0,07	0,06	0,04	0,05	0,07
C18:1trans9	0,00	0,00	n.a.	0,00	0,00	0,00	0,00
C18:1cis9	0,02	0,02	0,10	0,04	0,01	0,02	0,05
C18:2cis9,12	0,00	0,00	0,00	0,00	n.a.	n.a.	0,00

Table 8. FFA in the milk samples quantified by GC (Average of Trial 1 and Trial 3).

FFA	Strain 9	Strain 15	Strain 16	Strain 26	MB	M	M0
C4:0	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C6:0	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C7:0	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C8:0	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C9:0	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C10:0	0,01	0,01	0,01	0,01	0,00	0,00	0,01
C12:0	0,01	0,01	0,02	0,01	0,01	0,01	0,01
C14:0	0,02	0,03	0,06	0,03	0,02	0,03	0,06
C14:1cis9	0,00	0,00	0,01	0,00	0,00	0,00	0,00
C15:0	0,00	0,00	0,01	0,00	0,00	0,00	0,00
C16:0	0,08	0,13	0,14	0,09	0,09	0,07	0,12
C16:1cis9	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C17:0	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C18:0	0,05	0,07	0,06	0,05	0,05	0,04	0,06
C18:1trans9	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C18:1cis9	0,01	0,02	0,09	0,03	0,01	0,01	0,03
C18:2cis9,12	0,00	0,00	0,00	0,00	0,00	0,00	0,00

Appendix 4. **The volume (mL) of liquid and foam on all the milk samples from 0 – 30 min. (Trial 1, 2 and 3; and Average of Trial 1 and 3).**

Appendix 4.1. **The volume (mL) of liquid and foam of uninoculated pasteurized milk (M).**

Table 9. Liquid and foam volume (mL) of control sample M (Trial 1).

Incubation time	Sample	0 min	0 min	5 min	5 min	15 min	15 min	30 min	30 min
		liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)
Day 0	Milk	140	170	150	150	160	130	160	130
Day 1	Milk	140	185	160	160	165	135	170	140
Day 2	Milk	110	225	155	165	160	150	160	135
Day 3	Milk	120	170	150	180	160	140	160	137,5
Day 4	Milk	130	230	160	200	160	180	160	135
Day 5	Milk	130	210	160	170	160	150	160	140
Day 6	Milk	120	180	160	130	160	140	170	135
Day 7	Milk	120	230	165	170	165	122,5	170	112,5

Table 10. Liquid and foam volume (mL) of control sample M (Trial 2).

Incubation time	Sample	0 min	0 min	5 min	5 min	15 min	15 min	30 min	30 min
		liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)
Day 0	Milk	120	260	150	210	160	175	160	130
Day 1	Milk	120	270	160	210	170	160	170	150
Day 2	Milk	110	270	150	170	160	150	160	145
Day 3	Milk	110	175	155	185	160	135	160	153
Day 4	Milk	110	280	155	215	170	180	170	145
Day 5	Milk	110	280	160	170	170	160	160	108
Day 6	Milk	110	270	155	215	165	145	175	138
Day 7	Milk	115	250	160	195	160	165	165	128

Table 11. Liquid and foam volume (mL) of control sample M (Trial 3).

Incubation time	Sample	0 min	0 min	5 min	5 min	15 min	15 min	30 min	30 min
		liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)
Day 0	Milk	105	300	150	230	150	220	150	210
Day 1	Milk	120	270	160	205	170	160	170	150
Day 2	Milk	120	250	160	200	160	180	160	143
Day 3	Milk	105	290	160	205	160	185	170	160
Day 4	Milk	100	265	150	200	160	168	160	150
Day 5	Milk	120	270	150	230	160	183	160	165
Day 6	Milk	110	240	150	195	150	180	150	160
Day 7	Milk	110	230	160	200	170	180	150	160

Table 12. Liquid and foam volume (mL) of control sample M (Average of Trial 1 and Trial 3).

Incubation time	Sample	0 min	0 min	5 min	5 min	15 min	15 min	30 min	30 min
		liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)
Day 0	Milk	123	235	150	190	155	175	155	170
Day 1	Milk	130	228	160	183	168	148	170	145
Day 2	Milk	115	238	158	183	160	165	160	139
Day 3	Milk	113	230	155	193	160	163	165	149
Day 4	Milk	115	248	155	200	160	174	160	143
Day 5	Milk	125	240	155	200	160	166	160	153
Day 6	Milk	115	210	155	163	155	160	160	148
Day 7	Milk	115	230	163	185	168	151	160	136

Appendix 4.2. **The volume (mL) of liquid and foam of pasteurized milk with Bronopol (MB).**

Table 13. Liquid and foam volume (mL) of control sample MB (Trial 1).

Incubation time	Sample	0 min	0 min	5 min	5 min	15 min	15 min	30 min	30 min
		liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)
Day 0	Milk + Bronopol	130	160	160	140	160	140	160	140
Day 1	Milk + Bronopol	115	205	150	145	155	140	155	155
Day 2	Milk + Bronopol	120	225	155	170	160	160	160	155
Day 3	Milk + Bronopol	120	200	160	150	165	145	160	120
Day 4	Milk + Bronopol	140	180	150	150	170	120	170	120
Day 5	Milk + Bronopol	115	225	160	155	160	102,5	170	100
Day 6	Milk + Bronopol	110	200	160	170	160	135	160	100
Day 7	Milk + Bronopol	110	240	150	230	160	170	160	110

Table 14. Liquid and foam volume (mL) of control sample MB (Trial 2).

Incubation time	Sample	0 min	0 min	5 min	5 min	15 min	15 min	30 min	30 min
		liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)
Day 0	Milk + Bronopol	140	210	170	180	170	190	180	158
Day 1	Milk + Bronopol	110	270	160	220	170	190	180	160
Day 2	Milk + Bronopol	110	265	160	190	160	160	170	110
Day 3	Milk + Bronopol	110	195	160	190	165	160	170	120
Day 4	Milk + Bronopol	115	275	165	190	155	153	160	140
Day 5	Milk + Bronopol	115	275	160	200	165	118	170	143
Day 6	Milk + Bronopol	110	200	160	170	160	118	160	100
Day 7	Milk + Bronopol	115	230	165	170	170	145	165	110

Table 15. Liquid and foam volume (mL) of control sample MB (Trial 3).

Incubation time	Sample	0 min	0 min	5 min	5 min	15 min	15 min	30 min	30 min
		liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)
Day 0	Milk + Bronopol	130	305	170	230	170	195	180	160
Day 1	Milk + Bronopol	110	270	160	200	170	190	185	155
Day 2	Milk + Bronopol	110	260	150	200	160	180	160	165
Day 3	Milk + Bronopol	105	260	150	165	160	165	160	140
Day 4	Milk + Bronopol	100	255	150	185	160	160	160	145
Day 5	Milk + Bronopol	105	265	150	200	150	182,5	160	150
Day 6	Milk + Bronopol	115	265	165	200	150	170	160	160
Day 7	Milk + Bronopol	110	265	150	200	150	170	160	155

Table 16. Liquid and foam volume (mL) of control sample MB (Average of Trial 1 and Trial 3).

Incubation time	Sample	0 min	0 min	5 min	5 min	15 min	15 min	30 min	30 min
		liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)
Day 0	Milk + Bronopol	130	233	165	185	165	168	170	150
Day 1	Milk + Bronopol	113	238	155	173	163	165	170	155
Day 2	Milk + Bronopol	115	243	153	185	160	170	160	160
Day 3	Milk + Bronopol	113	230	155	150	163	168	160	160
Day 4	Milk + Bronopol	120	218	150	168	165	140	165	133
Day 5	Milk + Bronopol	110	245	155	178	155	143	165	125
Day 6	Milk + Bronopol	113	233	163	185	155	153	160	130
Day 7	Milk + Bronopol	110	253	150	215	155	170	160	133

Appendix 4.3. **The volume (mL) of liquid and foam of pasteurized milk inoculated with *Pseudomonas* strain 9.**

Table 17. Liquid and foam volume (mL) of milk sample inoculated with *Pseudomonas* strain 9 (Trial 1).

Incubation time	Sample	0 min	0 min	5 min	5 min	15 min	15 min	30 min	30 min
		liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)
Day 0	Strain 9	130	180	150	150	150	140	160	130
Day 1	Strain 9	130	190	150	160	160	140	160	130
Day 2	Strain 9	115	225	150	180	160	160	160	145
Day 3	Strain 9	110	255	160	170	170	180	165	155
Day 4	Strain 9	130	215	165	155	165	132,5	165	102,5
Day 5	Strain 9	95	205	160	175	170	112,5	175	95
Day 6	Strain 9	120	230	155	135	160	132,5	165	100
Day 7	Strain 9	130	220	160	190	170	150	170	97,5

Table 18. Liquid and foam volume (mL) of milk sample inoculated with *Pseudomonas* strain 9 (Trial 2).

Incubation time	Sample	0 min	0 min	5 min	5 min	15 min	15 min	30 min	30 min
		liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)
Day 0	Strain 9	120	280	160	220	170	180	170	160
Day 1	Strain 9	120	250	150	190	160	170	160	155
Day 2	Strain 9	110	290	150	230	160	173	165	135
Day 3	Strain 9	110	260	160	165	165	180	170	155
Day 4	Strain 9	115	270	160	200	165	170	165	85
Day 5	Strain 9	110	290	160	200	165	178	170	128
Day 6	Strain 9	110	290	160	220	170	200	170	120
Day 7	Strain 9	115	275	160	215	170	200	170	115

Table 19. Liquid and foam volume (mL) of milk sample inoculated with *Pseudomonas* strain 9 (Trial 3).

Incubation time	Sample	0 min	0 min	5 min	5 min	15 min	15 min	30 min	30 min
		liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)
Day 0	Strain 9	120	290	160	205	170	180	170	160
Day 1	Strain 9	110	265	165	190	160	170	165	160
Day 2	Strain 9	105	275	160	200	160	195	160	167,5
Day 3	Strain 9	110	215	160	200	160	167,5	162,5	162,5
Day 4	Strain 9	115	275	155	220	160	190	160	167,5
Day 5	Strain 9	105	260	150	200	160	180	160	145
Day 6	Strain 9	105	260	160	190	170	180	160	170
Day 7	Strain 9	115	270	160	205	160	175	165	165

Table 20. Liquid and foam volume (mL) of milk sample inoculated with *Pseudomonas* strain 9 (Average of Trial 1 and Trial 3).

Incubation time	Sample	0 min	0 min	5 min	5 min	15 min	15 min	30 min	30 min
		liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)
Day 0	Strain 9	125	235	155	178	160	160	165	145
Day 1	Strain 9	120	228	158	175	160	155	163	145
Day 2	Strain 9	110	250	155	190	160	178	160	156
Day 3	Strain 9	110	235	160	185	165	174	164	159
Day 4	Strain 9	123	245	160	188	163	161	163	135
Day 5	Strain 9	100	233	155	188	165	146	168	120
Day 6	Strain 9	113	245	158	163	165	156	163	135
Day 7	Strain 9	123	245	160	198	165	163	168	131

Appendix 4.4. **The volume (mL) of liquid and foam of pasteurized milk inoculated with *Pseudomonas* strain 15.**

Table 21. Liquid and foam volume (mL) of milk sample inoculated with *Pseudomonas* strain 15 (Trial 1).

Incubation time	Sample	0 min	0 min	5 min	5 min	15 min	15 min	30 min	30 min
		liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)
Day 0	Strain 15	125	180	160	140	160	140	160	120
Day 1	Strain 15	130	180	150	160	160	140	160	140
Day 2	Strain 15	110	230	150	170	160	150	160	140
Day 3	Strain 15	110	200	160	170	160	170	165	140
Day 4	Strain 15	125	215	160	160	165	135	165	125
Day 5	Strain 15	120	235	160	175	170	120	170	95
Day 6	Strain 15	115	240	160	165	165	150	170	115
Day 7	Strain 15	130	210	160	185	165	122,5	165	102,5

Table 22. Liquid and foam volume (mL) of milk sample inoculated with *Pseudomonas* strain 15 (Trial 2).

Incubation time	Sample	0 min	0 min	5 min	5 min	15 min	15 min	30 min	30 min
		liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)
Day 0	Strain 15	120	280	160	210	170	180	170	165
Day 1	Strain 15	120	250	160	195	170	160	170	150
Day 2	Strain 15	110	275	150	215	160	200	165	153
Day 3	Strain 15	110	285	160	140	160	170	170	150
Day 4	Strain 15	120	255	155	205	165	160	160	148
Day 5	Strain 15	115	285	160	200	170	163	160	118
Day 6	Strain 15	120	285	160	215	165	160	170	113
Day 7	Strain 15	115	275	160	215	165	158	170	120

Table 23. Liquid and foam volume (mL) of milk sample inoculated with *Pseudomonas* strain 15 (Trial 3).

Incubation time	Sample	0 min	0 min	5 min	5 min	15 min	15 min	30 min	30 min
		liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)
Day 0	Strain 15	120	290	160	210	175	200	170	160
Day 1	Strain 15	115	265	165	190	170	160	170	155
Day 2	Strain 15	120	275	160	170	160	160	160	160
Day 3	Strain 15	115	250	155	200	160	160	160	160
Day 4	Strain 15	100	270	150	195	155	185	160	157,5
Day 5	Strain 15	115	260	160	180	150	182,5	160	145
Day 6	Strain 15	110	265	160	190	160	180	160	155
Day 7	Strain 15	115	265	160	200	160	180	170	150

Table 24. Liquid and foam volume (mL) of milk sample inoculated with *Pseudomonas* strain 15 (Average of Trial 1 and Trial 3).

Incubation time	Sample	0 min	0 min	5 min	5 min	15 min	15 min	30 min	30 min
		liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)
Day 0	Strain 15	123	235	160	175	168	170	165	140
Day 1	Strain 15	123	223	158	175	165	150	165	148
Day 2	Strain 15	115	253	155	170	160	155	160	150
Day 3	Strain 15	113	225	158	185	160	165	163	150
Day 4	Strain 15	113	243	155	178	160	160	163	141
Day 5	Strain 15	118	248	160	178	160	151	165	120
Day 6	Strain 15	113	253	160	178	163	165	165	135
Day 7	Strain 15	123	238	160	193	163	151	168	126

Appendix 4.5. **The volume (mL) of liquid and foam of pasteurized milk inoculated with *Pseudomonas* strain 16.**

Table 25. Liquid and foam volume (mL) of milk sample inoculated with *Pseudomonas* strain 16 (Trial 1).

Incubation time	Sample	0 min	0 min	5 min	5 min	15 min	15 min	30 min	30 min
		liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)
Day 0	Strain 16	130	200	150	160	160	150	160	130
Day 1	Strain 16	135	200	160	160	160	150	170	130
Day 2	Strain 16	220	145	155	150	166	160	150	150
Day 3	Strain 16	120	185	155	180	160	160	165	117,5
Day 4	Strain 16	120	200	165	140	160	130	160	72,5
Day 5	Strain 16	110	220	150	180	160	120	170	130
Day 6	Strain 16	115	215	160	160	160	107,5	170	40
Day 7	Strain 16	130	200	160	135	170	80	175	35

Table 26. Liquid and foam volume (mL) of milk sample inoculated with *Pseudomonas* strain 16 (Trial 2).

Incubation time	Sample	0 min	0 min	5 min	5 min	15 min	15 min	30 min	30 min
		liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)
Day 0	Strain 16	120	275	160	220	160	200	160	190
Day 1	Strain 16	120	240	150	200	160	170	160	190
Day 2	Strain 16	120	240	150	205	160	150	160	153
Day 3	Strain 16	120	185	155	180	160	170	160	120
Day 4	Strain 16	115	265	160	185	165	140	165	108
Day 5	Strain 16	115	265	155	210	175	128	170	108
Day 6	Strain 16	115	275	165	215	170	120	170	125
Day 7	Strain 16	120	250	160	205	170	110	170	50

Table 27. Liquid and foam volume (mL) of milk sample inoculated with *Pseudomonas* strain 16 (Trial 3).

Incubation time	Sample	0 min	0 min	5 min	5 min	15 min	15 min	30 min	30 min
		liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)
Day 0	Strain 16	115	290	160	200	160	170	160	185
Day 1	Strain 16	105	265	150	200	160	170	160	190
Day 2	Strain 16	110	250	160	170	160	170	160	160
Day 3	Strain 16	115	260	160	180	160	170	170	150
Day 4	Strain 16	100	275	150	205	160	185	160	160
Day 5	Strain 16	120	225	150	115	165	90	170	0
Day 6	Strain 16	110	160	165	45	175	0	180	0
Day 7	Strain 16	150	70	160	40	180	0	180	0

Table 28. Liquid and foam volume (mL) of milk sample inoculated with *Pseudomonas* strain 16 (Average of Trial and Trial 3).

Incubation time	Sample	0 min	0 min	5 min	5 min	15 min	15 min	30 min	30 min
		liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)
Day 0	Strain 16	123	245	155	180	160	160	160	158
Day 1	Strain 16	120	233	155	180	160	160	165	160
Day 2	Strain 16	165	198	158	160	160	165	155	155
Day 3	Strain 16	118	223	158	180	160	165	168	134
Day 4	Strain 16	110	238	158	173	160	158	160	116
Day 5	Strain 16	115	223	150	148	163	105	170	65
Day 6	Strain 16	113	188	163	103	168	54	175	20
Day 7	Strain 16	140	135	160	88	175	40	178	18

Appendix 4.6. **The volume (mL) of liquid and foam of pasteurized milk inoculated with *Pseudomonas* strain 26.**

Table 29. Liquid and foam volume (mL) of milk sample inoculated with *Pseudomonas* strain 26 (Trial 1).

Incubation time	Sample	0 min	0 min	5 min	5 min	15 min	15 min	30 min	30 min
		liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)
Day 0	Strain 26	140	210	160	190	160	180	160	170
Day 1	Strain 26	135	185	152,5	155	160	140	165	125
Day 2	Strain 26	110	235	150	170	160	170	160	150
Day 3	Strain 26	125	170	160	170	160	137,5	165	135
Day 4	Strain 26	120	230	160	140	165	122,5	165	105
Day 5	Strain 26	120	210	160	160	160	110	160	102,5
Day 6	Strain 26	120	180	170	140	160	125	170	107,5
Day 7	Strain 26	135	215	165	170	170	140	175	102,5

Table 30. Liquid and foam volume (mL) of milk sample inoculated with *Pseudomonas* strain 26 (Trial 2).

Incubation time	Sample	0 min	0 min	5 min	5 min	15 min	15 min	30 min	30 min
		liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)
Day 0	Strain 26	120	270	160	190	160	170	170	120
Day 1	Strain 26	120	240	150	200	160	170	160	110
Day 2	Strain 26	110	260	155	185	160	170	125	110
Day 3	Strain 26	120	175	155	170	165	160	170	140
Day 4	Strain 26	110	275	155	215	165	205	165	170
Day 5	Strain 26	110	275	160	205	165	115	160	110
Day 6	Strain 26	115	265	160	205	170	160	170	120
Day 7	Strain 26	120	260	170	195	170	128	170	110

Table 31. Liquid and foam volume (mL) of milk sample inoculated with *Pseudomonas* strain 26 (Trial 3).

Incubation time	Sample	0 min	0 min	5 min	5 min	15 min	15 min	30 min	30 min
		liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)
Day 0	Strain 26	115	300	150	230	150	220	150	210
Day 1	Strain 26	105	275	155	200	160	170	160	190
Day 2	Strain 26	120	230	155	180	160	170	160	170
Day 3	Strain 26	115	260	160	200	160	185	160	150
Day 4	Strain 26	105	265	150	200	160	177,5	160	147,5
Day 5	Strain 26	105	260	150	190	155	170	160	137,5
Day 6	Strain 26	100	260	150	200	160	160	160	160
Day 7	Strain 26	120	220	160	140	160	55	170	40

Table 32. Liquid and foam volume (mL) of milk sample inoculated with *Pseudomonas* strain 26 (Average of Trial and Trial 3).

Incubation time	Sample	0 min	0 min	5 min	5 min	15 min	15 min	30 min	30 min
		liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)	liq (ml)	foam (ml)
Day 0	Strain 26	128	255	155	210	155	200	155	190
Day 1	Strain 26	120	230	154	178	160	155	163	158
Day 2	Strain 26	115	233	153	175	160	170	160	160
Day 3	Strain 26	120	215	160	185	160	161	163	143
Day 4	Strain 26	113	248	155	170	163	150	163	126
Day 5	Strain 26	113	235	155	175	158	140	160	120
Day 6	Strain 26	110	220	160	170	160	143	165	134
Day 7	Strain 26	128	218	163	155	165	98	173	71

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