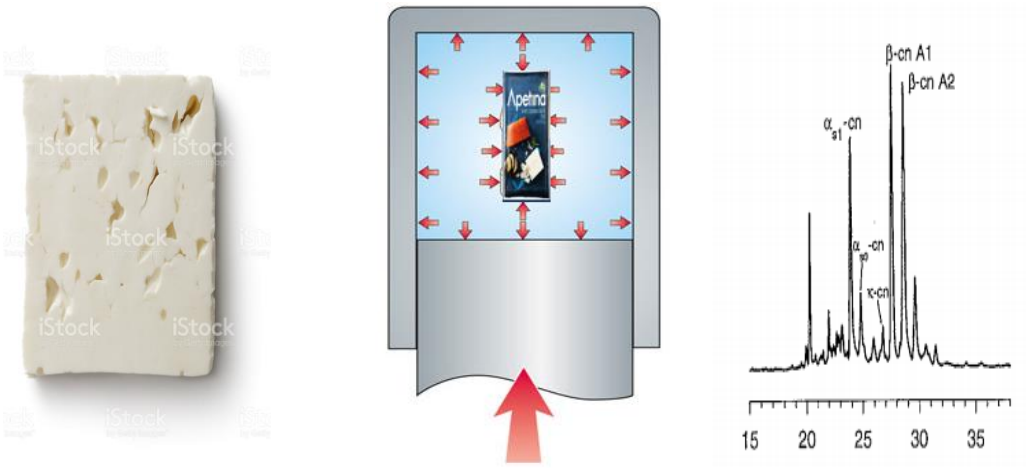
UNIVERSITY OF COPENHAGEN

FACULTY OF SCIENCE

High Pressure Preservation (HPP) for extension of shelf life of commercial packed White brined cheese in terms of microbial quality and physio-chemical properties



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Master Thesis

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Preface

The present study is a master thesis of 30 ECTS-credits which is written as a part of the MSc in Food Science and Technology with specialization in Dairy Science and Technology. The project has been performed in collaboration with Arla Foods, Krusa Dairy, Denmark. The experiments on which the study is based is on was carried out in Department of Food Science, Section of Ingredient and Dairy technology, University of Copenhagen. The project has been conducted from the 4th of September 2017 to the 5th of March 2018 under the supervision of Prof Lilia Arhné, Department of Food Science, University of Copenhagen.

I would like to acknowledge my supervisor Lilia Arhné for her valuable guidance, support, and motivation throughout the entire period of the thesis. I am really grateful to Michael Crafack, Co-supervisor from Arla Foods for giving me this opportunity.

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Abstract

The objective of this study was to extend the shelf life of white brined cheese using High-Pressure technology. Samples were treated at 200,300, and 400 MPa. Treated and untreated samples were stored at 5°C /20°C for 16 weeks. The samples were plated on suitable media to identify yeast and mesophilic aerobic bacteria. Texture analysis was conducted using a texture analyzer was done. Casein fraction were analyzed using Capillary Electrophoresis (CE) and degree of acidity (DOA) was determined for week 0 and 4 to understand the proteolysis in cheese. pH, moisture and sensory analysis was also done to understand the effect of HPP over control samples.

In case of Total aerobic mesophilic bacteria (TAMB), 3 log reduction was observed for samples treated at 400 MPa as compared to 2 log reduction for 200 MPa treatment for 5 min. The effect on microbial inactivation diminished during the storage due to recovery of (TAMB). On the other hand pressuring the cheese samples had detrimental effects on the growth of yeast and molds when treated at 400 MPa for 5 min, as compared to the control irrespective of storage temperature. The effect of HPP on texture (Hardness) and pH diminished significantly over the storage time.

HPP treated samples showed significant degradation of α_{s1}-CN-8P in 4 weeks for both the storage temperatures. While β-CN-A2 degradation was not affected ($P<0.05$) by the pressure but by the storage temperature. In case of lipolysis, pressure treatment increased ($P>0.05$) Degree of Acidity significantly after a month of storage. This effect was more pronounced for samples stored at 20°C. This can show that HPP can accelerate proteolysis in white brined cheese samples.

Industrial relevance: HPP treated sample at 400 MPa prevented late blowing defect as compared to control after storage at 20°C for 16 weeks. Sensorial parameter were improved at the same treatment as compared to the control for both storage temperatures. Thus, it is possible to produce safe and commercially acceptable white brined cheese with extended shelf using HPP.

Abbreviations and symbols

α – Alpha

β – Beta

κ – Kappa

γ – Gamma

ANOVA – Analysis of Variance

CE – Capillary Electrophoresis

CFU – Colony forming units

CN – Casein

DNA – Deoxyribonucleic acid

DOA – Degree of Acidity in fat

EDTA – Ethylene diamine tetraacetic acid

GMP – Glyco-macro-peptide

HCl – Hydrochloric acid

HPLC – High-Performance liquid chromatography

HPP – High-Pressure Preservation/ High-Pressure Processing

ISN – Insoluble

KOH – Potassium Hydroxide

LAB – Lactic Acid Bacteria

MPa – Mega Pascal

PGE – Pregastric esterase

PTA – Phosphotungstic acid

SDS – Sodium dodecyl sulfate

SN – Soluble

TAMB – Total aerobic mesophilic bacteria

TCA –Trichloroacetic acid

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1 Introduction

1.1 Brined cheese

Cheese is a versatile dairy product which is made from buffalo, cattle, goat or sheep's milk in a wide range of flavors and forms across the world. The cheese-making process is to convert milk (nutritious and less stable product) into a concentrated form with improved shelf-life and sensorial properties. It has been reported that there are more than 1000 different kinds of cheese varieties, which can be classified on the basis of three parameters: 1) Texture (Soft, Semi-hard, Hard, Very hard with > 40%, 36-40 %, 25-36 %, <25 % Moisture respectively). 2) Method of Coagulation as primary criteria (Rennet, Acid, Heat), coupled with other criteria 3) Ripening indices (Fox & McSweeney, 2004).

Brined cheese is white in color, have closed texture without any rind, falls in soft to the semi-hard category, rennet coagulated and ripened in brine (Ei-salam, 2004). They are characterized by salty and mildly acidic taste which are popular in the Balkan countries, although they may have a common origin. The cheese variety differs depending on the climatic conditions and dietary habits of the inhabitants of the particular region. Traditionally they are known to be produced from sheep's milk and currently also with cow's or goat's milk or a combination of both is also used (Anifantakis & Moatsou, 2007). Feta (Greek), is the most popular in terms of production volumes, worldwide acceptance, and economic viability, while Domiati (Egypt), Halloumi (Turkey) and Beyaz peynir (Turkey) along with 11 others are comparatively less popular varieties (Ei-salam, 2004).

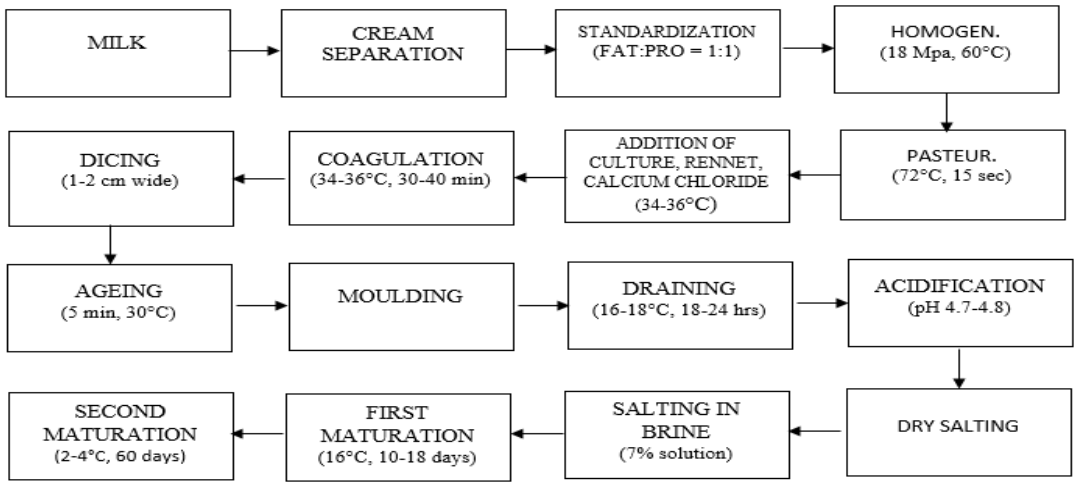

Figure 1.1 – Industrial production of white brined cheese (Ei-salam, 2004; R.K. Robinson and Tamime, 1996)

Chlorophyll and lipase are added to cow's milk before addition of rennet to achieve organoleptic properties of traditional feta cheese (Robinson 1991). Yogurt starter was used for acidification traditionally which is now replaced by cultures with high acidification capabilities. The draining of the curd in the perforated molds leads to the formation of small holes in the cheese curd which is highly desirable in the end product. The cheese curd is periodically turned for optimum drainage and pH development. Dry salting is done to achieve a salt concentration of 3% in the cheese. Then it is kept in brine with 7-8% concentration solution to avoid further softening. At the first stage of maturation, pH reached 4.6 and basic characteristic flavor formation occurs. By the end of the first stage, the moisture level is less than 56% and pH is between 4.4 and 4.6 (Ei-salam, 2004).

Over the last 40 years, major developments have happened, that has influenced production and consumption of feta cheese, which can be summarized as follows:

- The concentration of Milk by Ultra-filtration (UF), resulted in altering of milk composition prior to further processing.
- Increase in production volumes by automation and mechanization of cheese making process.
- Improvements in bacterial cultures.
- Marketing of cheese at global platform along with the rise of consumer awareness regarding health and safety aspect. (Anifantakis & Moatsou, 2007; Ei-salam, 2004; Fox & McSweeney, 2004).

Currently, consumer demands products which are microbiologically safe, minimally processed, highly nutritious, with superior sensorial attributes. On the other hand, cheese manufacturers are investigating to reduce processing cost and extend the shelf life of cheese to ship more products overseas (Gingras, 2016). Shelf life can be limited by physiochemical (syneresis of curd, textural changes), chemical (oxidation of fat) and biochemical changes(ripening, microbial growth, enzymatic degradation) (Terpou et al., 2018). Various non-thermal innovative technologies have been explored like High Pressure, ultra-violet, and pulsed electric field processing has been explored to meet the consumer and manufacturers demands (Portfolio, 2008). High pressure (HP) has been investigated quite extensively over past two decades to produce products that are microbiologically safe without comprising on sensorial and nutritional properties. Application of High-Pressure Preservation has been reported to achieve: 1) Extension of shelf-life which deactivating the spoilage microorganisms. 2) Improve the texture of texture and flavor, and 3) Acceleration of ripening

(Chawla, Patil, & Singh, 2011; Huppertz, 2010; Martínez-Rodríguez et al., 2012; Tewari, 2007).

Effect of High Pressure has been studied for its effects on texture, pH, moisture, and microflora for brined cheese varieties. There is lack of literature for effect of HPP on cow's milk and pressurizing of cheese after packging in brine. Effect of HPP on proteolysis and lipolysis during the ripening of HPP treated cheese in brine from cow's milk needs to further investigate. (Ávila, Gómez-Torres, Delgado, Gaya, & Garde, 2017; Delgado, González-Crespo, Cava, & Ramírez, 2012; Evrendilek, Koca, Harper, & Balasubramaniam, 2008; Koca, Balasubramaniam, & Harper, 2011; Maniou et al., 2013; Moschopoulou, Anisa, Katsaros, Taoukis, & Moatsou, 2010)

1.2 Objective

This work will explore the effects of HPP on commercially available white brined cheese on shelf life and physio-chemical properties.

The work will include:

- Update review literature with a focus on the effect of HPP on brined cheese matrix.
- Perform HPP in range of 200-400 MPa with commercially available classic block (Deep-drawn foil pack) at a storage temperature of 5°C and 20°C. Testing samples at 0,2,4,8 and 16 weeks.
- Selection and set up of methods to evaluate changes in cheese during the shelf life, for example, microbial development, texture analysis, and pH, proteolysis, and lipolysis.
- Determination of critical parameters and potential for improvement.

2 Theory and Literature Review

2.1 White brined-Cheese

Ewe's or goat milk or a mixture of both (70:30 ratio) has been traditionally used to prepare white brine cheese. Casein (CN) constitute about 75-80% of milk protein and basic structure forming an element of the cheese curd. Casein comprises of κ-CN β- CN, α_{s1}-CN, α_{s2}-CN, γ-CN which is insoluble at pH 4.6.Due to higher fat content and casein (α-s1 fraction) availability makes it suitable to impart the desired properties to the cheese (Moatsou & Govaris, 2011).

Table 2.1- Composition of milk (g/100g) from different species (Bylund, 2015)

Species	Moisture	Fat	Casein	Lactose	Ash	Whey
Ewe	82	7.6	3.9	4.8	0.9	0.7
Cow	87.3	4.4	2.8	4.6	0.7	0.6
Buffalo	82.2	7.8	3.2	4.9	0.8	0.6
Goat	86.7	4.5	2.6	4.4	0.8	0.6

The brined cheese is coagulated by rennet and rennet, which destabilizes the caseins to form three dimensional network trapping milk constituents. Rennet is responsible to cleave κ-Casein (CN) which results in aggregation of para-κ-CN and release of Glyco-macro-peptide (GMP) the system. Acid production is due to conversion of lactose to lactic acid by the starter culture. This results in a decrease in pH, which causes neutralization of overall charge on casein micelle. When the iso-electric point is reached (Net charge is zero at pH 4.6) aggregation of casein occurs. This combined aggregation results in entrapment of fat globules and milk serum (Bylund, 2015; Walstra, Wouters, & Geurts, 2006).

The final properties of the cheese are determined by the composition of milk, processing and the ripening conditions. The treatment of the cheese curd-like heating, cutting and brining will affect the moisture content and acid development. This, along with salt distribution will determine the overall condition for the biochemical changes happening in the cheese. Also,

the heat treatment will determine the type of microbial growth and enzymatic activity in the cheese which is crucial for proteolytic activities in cheese (Moatsou & Govaris, 2011).

2.1.1 Proteolysis in white brined cheese

Proteolysis is one of the essential steps in the cheese making process which converts the fresh curd into ripened cheese. There are four important agents which cause proteolysis: milk proteases, coagulants, and microbial proteolytic and pedtidolytic enzymes. Generally, it involves casein breakdown by plasmin and chymosin. The degraded peptides are further hydrolyzed by Lactic acid bacteria (LAB) into smaller peptides. The intracellular LAB peptidases are responsible to release amino acids from the smaller peptides (Sousa, Ardö, & McSweeney, 2001). White brined cheese proteolytic is majorly due to chymosin and proteolytic enzymes from the bacteria. Low pH and absence of heat treatment promote chymosin activity as compared to plasmin. Major biochemical changes are observed during the first stage of maturation, 30-50% α-s1 fraction of casein is degraded while 66% of soluble nitrogen is medium to small peptides or amino acids. (Moatsou & Govaris, 2011). The degraded peptides have free amino and carboxyl groups which improves water binding capacity resulting in hydration of protein matrix. This results in changes in cheese texture (McSweeney, 2011). Also, the free amino acids act as a precursor for flavor development of the cheese (Ardö, Magboul, & Upadhyay, 2017).

Table 2.2- General mechanism in cheese ripening (Mcsweeney, 2007; Sousa et al., 2001)

Enzyme	Origin	Substrate	Product
Chymosin	Rennet	α_{s1}-Casein (CN)	α_{s1}-I-CN and α_{s1} (f1-23)
Plasmin	Milk	β – CN	γ_1, γ_2, γ_3, and protease-peptones
Lactocepin	LAB cell envelope	α_{s1}(f1-23), protease-peptones	Depend on strain α_{s1}(f1-16), α_{s1}(f1-14), α_{s1}(f1-13)
Peptidases	LAB intracellular	Peptides up to about 8 amino acids	Amino acids and small peptides

Plasmin is an alkaline serine protease present in milk which cleaves β-CN at three different sites. The enzyme has trypsin specificity having an optimal pH of 7.5-8 and 37°C. It can also cleave α_{s2}-CN while α_{s1}-CN and κ-CN have limited or no degradation. Although plasmin activity is improved by 2% or more salt concentration it has lesser activity when cheese is made with mesophilic cultures and lower cooking temperatures.

Chymosin is an important proteinase in the rennet which is responsible for milk coagulation and proteolysis in brined cheese. It cleaves κ-CN at Phe_{105}-Met_{106} which results in loss of colloidal stability, resulting in gelation at temperatures above 20°C. It was found that up to 15% of rennet activity remains in the curd while the rest is lost in whey. This will depend on the type of coagulant (animal, microbial and plant). In brined cheese chymosin hydrolyze β-CN is predominant in the first stage of maturation (Figure 1.1), when the salt concentration is less in the cheese interiors. Residual chymosin slowly hydrolyze α_{s1}-CN and the activity is higher in non-scalded cheese varieties. Para-κ-CN and α_{s2}-CN are resistant to chymosins activity during the cheese maturation. Chymosin which has a high ratio of clotting to proteolysis activity is preferred in brine cheese making (McSweeney, 2011).

Lactococcus lactic and cultures from yogurt (*Streptococcus thermophilus, Lactobacillus delbrueckii subsp. bulgaricus*) are ideally used for brined cheese varieties (Özer, 2014). They mainly degrade large and medium size peptides from chymosin and plasmin into oligopeptides. The degraded oligopeptides are transported inside the bacteria for further degradation.

In the intracellular there various aminopeptidases (di- and tri-peptidase, prolinase, endopeptidases) which are highly specific for oligopeptides to form amino acids. When the bacterial cell wall is lysed these enzymes are liberated into the cheese matrix along with amino acids which will act as a precursor for the flavor formation in the cheese (Mcsweeney, n.d.).

White brined cheese types are produced at lower cooking temperatures to be able to keep high moisture in the curd. This lower temperature of cooking a large amount of viable starter culture which results in higher protease activity as compared to scalded cheese. The lactococcal proteinase lactocepin produces peptides degraded by plasmin and chymosin. Intracellular peptidase further degrades the oligopeptides to amino acids for utilization. This results in an excess concentration of amino acids which are not utilized which are not utilized by the bacteria. Type and concentrations of amino acids are considered to be important criteria in monitoring degrees of proteolysis and in deciding the suitability of starter cultures

for white-brined cheese. Glutamic acid, leucine, phenylalanine, valine, and serin are present in relatively higher concentrations in white brined cheese varieties (Mcsweeney, n.d.; Özer, 2014).

In Feta cheese, the peptides are mainly coming from α_{s1}-casein (α_{s1}-CN(f1–14), α_{s1}-CN(f4–14), α_{s1}-CN(f24–30), α_{s1}-CN(f24–32), α_{s1}-CN(f40–49), α_{s1}-CN(f91–98), α_{s1}-CN(f102–109)), 2 peptides originated from the Carboxyl end of β-casein (β-CN(f164–180), β-CN(f191–205)) and 1 peptide from κ-casein (κ-CN(f 96–105)). Most of the peptides could be explained on the basis of known specificity of chymosin and the *lactococcal* proteinase (Ardö et al., 2017).

To evaluate proteolysis in cheese involves separation and identification of different Nitrogen (N) content in the cheese. This involves fractionation of cheese in different solvents and pH to identify and quantify different N component in the cheese. Detailed information on enzymatic and microbial processes behind proteolysis is done by analyzing casein in capillary electrophoresis and amino acids by high-performance liquid chromatography (HPLC) (Miralles et al., 2001).

Table 2.3 - Table 2.4- Different fractions of N compounds extracted from cheese fraction (Ardö, 2001) (ISN – Insoluble, SN – Soluble, TCA – Trichloroacetic acid (12%), PTA – Phosphotungstic acid (5%))

Fraction	**N compounds in cheese**
Citrate dispersion	Casein, whey protein, peptides, amino acids, smaller N compounds (urea, amines, and ammonia)
pH 4.6-ISN	Casein components
pH 4.6-SN	Whey proteins, peptides, amino acids, smaller N compounds
TCA-ISN	Primary breakdown products from casein
TCA-SN	Medium-sized to small peptides and small N compounds
PTA-SN	Very small peptides, amino acids, and ammonia

2.1.2 Lipolysis in white brined cheese

Lipolysis in cheese is an important biochemical reaction that happens during the ripening. The reaction is characterized by the breakdown of milk fat (triglycerides) which happens to a lower extent in brined cheese varieties. Lipolytic agents result in hydrolysis of triglyceride resulting in the release of free-fatty acids (FFA) which reacts with products from proteolysis and other volatile compounds to impart the characteristic cheese flavor. The degree of this reaction depends on the lipolytic agents (Milk lipase, rennet) and microflora of the cheese. Although commercially available rennet are known to be free of lipolytic activity. Quantification of medium and short chain fatty acids can be used to characterize the degree of lipolysis in cheese (Georgala et al., 2005; Wilkinson, 2007).

The starter culture in the cheese is mainly responsible to the degree of lipolysis during the ripening. The traditional Greek feta is known to have lipase called pregastric esterase (PGE) which is specific for short chain fatty acid. They cleave at the sn-3 position of triglyceride which results in characteristic piquant flavor in cheese. Although, the white brined cheese is low at lipolytic activity additional lipases can be used to accelerate the ripening process (Thierry et al., 2017).

Commercial white brined cheese is made with starter cultures with lower lipolytic activity. It is common to find long-chain free fatty acids (FFAs), like myrstic (C14), palmitic (C16), stearic (C18), and oleic (C18:1) acids, but short- chain FFAs (SCFFAs, C4:0-C8:0), contribute to the white brine cheese flavor development directly or indirectly. The Degradation products of FFAs by starter culture results in the formation of volatile compounds, such as esters, alcohols, aldehydes, ketones, and lactones (Özer, 2014).

The free fatty acids released in the cheese matrix can is used to find the degree of acidity (DOA) of cheese. This gives property can quantify to give the extent of lipolysis in the cheese during the maturation. The further FFAs profile can evaluate using gas chromatography to give a deeper understanding of the reaction in the cheese (Karami, 2017).

2.1.3 Microflora of white brined cheese

Lactic Acid Bacteria (LAB) are the predominant microorganism found in white brine (Feta style) cheese produced. The combination and composition of different bacteria are ideal to optimize the acidification rate, flavor, and texture of the cheese. Currently, a combination of *Lactococcus lactic* and cultures from yogurt (*Streptococcus thermophilus, Lactobacillus delbrueckii subsp. bulgaricus*) are ideally used. The ideal culture for white brined cheese is

decided based on the enzymatic activity, tolerance to low pH and high salt content. The cheese composition favors the growth of lactobacilli over the others making it the predominant microflora of the cheese (around 90%).

Table 2.5 Table 2.6 Different Starter culture combination used in the production of White brined cheese (Özer, 2014)

Cheese type	**Starter Culture combination used in the production**
White brine (Feta style)	*Lc. lactis subsp. lactis* + *Lactobacillus casei* + *Leuconostoc mesenteroides subsp. cremoris* (3:1:1)
	Lc. lactis subsp. lactis + *Lc. lactis subsp. cremoris* (1:1)
	P. pentosaceus + lactic starter
	Yogurt culture + *Lc. lactis subsp. lactis* + *Lb. casei*
	Lc. lactis subsp. lactis + *Lb. casei*+ *Enterococcus durans*+ *Ln. mesenteroides subsp. cremoris* (6:2:1:1)
	Lc. lactis subsp. lactis + *Lb. casei* + *E. durans* (6:2:2)

The strain combination that is decided is dependent on each other to produce optimum acid and proteolytic activity in cheese. Conversion of lactose to lactic acid results in the formation of the characteristic crumbly texture of the cheese. On the other hand, mild proteolytic and lipolytic activity will result in characteristic flavor and aroma formation. When only thermophilic starters are used in the cheese it will not only lack in desired flavor (bitter taste) and aroma but also textural (fragile) problems will be significant. *E. durans* are known to solve these problems to a great extent and thus used in combination with the yogurt culture. The combination of *Lc. lactis subsp. lactis* and *Lc. lactissubsp. cremoris* with yogurt culture has been found to produce optimum results (Özer, 2014).

2.1.4 Microbial Contaminants and defects

The microbial quality of cheese depends on the microbial load in the milk, a method of processing and storage/ripening conditions.

The counts of psychrotrophic bacteria tend to white brined cheese will increase during the initial weeks of ripening. Then the growth will depend on the initial microbial load and contaminants added during the processing of cheese. *Pseudomonas spp., Aeromonas spp.*, and *Acinetobacter spp.* are predominant in white brined cheese varieties. Coliforms which are also present in high numbers during early stages of ripening reduces in number at later stages. Their population is quite significant in cheese made from raw milk and cheese processing with poor hygienic conditions.

Pathogens like *Yersinia enterocolitica, Staphylococcus aureus,* and *Listeria monocytogenes* may be associated with white brined cheese. The risk of *Yersinia enterocolitica* increases when the pH development in cheese is slow and the final pH is higher than 4.5. In case of *Listeria monocytogenes* which has relatively higher tolerance towards high salt and low pH can remain viable in the cheese up to 90 days, but concentration depends on initial contamination. While the growth *Staphylococcus aureus* is when the population of lactic acid bacteria decreases. It has been known to grow in symbiosis with yeasts.

In brined cheese yeasts are known to be present in low numbers, while Molds grow more easily as compared to yeasts in the brine. Yeasts are added to starter culture in some brined cheese varieties. They are not considered as potential risk for consumption but they are known for degrading the product by affecting the texture and production of undesired aroma, flavor compounds. The genera *Penicillium*, *Mucor*, *Aspergillus*, *Cladosporium*, and *Fusarium* have been associated with Feta and Turkish brined cheese varieties. Depending on the genera and strain they have zero to high proteolytic activity and some varieties are able to produce aflatoxins. Aflatoxin production depends on the storage temperature of cheese. They are produced in very less amount around 5°C and can be detected at 15-20 mm below the cheese surface. (Özer, 2014).

2.2 High-Pressure Processing (HPP) technology

High-Pressure processing technology is the most popular non-thermal technology to produce microbial safe food products without significantly altering the physio-chemical properties. Although there are various physical and chemical methods to preserve food products, they

are known to undesirably change nutrient quality, taste, and flavor. As the consumer demands more natural, minimally processed and nutritious products, various research has been to explore various non-thermal techniques of preservation. In HPP the shelf life is extended by subjecting it to a uniform pressure ranging from 200-1000 MPa for few seconds to several minutes. (Okpala, Piggott, & Schaschke, 2009; Tewari, 2007).

HPP has been discovered in the 19th Century, but its commercial importance was realized during 2005-2010 where the total number of equipment's in use doubled. Groundbreaking development in different technologies happened during 1860-1890. It all started with when Lois Pasteur was able to deactivate micro-organism by the application of heat, followed the filing of the first patent for the prototype of the homogenizer. This innovation became indispensable for the dairy industry development in the next century. At the same time, a patent was filed where the shelf life of raw milk was extended for several days when subjected to a pressure of 700 MPa in a manually operated steel cylinder. Due to technical and economic challenges this technology remained a topic of exploration only for the researchers in the coming century (Tewari, 2007).

Various independent research happened between 1980-1990 in Japan and USA. This lead to the availability of commercial products in Japan by Meidi-ya company (fruit jam, jellies, sausage and salad dressing), while in the USA - sliced cooked ham, avocado puree, and oysters were available between 1990-1998. European countries like France and Spain also started processing fruits and vegetables by using the same technology during the same period (Portfolio, 2008). Various research on application of HPP on cheese (shelf life, physiochemical properties) has been conducted between 1988-2017 (Ávila et al., 2017; Chawla et al., 2011; Costabel et al., 2016; Delgado et al., 2012; Evrendilek et al., 2008; Giannoglou et al., 2016; Koca et al., 2011; Maniou et al., 2013; Moschopoulou et al., 2010; Okpala, Piggott, & Schaschke, 2010; Portfolio, 2008; Reps, Kolakowski, & Dajnowiec, 1998; Wium, Kristiansen, & Qvist, 1998).

Currently, there are 400 HPP industrial scale units installed all over the world of which dairy sector represents 2% of the total. Current commercial processing capacities range from 250-3000 kg/hr. Various improvements and innovations are being carried out to make the process continuous instead of semicontinuous or batch mode. Commercially application of HPP for fresh cheese in a brine made from goat and buffalo milk is being implemented in Lebanon and Spain, where the shelf life has been extended by an extra month. (Hyperbaric Spain Spokesperson, 2017).

2.2.1 Principle and Process

The HPP technology involves subjecting food products to pressure ranging from 200-1000 MPa. This is governed by two main principles, firstly Le Chalteliers principle which is based on the second principle of thermodynamics states that when the equilibrium of a system is disturbed it reacts in opposite manner to reduce the change. Secondly, there is the isostatic principle which states that the pressure is instantaneously and uniformly transmitted through food irrespective of shape and size of the object. The covalent bonds are not altered and thus the primary structure of the protein is still intact of the product.The technology is known to alter Van der wall forces of interactions, hydrogen bond, electrostatic and hydrophobic interactions in the system. Compounds with lower molecular weight do not any significant changes while the functional properties of macro compounds like proteins and polysaccharides are altered. Pre-packed products in flexible packaging undergo uniform treatment, retains sensorial properties and nutrients in HPP making it more suitable as compared to thermal processing (Balasubramaniam, Barbosa-Cánovas, & Lelieveld, 2016; Huppertz, 2010; Tewari, 2007).

HPP system can be batch or semi-continuous which consists of the pressure vessel, pressure generating system, transmitting fluid, cooling system and material handling as basic components. The vessel is made of stainless steel and the thickness will depend on the pressure that will be intended to apply in the system. Water is the most transmission fluid used since its adiabatic heating due to compression per 100 MPa is only 2-3°C as compared to 4-9°C for oil-based fluids. Due to this rise in temperature during the processing cooling system is essential to avoid heat damages to the product. Once the desired pressure is achieved the system is held under that pressure for the desired amount of time. This time of pressurizing and holding the product for a certain time is called one cycle. Processing parameters like systems pressure, holding time, temperature and number of cycles are considered to achieve the desired quality of the final quality of the product (Balasubramaniam et al., 2016; Tewari, 2007).

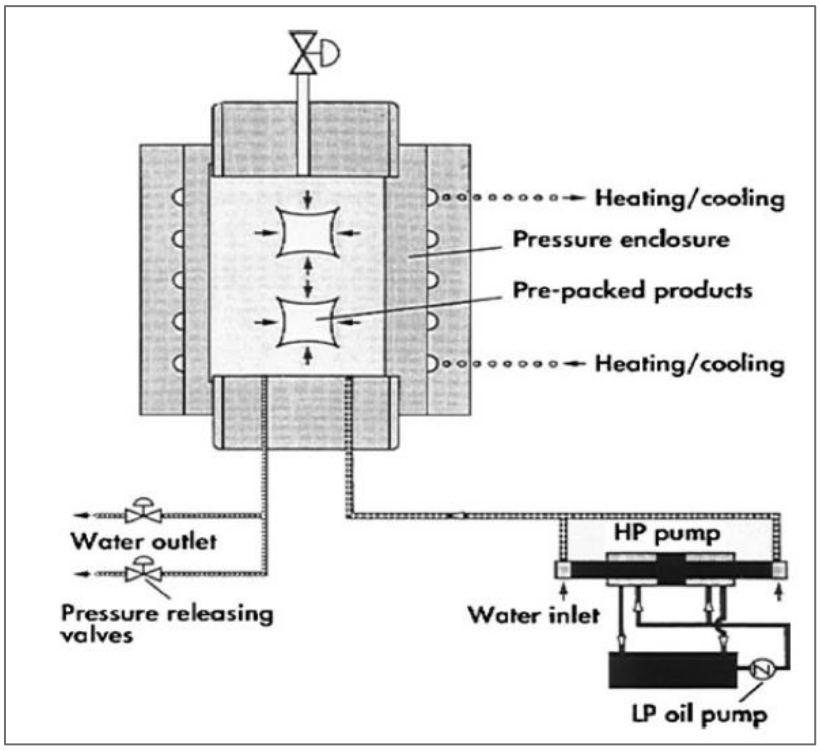

Figure 2.1- Batch mode HPP system for processing of packaged food (Chawla et al., 2011)

2.3 Effect on High Pressure on White brined Cheese

Considerable research has been done on the effect of HPP on casein micelle model (Balasubramaniam et al., 2016). It has been found that HPP causes casein micelle disruption. Changes in intermolecular hydrophobic interactions and solubilization of calcium phosphate are two principles behind the same. Effect of HPP on milk and cheese made from it has been studied extensively (Balasubramaniam et al., 2016; Chawla et al., 2011; Okpala et al., 2009). While the effect on cheese by using HPP treated started has also been studied extensively due to less processing costs (Giannoglou et al., 2016).

2.3.1 Effect of High Pressure on Ripening

The application of HPP for acceleration of ripening has been patented in 1993, where ripening times of cheddar and parmesan type cheese where reduced significantly. Since then a lot of studies has been done on other cheese varieties, there is lack of literature on the effects of the technology on white brined type cheese. Other than that proteolysis and lipolysis has been studied for over 15 years for a different kind of cheese, the effect on glycolysis is yet be evaluated (Chopde, Deshmukh, & Kalyankar, 2014; Huppertz, 2010; Martínez-Rodríguez et al., 2012; O'Reilly, Kelly, Murphy, & Beresford, 2001; Portfolio, 2008; Reps et al., 1998).

The key changes induced by the process are conformational changes in the enzyme structure, alteration of casein matrix, and release of microbial enzymes through cell wall lysis, and modification of water distribution (Chawla et al., 2011; Martínez-Rodríguez et al., 2012).

Plasmin which is responsible for cleavage of α_{s2}- and β-casein is highly pressured resistant and thus its activity remains unchanged after HPP treatment. On the other hand, chymosin has shown 50-62% reduction in residual coagulant activity when semi-hard ewes milk cheese was treated at 400MPa/10min/12°C at 15 days of ripening (Martínez-Rodríguez et al., 2012). Also, this reduced chymosin activity has been reported to have no significant effect on the primary maturation of cheese (Okpala et al., 2009)

Peptidases released by the starter culture is important for the secondary proteolysis. HPP treatments induce cell lysis of the start culture, the effect is highly dependent on the strain. Also, the aminopeptidase activity has been reported to improve when treated in the range of 200-500 MPa/15min/12°C. Significant changes were observed on day 1 after processing while no changes were observed in the enzyme activity after 15 days of ripening. In the same study, the fate of lipolysis in the cheese was also similar i.e. high FFA concentration during day 1 which reduced significantly over few weeks. The changes in lipolysis process are due to changes in protein-fat network and water availability for enzymes. (Martínez-Rodríguez et al., 2012).

2.3.2 Texture and Microstructure

HPP treatment undergoes changes in ripening and colloidal state, the combination of both results in unique texture in cheese (Koca et al., 2011). HPP treatment is known to create structural changes in the Calcium-caseinate complex. These changes are reversible once the applied pressure is removed. It has been suggested that some minor changes have been found in the complex once the equilibrium has been re-established (Cadesky, Walkling-Ribeiro, Kriner, Karwe, & Moraru, 2017). This induces significant changes in texture right after the pressure is applied, resulting in relatively more resistant cheese to deformation (Evert-Arriagada, Hernández-Herrero, Guamis, & Trujillo, 2014).

Significant changes have been observed in the microstructure of the pressurized cheese samples above 200 MPa. Control samples were observed to have a sponge-like structure with the non-uniform shape of fat globules and cavities. On the other hand, pressurized samples had more uniform and homogeneous protein matrix with few cavities (Evrendilek et al., 2008).

Ewe milk cheese was treated from 200-500MPa/10min/12℃ on day 1 and 15 after manufacture. The pressure of 200-300 MPa resulted in increased firmness with a more homogenous cheese structure (Martínez-Rodríguez et al., 2012).

2.3.3 Inactivation of Micro-organisms

Application of High-pressure technology for microbial inactivation has been studied extensively over past two decades. For some food matrixes, HPP technology has been found effective against pathogenic micro-organisms like *E coli, L. monocytogenes, Staphylococcus aureus* etc. along with spoilage microorganisms like coliform, yeast, and molds. The high pressure results in structural changes in the vegetative cells leading to injury or death. The cell wall is either ruptured or becomes more permeable leading to decrease in population. Along with that the key components of the cells like enzymes involved in Deoxyribonucleic acid (DNA) replication, membrane protein are also deactivated by HPP. The reduction in a microbial population depends upon the type of micro-organism, process condition, and product.

In a recent study, it was found that HPP at 300,400 and 500 MPa at 14°C for 10 min prevented late blowing effect by *Clostridium tyrobutyricum* of semi-hard cheese made from raw ewe's milk cheese. (Ávila et al., 2017). For feta-type cheese, it was found that HP treatment at 200 MPa at 20°C for 20 min did not significantly affect the bacterial count (Giannoglou et al., 2016). In Turkish white brined cheese when treated at 600 MPa/10min/25℃ there was 4.9 log reduction in the population of *Listeria monocytogenes.* While *Lactococcus* and *Lactobacillus* at same condition had 5.2-5.5 log reduction (Chawla et al., 2011) There was a study in which Turkish white brined cheese where pressurized from 300-600 MPa resulted in 2 to 5 log CFU/ g of Enterobacteria and *L. monocytogenes*. While lower reduction was found at 200 MPa (Koca et al., 2011).

2.3.4 Effect on Physiochemical properties

HPP treatment is known to have no effect on total solids, ash, fat, protein, moisture and nutrient content in the cheese. The changes in pH have been observed depending on the treatment conditions and cheese storage time. The pH value has been observed in different varieties of cheese at a higher pressure which was due to release of colloidal calcium phosphate in the aqueous phase. Also, the effect is reduced with the storage time of cheese.

Similarly, HPP has been found to alter water and salt distribution. The HPP leads to changes to water molecules in the matrix, which results in more water bound to protein than free water in the system. Salt diffusion was improved in ewe milk cheese treated at 300MPa/10min/12℃. Also, salt in moisture content was improved for the same treatment during the 15th and 60th day of ripening (Martínez-Rodríguez et al., 2012). When the cheese was pressurized in brine at 300 MPa has been known to disrupt the para-casein micelle structure which leads to more soluble nitrogen in the serum phase. Also, salt uptake in cheese was not affected and the water loss in cheese was reduced by the HPP treatment from 200 MPa onwards as compared to the control samples of gouda cheese (Okpala et al., 2009).

2.4 Capillary Electrophoresis

Capillary electrophoresis (CE) which is used to analyses casein components utilizes gel electrophoresis and column chromatography principle. Casein micellar disruption is achieved by the addition of urea or sodium dodecyl-sulfate (SDS), whereas the addition of calcium-chelating agents ethylenediamine tetraacetic acid (EDTA) results in the disruption of micelles through solubilization of the micellar calcium present in the nanoclusters (Patel & Huppertz, 2014). The separation of casein components happens in the capillary which is filled with carrier electrolyte. Then there is a voltage difference applied between ends which results in migration of compounds in the capillary based on charge, size and mass (Electrophoresis & Food, 1998; Kilara & Vaghela, 2018; Miralles et al., 2001). Ionic strength, pH, the concentration of electrolyte buffer and applied voltage will crucial parameters that will affect the casein analysis.

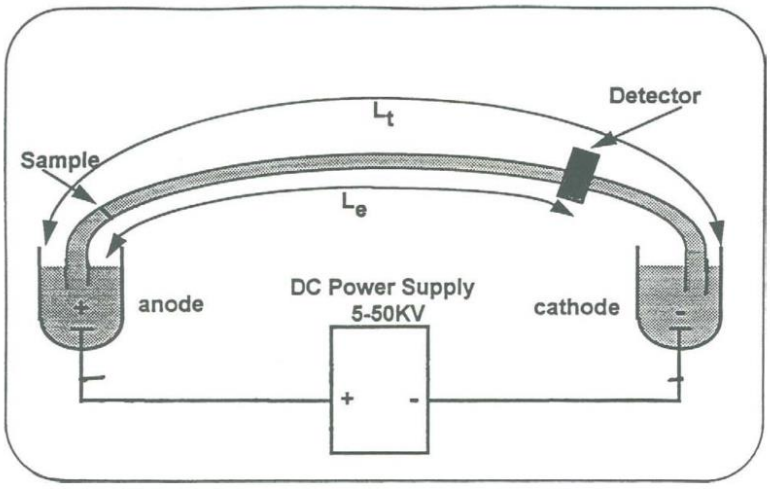

Figure 2.2 – Basic elements of CE (Electrophoresis & Food, 1998)

The sample passes through a hydrophilic coated capillary of total length (Lt). They pass through an optical detector which operates at a certain frequency. The time interval between initiation and detection is the migration time which is different for different peptide-type. The graph of absorbance vs migration time is plotted (Electrophoresis & Food, 1998).

When caseins eluted from the CE column para-κ-CN peak is observed first which is followed by several peaks of different α_s-and β-CN fragments. Starting with α_{s2}-CN, which is identified by 4 peaks representing different phosphorylation patterns which cause different migration times. Then α_{s1}-CN is eluted which has two peaks with 8 and 9 phosphorylation groups. Followed by β-CN having two or three genetic variants (A1, A2 and maybe B). At the end of the migration curve. Chymosin derived α_{s1}-CN-I (α_{s1}-CN cleaved from 24/25-199). The four γ-CN (fraction of β-CN) are normally shown in between α_{s2}-CN and α_{s1}-CN peaks. Depending on cheese type and processing, the activity of chymosin and plasmin is either promoted or inhibited along with composition (pH, water, fat, and salt). This can be used as a characterization of particular cheese variety and quality. CE profile of white brined cheese appears similar to those of acid casein with dominant β-CN peaks (Ardö et al., 2017; Otte, Zakora, Kristiansen, & Qvist, 1997).

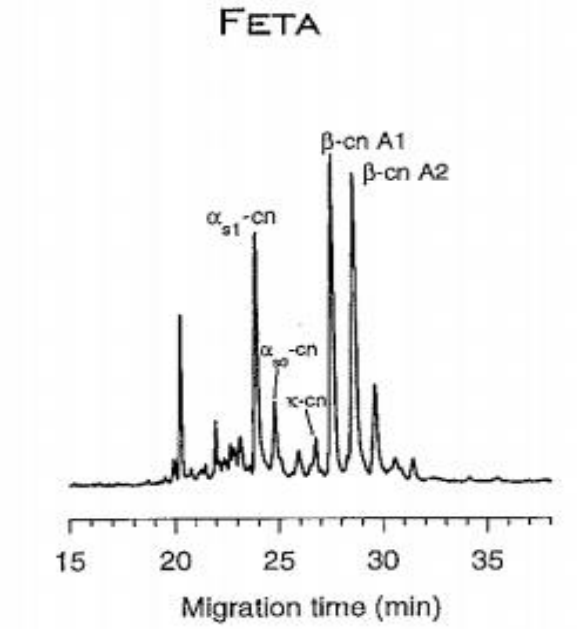

Figure 2.3 – Typical CE profile of 6 weeks old UF-White brined cheese (Feta Style) made with cow milk (Otte et al., 1997)

3 Materials and Methods

3.1 Cheese Samples High-Pressure Treatment and Preparation

The commercially available white-brined cheese was supplied by Krusa dairy (Arla Foods Amba) in deep-drawn foil packages of 200 gm each. The cheese samples were kept at below 5°C all the time before the HPP treatment. Duplicate sealed cheese samples were vacuum packed using PlusVac 20 (Komet, Plochingen, Germany), then the samples in placed 0.9 Liter HPP system (ABB Pressure Systems, Västerås, Sweden) and pressurized at 200/300/400 MPa for 5 min at 5°C. Distilled water was used as the transmission fluid to maintain the temperature of the system. After the pressure treatment samples were labeled and stored in quadruplicate at 5/20℃. Microbial, texture, sensory, pH and moisture analysis were performed at 0, 2, 4, 8 and 16 weeks were done for control and pressurized samples.

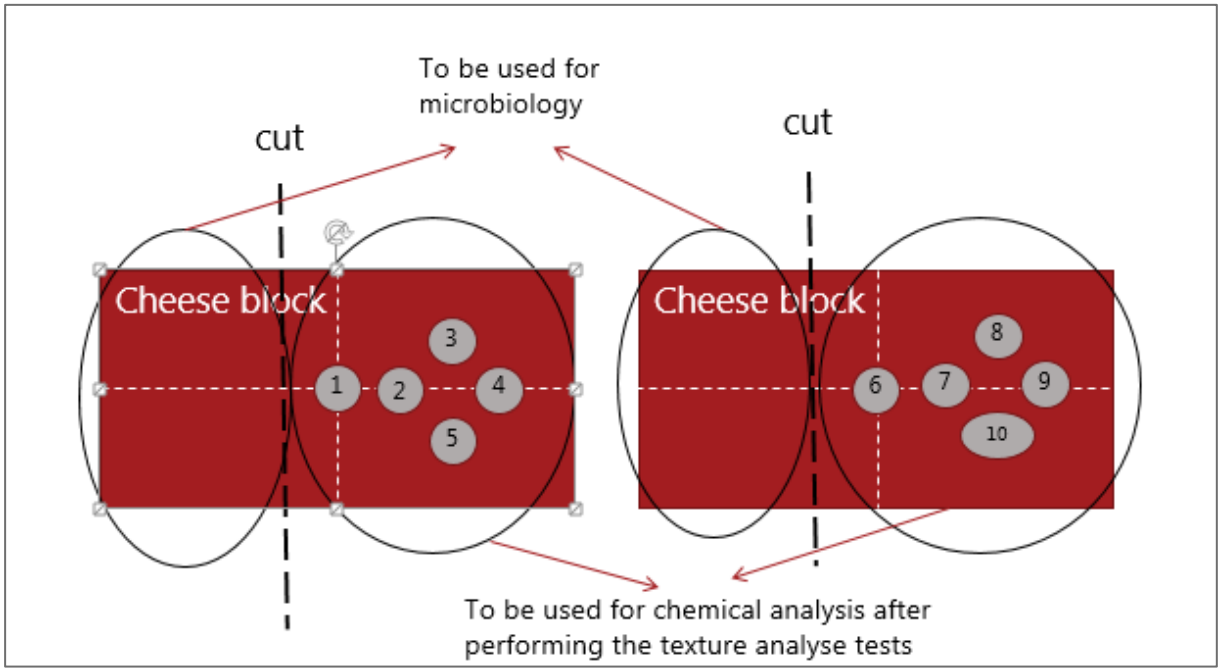

Figure 3.1 - Preparation of cheese sample for analysis (Ref: Arla Foods Amba)

The cheese packaging was wiped with 70% ethanol and the whey was drained before conducting the analysis. Individual cheese block (200gm) was then divided as shown in the picture. Duplicate samples were used for analysis, while duplicate samples were sent back to the dairy in an insulated box for sensory analysis.

3.2 Microbial Analysis

10 gm of representative cheese sample from duplicate pressurized and control group in stomacher bag and made 10 times of weight using 0.9% saline solution. The sample was homogenized by using Stomacher 400 (Seward, West Sussex, UK) for 1 min at max speed. Using the solution, 10^{-2}, 10^{-3}, 10-^{4}, 10^{-5} dilutions were made. total aerobic mesophilic bacteria (TAMB) were calculated by spread plate technique (0.1 mL inoculum) in triplicate on Plate Count Agar (PCA CMO325, Oxoid, UK) and incubated at 37°C aerobically for 5 days. Yeast and mold count was also calculated using the same on Yeast Extract Glucose Agar (YGC 95765, Sigma Aldrich GmbH, Steinheim, Germany) and incubated at 25°C aerobically for 5 days.

3.3 Moisture analysis and pH

The remaining of the sample from the microbial analysis was used to measure pH and Moisture. The cheese sample was kept in a beaker and pH meter probe (PHM 240, Radiometer Analytical, USA) was inserted. For moisture analysis, pre-weigh samples were kept in the oven 3 hours at 100°C. After the drying, the samples were weighed again and the moisture content was calculated (Difference in weight/Original weight)*100.

3.4 Texture Analysis

Hardness and Work of penetration were analyzed using a Puncture test of control and pressurized samples conditioned at room temperature of 20°C. 6mm diameter cylindrical probe was inserted into 5 different sections as shown in Figure 3.1 of duplicate samples by TA-XT2 texture analyzer (Stable Microsystems, London, UK). The probe was inserted at a distance of 15 mm with pre-test and a test speed of 1 mm/sec and was withdrawn at the speed of 10 mm/sec. After the analysis the duplicate cheese samples were stored at -20°C

3.5 Sensory Analysis

Remaining duplicate samples of control and the pressurized group were delivered to Arla Krusa Dairy in refrigerated conditions for sensory analysis. The analysis was done for week 0, 2, 4, and 16. Each cheese product is evaluated from the scale of 1-13 (2 point increment) on parameters like Appearance, Taste/Smell, Consistency, Structure, and Color. The final

score is given on the basis of each category. A sensory score of below 9 is not commercially acceptable.

3.6 Assessment of proteolysis by Capillary Electrophoresis

After the cheese has been dispersed in tri-sodium citrate solution, pH is adjusted to the isoelectric point of casein (pH 4.6). This precipitated fraction is analyzed to predict the degree of proteolysis in cheese.

Frozen cheese samples (-20°C) of week 0 and 4 were conditioned at room temperature before analysis. 12.5 gm of grated cheese in triplicates was weighed out with 4 decimal accuracy in a beaker. 50 mL of 0.5 M tri-sodium citrate solution was added. The solution was heated (40-50°C) and stirred frequently for 1 hour. The solution was cooled down to room temperature and diluted till final volume was 250 mL. 50 mL of the sample was used for Capillary Electrophoresis analysis.

1.5 mL of the prepared citrate dispersion is placed in Eppendorf tubes. The samples are centrifuged at 5°C at 1500 rpm for 30 min to remove fat. 1 mL of fat-free sample is filtered through 0.2 µm syringe filter. 600 µl of the filtered sample is mixed with 600 µl of sample buffer (505 mg hydroxymethyl aminomethane, 620 mg ethylenedinitrilo tetraacetic acid disodium salt, 220 mg 3-morpholino-propanesulphonic acid, 64 mg of DL- dithiothreitol in Urea 25 mL) and it is incubated for 1 hour at the room temperature. Capillary electrophoresis is performed at 45°C with a capillary electrophoresis system (G1600AHP3D, Hewlett-Packard A/S, Bikerod, Denmark) containing bare fused silica coated capillary column of effluent length 56.0 cm and 50 micrometers in diameter. Migration starts with a linear voltage gradient from 0 to 25kilo Volt for 3 min and then continues at 25 kilo Volt for 40 min.

3.7 Assessment of Lipolysis

Para-casein is dissolved by addition of sodium hydroxide. Sodium hexametaphosphate is added to liberate the fat. The free fat is titrated using alkali solution. The degree of acidity is expressed in mL of used Potassium hydroxide (KOH) per 100 gm of fat.

Frozen cheese samples (-20°C) of week 0 and 4 were conditioned at room temperature before analysis. 1.2 gm of sodium hexametaphosphate was added to 30 gm of duplicate grated cheese in a mortar. 3.0 mL of 1 M Sodium Hydroxide along with distilled water (≤ 100 mL

at 40-50°C) was added to make a thick paste and transferred to 250 mL measuring flask. The open flask was kept in the boiling water bath for 15-20 min until cream/fat layer was separated. 15 mL of 1 M Hydrochloric Acid (HCl) was added and was stored overnight at refrigeration temperature (≤4°C).

50 mL B.D.I. reagent (30 gm Triton X-100 + 70 gm sodium hexametaphosphate in 1000 mL of distilled water) was added and kept in the boiling water bath until the fat layer appears clear and separated. Then the 250 mL flask is filled with boiling distilled water. The level of water in the water bath was above the liquid during the entire analysis. Clear samples in triplicate were weighed in a small Erlenmeyer flask. 15 mL of fat dissolving mixture (Petroleum ether with 99.8% ethanol in 2:1) is added to the samples and titrated against 0.0082 M KOH solution under nitrogen atmosphere till the color changes from yellow to red/pink.

3.8 Statistical Analysis

Statistical analysis was evaluated by using the software IBM SPSS statistics Version 24 for Windows (IBM Corporation, NY, USA). Two way ANOVA (Pressure level and storage temperature) and three-way ANOVA (Pressure level, storage temperature and ripening time) were used to analyze the data.

4 Results and Discussion

4.1 Microbial Analysis

Macro compounds in the cheese like proteins, carbohydrates, lipids, along with minerals can have a shielding effect on the microflora, usually known as a 'baroprotective effect'. pH and pressure can work in synergy to deactivate the microorganism and also prevent the recovery of the same during the storage (Portfolio, 2008).

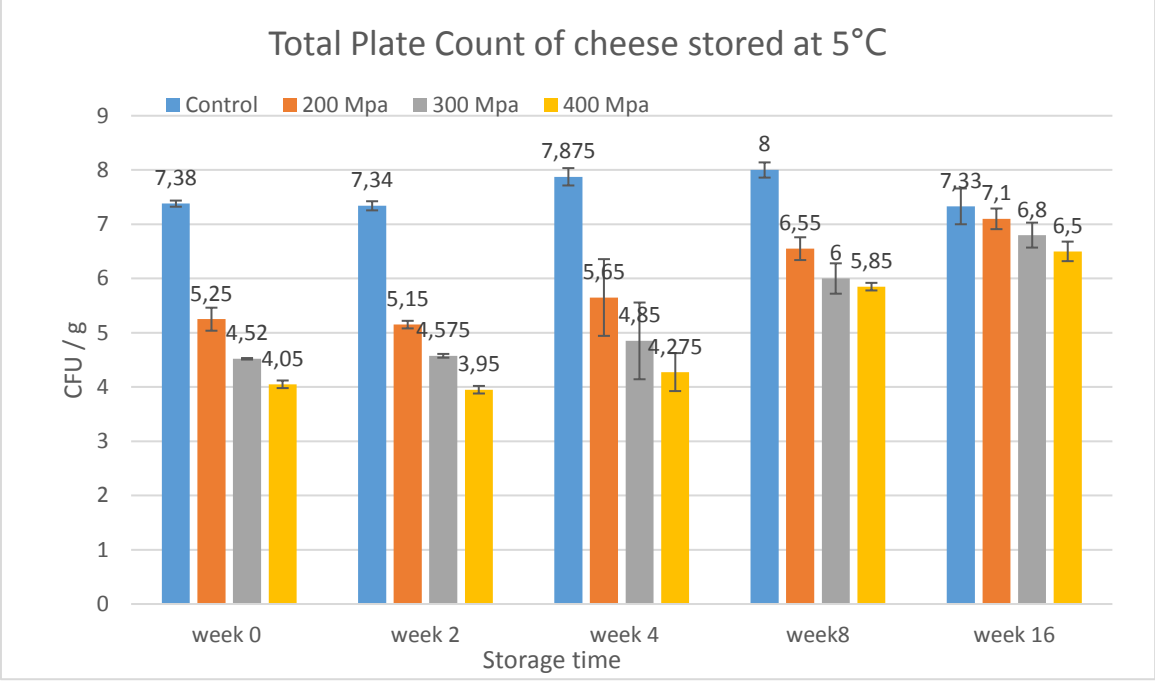

Figure 4.1: Total Plate Count of cheese samples stored at 5℃ for week 0, 2, 4, 8 and 16. The error bars represent the standard deviation of triplicate samples.

Figure 4.1 represents the data of total aerobic mesophilic bacteria (TAMB) in the cheese samples stored at 5℃. Both pressure and storage time had a significant effect on the values ($P<0.01$). During week 0, 3 log reduction was observed for samples treated at 400 MPa as compared to 2 log reduction for 200 MPa treatment for 5 min. It has been reported that no significant difference ($P<0.05$) was found when the brined cheese samples were treated at 200 MPa, while 4-5 log reduction at pressure treatment of 500 MPa (5 and 15 min) from 8 log CFU/gm (Koca et al., 2011). Similar results were found for ovine cheese and Turkish white brined cheese (Evrendilek et al., 2008; Moschopoulou et al., 2010). The difference in the pressurized samples was significant until week 2.

After 4 weeks, TAMB has recovered 1 log CFU/gm in with no significant difference (P>0.05) in the pressurized samples. It has been reported that TAMB recovered by reversibly by 2 log cycles during the ripening in ovine cheese, due to the sub-lethal effect of HPP treatment and protective effect from the cheese matrix (Moschopoulou et al., 2010). Until week 8 there was the recovery of 1.5 log CFU/gm for 200 and 300 MPa samples, while 2 log CFU/gm for 400 MPa. At week 16 statistically no difference (P>0.05) between the control and pressurized samples suggesting complete recovery of TAMB in the samples.

The control samples had 7 log CFU/gm throughout the storage, except for week 8. In the literature control samples started with 9 log CFU/gm and decreased by 1-2 log CFU/gm during ripening and remained constant during further storage (Evrendilek et al., 2008; Koca et al., 2011; Moschopoulou et al., 2010).

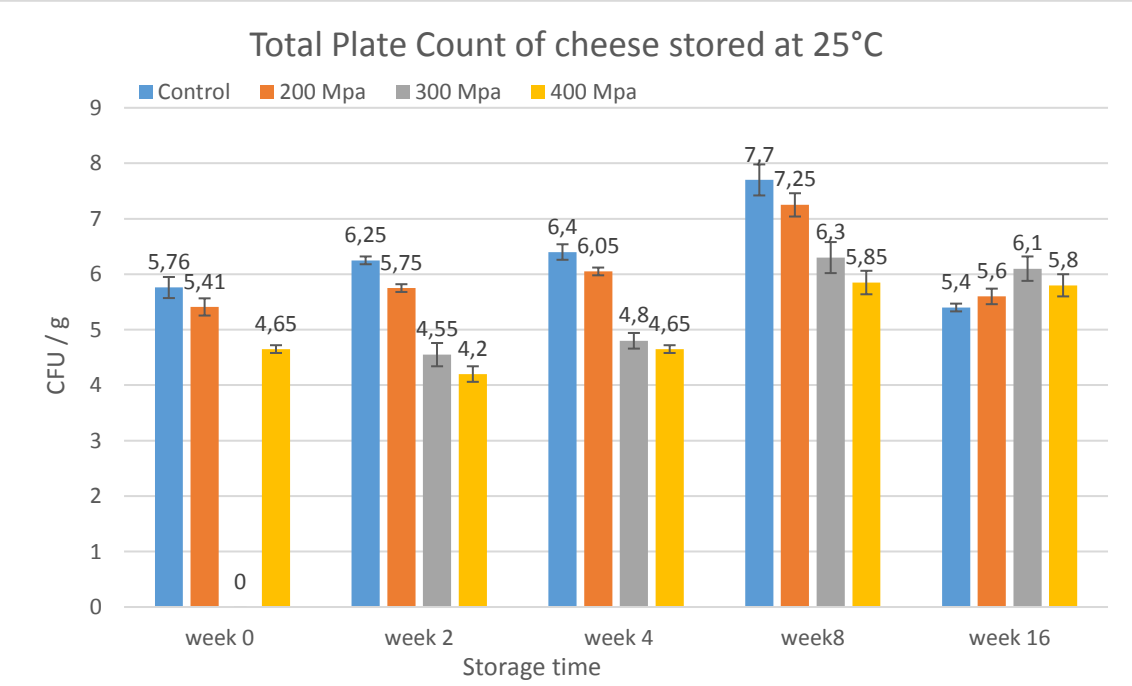

Figure 4.2: Total Plate Count of cheese samples stored at 20℃ for week 0, 2, 4, 8 and 16. The error bars represent the standard deviation of triplicate samples. (Note: Zero value in week 0 for 300 MPa treatment was due to the sampling error).

Figure 4.2 represents the data of (TAMB) in the cheese samples stored at 20℃. During week 0, there was a significant difference only for the samples treated at 400 MPa. After comparing with log reductions of the week 0 in Figure 4.1, it was found that the values for 200 MPa and 400 MPa were similar at both storage temperatures. The odd behavior was due

to low detection in control sample of 20℃ which could be associated with sampling error. This behavior in the control samples was continued till week were the log values remained around 6 CFU/gm for control samples.

High fluctuations in the data were observed for week 8 and week 16. High storage temperature and time could be responsible for this behavior. High level of yeast and molds were detected in the samples for week 16. A complete analysis of non-starter LAB and psychrotropic bacterias need to investigate further to completely understand the behavior of the data. There is lack of literature on accelerated growth of a microorganism in white brined cheese.

Gas production was observed in the week 16 control samples, unlike pressurized samples. This is associated with *Clostridium tyrobutyricum* and *Cl. butyricum* which converts lactate to acetate, carbon dioxide, and butyrate. This is mainly due to low starter activity in the cheese (Mcsweeney, n.d.). This could also justify low starter values in the control samples.

High pressure has been proved quite effective against yeast and molds (Martínez-Rodríguez et al., 2012). For yeast and molds in the cheese samples, no signs of growth were observed in control and pressure treated samples till week 8 for samples stored at 5℃. After week 16 the yeast and molds showed some sign of growth except for 400 MPa samples. For samples stored at 20℃ yeast growth was observed from week 4 in all samples except for 400 MPa and at the end of week 16, there 2-5 log CFU/gm (400MPa - Control) of yeast and molds.

There is a lack of data on the effect of HPP on the growth of yeast and molds in brined cheese. In Turkish white brined cheese made from raw pressure treatment above showed 3 log reduction from 200 MPa (Moschopoulou et al., 2010).

4.2 Moisture analysis and pH

*Table 4.1: pH values of the cheese samples stored at 5℃ and 25℃ for week 0, 2, 4, 8 and 16. The error values represent the standard deviation of 2 measurements each from duplicate samples. *C – Control. Rows with the same superscript are no different (P> 0.05)*

Storage time	**pH of the cheese samples**							
	Samples stored at 5°C				**Samples stored at 20°C**			
	C*	200 MPa	300 MPa	400 MPa	C*	200 MPa	300 MPa	400 MPa
Week 0	4.60 ± 0.02	4.60 ± 0.02	4.65[a] ± 0.01	4.65[a] ± 0.01	4.35[b] ± 0.02	4.51[c] ± 0.04	4.59[c] ± 0.02	4.55c ± 0.06
Week 2	4.58 ± 0.03	4.61 ± 0.04	4.62 ± 0.05	4.63 ± 0.05	4.36[a] ± 0.03	4.40[a] b± 0.07	4.47[a] ± 0.06	4.55[a] ± 0.04
Week 4	4.53 ± 0.05	4.58 ± 0.05	4.58 ± 0.07	4.62 ± 0.06	4.34[a] ± 0.05	4.30[a] ± 0.03	4.31[a] ± 0.05	4.30[a] ± 0.05
Week 8	4.67 ± 0.02	4.71 ± 0.03	4.69 ± 0.00	4.69 ± 0.01	4.56[a] ± 0.05	4.60[a] ± 0.04	4.61[a] ± 0.02	4.63[a] ± 0.02
Week 16	4.87 ± 0.02	4.82 ± 0.06	4.83 ± 0.02	4.85 ± 0.02	4.98 [a] ± 0.04	4.91[b] ± 0.04	4.84 ± 0.03	4.92[b] ± 0.03

During the initial week, a significant difference (P<0.04) was observed between control samples and pressurized samples at 200-300 MPa at 5° and 20°C storage. In the research pH of the cheese was not altered by the high-pressure treatment, except for initial week where the pH of pressurized samples was slightly higher. This increase in pH is associated with the release of colloidal calcium phosphate into the aqueous phase of the cheese. (Avila and others 2006; Garde and others 2007; Juan and others 2007; Messens and others 1998; Saldo and others 2000). When gouda cheese pressurized (100-300 MPa for 30 min) under brine

the pH difference between the control and the pressurized samples diminished during the ripening. The pH difference diminished from week 2 of storage. Significant variations were found between the samples stored at 5° and 20°C storage. This could be attributed the difference in the microflora composition. The pH increases during the ripening due to the utilization of lactic acid to form other non-acid compounds and degradation of amino acids to release basic groups in the system (Messens, Dewettinck, Camp, & Huyghebaert, 1998). pH range of 4.6-4.8 is necessary to keep the quality of white brined cheese (R.K. Robinson and Tamime, 1996). In week 16 for samples stored at 20°C had really high pH close to 4.9 which can lead undesirable textural properties and microbial growth in the cheese (Avila, Gomez-Torres, Delgado, Gaya, & Garde, 2016; Ávila et al., 2017).

*Table 4.2: Moisture content of the cheese samples stored at 5°C and 20°C for week 0, 2, 4, 8 and 16. The error values represent the standard deviation of 2 measurements each from duplicate samples. *C – Control. Rows with the same superscript are no different (P> 0.05)*

Storage time	**The moisture content of the cheese samples (%)**							
	Samples stored at 5°C				**Samples stored at 20°C**			
	C*	200 MPa	300 MPa	400 MPa	C*	200 MPa	300 MPa	400 MPa
Week 0	54.2 ± 0.51	55.5 ± 0.32	55.4 ± 0.47	56.2[a] ± 0.59	54.6 ± 0.05	55.3 ± 0.04	56.7[a] ± 0.02	54.1 ± 0.06
Week 2	53.3 ± 0.37	55.5[a] ± 0.46	55.7[a] ± 0.52	55.5[a] ± 0.26	50.7[b] ± 0.5	51.2[b] ± 0.7	52.4 ± 0.66	53.0 ± 0.14
Week 4	54.6 ± 0.50	55.7 ± 0.35	55.7 ± 0.17	56.3[a] ± 0.6	49.6[b] ± 0.12	51.0[b] ± 0.53	49.1[b] ± 0.69	52.3[c] ± 0.22
Week 8	54.7 ± 0.19	54.9 ± 0.03	55.5 ± 0.60	55.6 ± 0.91	49.7[a] ± 0.18	49.9[a] ± 0.04	49.7[a] ± 0.02	51.7[a] ± 0.72

Week 16	53.2 ± 0.20	54.2 ± 0.65	55.5 ± 0.25	55.9 ± 0.32	49.8[a] ± 0.10	51.0[a] ± 0.23	50.6[a] ± 0.47	51.0[a] ± 0.80

Moisture content ranging from 51.54% to 55.80% after 60 days of ripening are typical for white brined cheese varieties (Alizadeh, Hamedi, & Khosroshahi, 2006; Ávila et al., 2017; Koca et al., 2011; Maniou et al., 2013; Moschopoulou et al., 2010) For week 0 the moisture content remained the same except for 400 MPa (5℃) and 300 MPa (25℃). According to the literature, lower moisture values are expected in cheese samples due to changes in κ-CN (Moschopoulou et al., 2010). Due to pressure treatment in brine could have to lead to increasing moisture changes in the cheese samples. During the week 2, the moisture content increased in the pressure treated samples at both the temperatures. Pressure treatment results in more water bound to the protein which results in less moisture loss (Messens et al., 1998). This could be responsible for high moisture content in the pressure treated samples.

In week 4, 400 MPa sample (5℃) showed significantly high moisture ($P<0.05$) which reduced in week 8. In the literature 400 MPa/15 min treated cheese samples showed higher moisture retention as compared to the others (Evrendilek et al., 2008). While the samples stored at 25℃ showed a significant reduction in the moisture content. This could be due to accelerated moisture migration from the cheese to the brine. No significant change ($P>0.05$) in the pH was observed for the samples Figure 4.1.

From 8 weeks onwards the moisture content did not change significantly ($P>0.05$) between the control and pressurized samples stored at 5℃ and 25℃. This could be due to fading effects of HPP and equilibrium of moisture migration between the cheese-brine.

4.3 Texture Analysis

The texture development in cheese is affected by proteolysis (release of amine and carboxyl groups), pH changes (lactic acid production/utilization)and moisture content (Terpou et al., 2018). Hydrolysis of a_{s1}-CN to a_{s1}-CN-I is responsible for the early softening of high-moisture cheese (Wium et al., 1998). Since the cheese block are stored in brine there is a possibility of moisture migration which can highly affect the outer surface of the cheese. This migration is also known to create moisture gradient which results in some sections of cheese becoming too soft and pasty. Also, the cheese is known to have porous structure

making it challenging for a representative sampling (Johnson, 2003). That's why along with peak hardness, work of penetration (Total area under the graph) was evaluated for the samples.

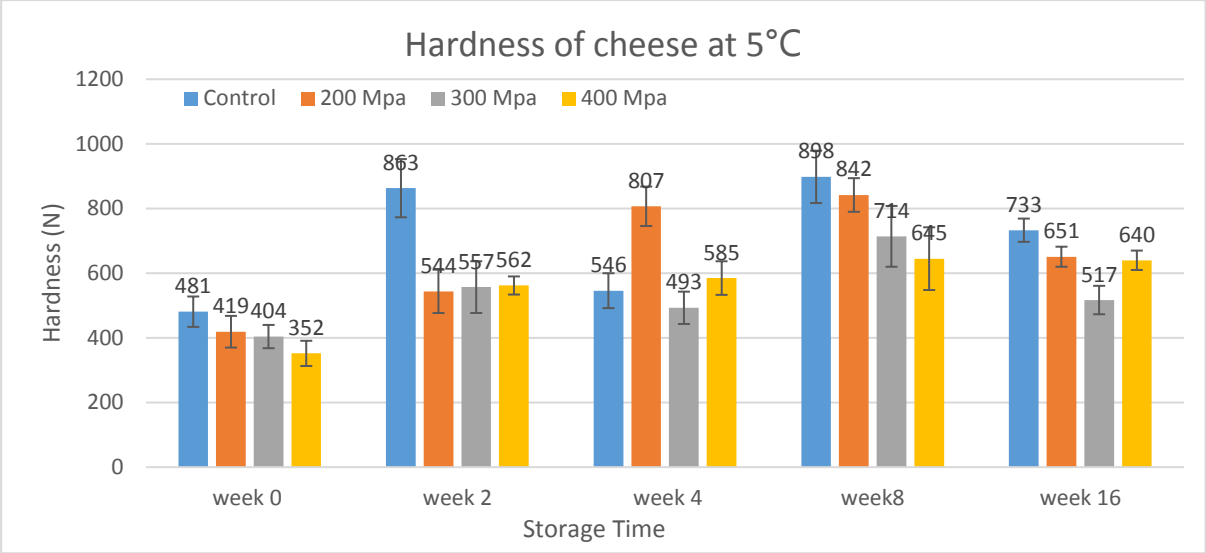

*Figure 4.3: Hardness of cheese samples stored at **5°C** for week 0, 2, 4, 8 and 16. The error bars represent the standard deviation of 5 measurements each from duplicate samples.*

Both storage time and pressure had an effect on the hardness ($P<0.04$) of the sample. For the control sample, the peak hardness had a cyclic pattern as it increases significantly in week 2 followed by a decrease in week 4. This cycle was continued in the following week. The moisture and pH did not vary significantly for the control sample. This effect could be due to proteolysis in the cheese, solubilization of remaining calcium phosphate in the cheese, changes in the ratio of intact casein in the cheese affects the texture development in the cheese. The sudden high value of hardness could be due to the pH as the cheese samples had pH very close to 4.6 (isoelectric point) as shown in Table 4.1 (Lawrence, Creamer, & Gilles, 1987).

During week 0 there was a significant difference ($P<0.05$) between the control sample and 400 MPa treated sample. Microstructure reconfiguration could be responsible this and the cheese was also perceived at soft when the sensory test was conducted.

In pressurized samples, the hardness value for 200 MPa increased until week 8 and then decreased in week 16. In 300 MPa samples, the values increased till week 2 and then remained the same till week 16 (except for week 8 where it increased significantly). While for 400 MPa the hardness values increased throughout the storage period. It was reported that hardness of the pressurized cheese increase with storage in white brined cheese. Also, it

was suggested that there close co-relation between cheese microstructure and texture. Combination of changes in microstructure and calcium-casein complex interaction has resulted in a difference in texture as compared to the control (Koca et al., 2011).

These changes in the cheese samples can be explained by studying calcium-casein complex during the cheese storage.

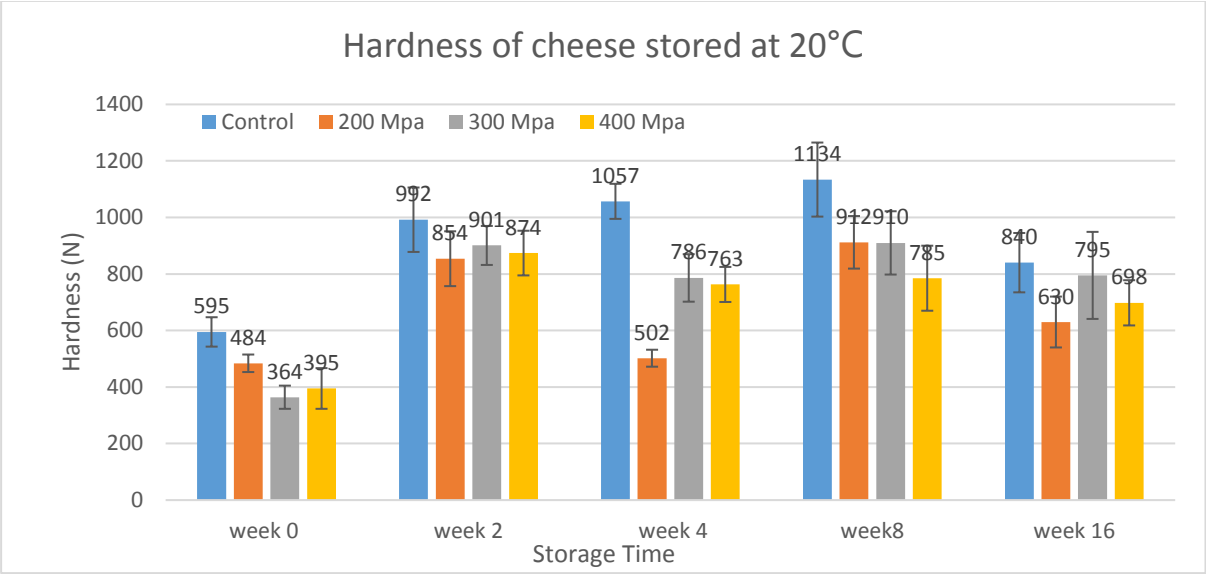

*Figure 4.4: Hardness of cheese samples stored at **20°C** for week 0, 2, 4, 8 and 16. The error bars represent the standard deviation of 5 measurements each from duplicate samples.*

Both storage time and pressure had an effect ($P<0.04$) on the hardness of the sample stored at 20°C. For the control sample, the peak hardness had an increased during week 4 and remain constant until week 8 followed by a significant drop in week 16. High pH values of above 4.9 as shown in Table 4.1 could have to this drop in hardness of the samples (Lawrence, Creamer, & Gilles, 1987).

During week 0 there was a significant difference between the control sample and 400 MPa treated sample just like the samples stored at 5°C. Microstructure reconfiguration could be responsible this and the cheese was also perceived at soft when the sensory test was conducted.

In pressurized samples, the hardness value for 200 MPa has shown the cyclic pattern where it increases and decreases in alternate weeks. In case of 300 MPa treatment has shown no significant changes in the hardness values after week 2, high range of error bars suggests higher moisture migration rate in the cheese sample. While for 400 MPa the behavior was similar it increases during week 2 and remained non-significant thereafter. These changes in

the cheese samples can be explained by studying calcium-casein complex during the cheese storage.

The complex combination of pH, moisture, fat distribution (microstructure), and proteolysis has had balancing effect on the cheese texture. Except for few exceptions, pH and moisture did not affect the hardness of the cheese samples. Thus microstructure, proteolysis and calcium casein complex needs to be further investigated to get an overall perspective of the data.

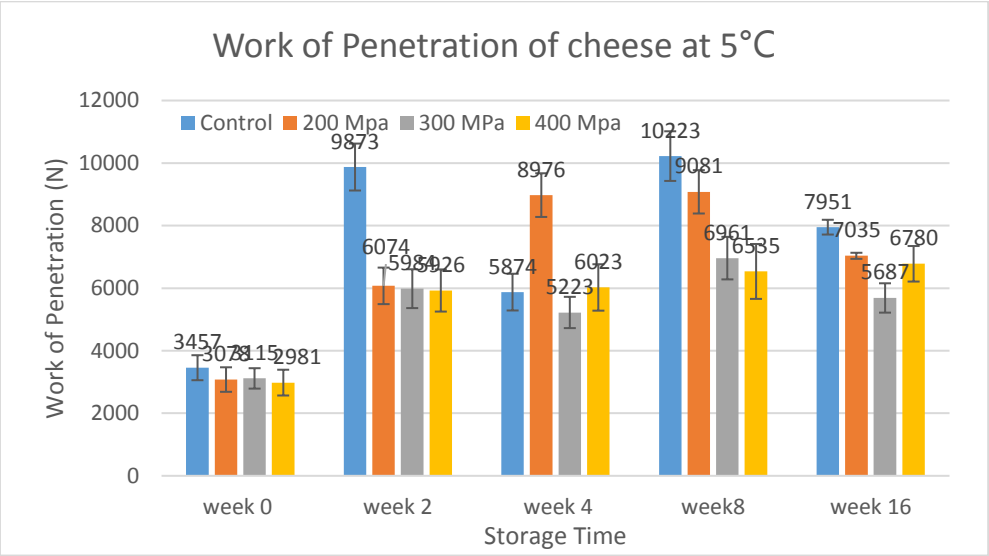

*Figure 4.5: Work of Penetration of cheese samples stored at **5°C** for week 0, 2, 4, 8 and 16. The error bars represent the standard deviation of 5 measurements each from duplicate samples.*

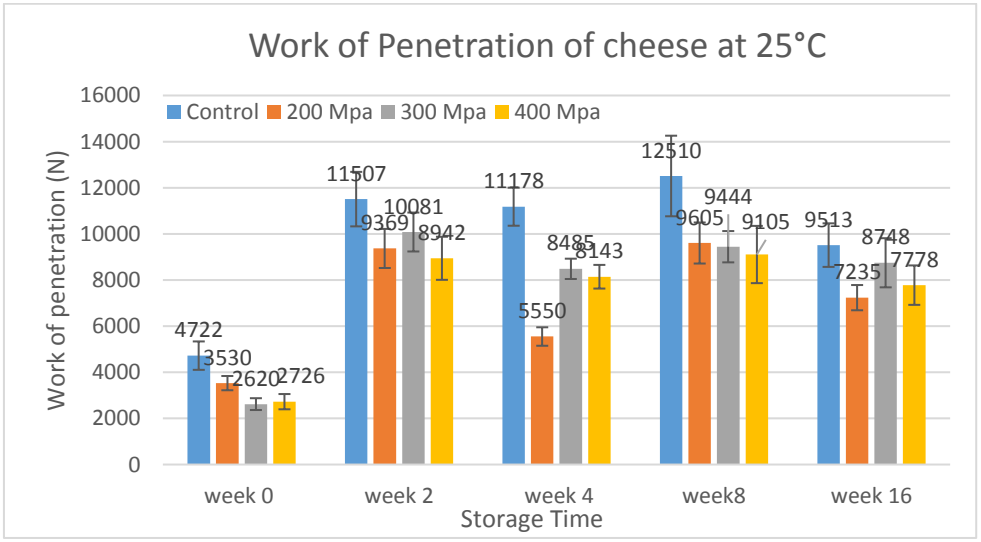

*Figure 4.6: Work of Penetration of cheese samples stored at **25°C** for week 0, 2, 4, 8 and 16. The error bars represent the standard deviation of 5 measurements each from duplicate samples.*

After comparing work of penetration data (Figure 4.5 and 4.6) with the peak hardness (Figure 4.3 and 4.4) similar pattern in the nature of the graph was observed. Which shows that moisture gradient in the cheese was not significant enough and the data obtained was representative of all the cheese block.

4.4 Sensory Analysis

*Table 4.3: Sensory analysis for cheese samples stored at 5℃ and 25℃ for week 0, 2, 4, and 16. The table indicates the final score of the samples on a scale of 1-13 (with 2 point increment) which are analyzed for Appearance, Taste/Smell, Consistency, Structure, and Colour. A score of 9 or above is commercially acceptable. (*L – Lipase taste. **S- Sour taste. ***G- Gas formation)*

Storage time	**Sensory Analysis**							
	Samples stored at 5°C				**Samples stored at 20°C**			
	C*	200 MPa	300 MPa	400 MPa	C*	200 MPa	300 MPa	400 MPa
Week 0	9	13	11	13	9	11	11	13
Week 2	9	11	11	11	9	11	11	11
Week 4	11	13	13	13	11^{L}	11^{L}	11^{L}	11
Week 16	13	11	13	13	$5^{S,G}$	7^{S}	9^{S}	7^{S}

Appearance, Taste/Smell, Consistency, Structure, and Color were evaluated for the samples and a final score was given. Overall as we go higher in pressure there was less brine in the package. In case of storage temperatures, there was more brine in the samples stored at 20°C as compared to 5°C. No significant color change was observed in the samples.

The moisture migration is a common phenomenon in packed brined cheeses. The difference in the salt concentration is responsible for the same (Johnson, 2003). The brine concentration

is around 7-8% while the cheese is around 3-3.5% (Mann, 1996). HPP treatment (100-500 MPa/15 min) results in the conversion of free water into protein-bound water, this resulted in the reduced loss of moisture from Gouda cheese when it was pressurized under the brine. Also the no effect of high pressure on salt uptake was found. (Messens et al., 1998). No significant difference in salty taste was observed in all the samples.

For week 0, samples stored at 5°C and 20°C received a better overall score for the pressurized samples than the control. For samples at 5°C, the gritty texture was noted for the control samples leading to a score of 9. While the texture improved with the pressure and it was perceived to be better at 200 and 400 MPa. In case of samples stored at 20°C the structure of the cheese (less gritty) improved as you go higher in pressure. Also, the low values of the hardness of cheese as shown in Figure 4.1 and 4.2 was not observed, this could be either due to the delay between the texture and sensory test. Also, the samples were refrigerated and delivered to the dairy leading to temperature abuse of the samples.

For week 2, just like week 0 pressurized samples received a better score than the control. Suggesting HPP does improve the sensory profile of the cheese. For samples stored 5°C softer consistency was observed, which resulted in an overall higher score. Which goes with the data provided by a figure 4.3 so and so as the hardness value was half of the control. Also, for samples stored at 20°C results were similar as compared to week 0.

For week 4, Samples stored at 5°C was perceived better than the samples stored at 20°C. For samples stored at 5°C, pressurized samples had less brine as compared to the control which suggests moisture migration in pressurized samples. Overall pressure treated samples received a better score than the control. On the other hand, for samples stored at 20°C more brine was observed as compared to 5°C. Also, lipase taste was observed for all the samples except for 400 MPa stored at 20°C. Yeast growth was observed for those samples. This shows that 400 MPa was effective in deactivating the yeast. Overall all the samples at 20°C received an equal score and were commercially acceptable.

For week 16, Samples stored at 5°C were scored higher as compared to the samples stored at 20°C. For 5°C lipase taste was observed in control, 200 MPa and 300 MPa samples. Sample 200 MPa was rated lower due to higher lipase taste in the sample. All the samples were soft in texture and were rated highest on the scale on 13 (except for 200 MPa). While for the samples stored at 20°C received scores which are not acceptable for commercial

products. Excess gas production in the control sample lead to the puffing of the packaging, followed by sour taste and high yeast growth was observed. The sample got the least score of 5. The butyric acid bacteria like *Clostridium tyrobutyricum, Clostridium butyricum* and *Clostridium beijerinckii*, which are a group of spoilage bacterias have been associated with late blown package phenomenon in semi-hard cheese from ewe milk (Avila et al., 2016). In case of pressurized samples, a sour taste was observed due in all samples. 300 MPa samples had lesser sour taste as compared to 200 MPa and 400 MPa. The pressurized samples at 300 MPa and 400 MPa had a desirable consistent texture. High-pressure treatment has been proved to be very effective against yeast. Although the effects were found to less significant and diminishing during the storage (Martínez-Rodríguez et al., 2012). The application of HPP after the product has been packed can be the factor responsible for the same. As in the research the HPP treatment is either applied to milk, starter culture or cheese block before packaging (Ávila et al., 2017; Evert-Arriagada et al., 2014; Evert-Arriagada, Hernández-Herrero, Juan, Guamis, & Trujillo, 2012; Evrendilek et al., 2008; Giannoglou et al., 2016; Koca et al., 2011; Maniou et al., 2013; Martínez-Rodríguez et al., 2012; Moschopoulou et al., 2010; Okpala et al., 2009; Portfolio, 2008).

The greenish and yellowish color was observed in the white brined cheese as the pressure increased from 50 to 400 MPa when treated at 15 min. These changes were attributed to structural changes which tend to alter the light scattering properties of the cheese (Koca et al., 2011). Casein micelle disintegration by HPP is due to disruption of non-covalent bonds which are significantly higher at room temperature or higher temperatures (Chawla et al., 2011). Since in the current experiment the samples were pressurized for 5 min and at 5℃ the changes induced on the structure were not significant enough to give appreciable color change in the cheese samples through storage.

HPP has been known to retain the sensory parameters of cheese if the processing temperature and pressure are low. In other cheese varieties, HPP was applied to milk and a starter culture, in which resulted in effects of HPP reduced or diminished significantly (Martínez-Rodríguez et al., 2012). In the given experiment HPP did not influence the characteristic color and smell of the product. While the texture, taste, and structure of the product were altered positively along with deactivation of yeasts at a pressure of 400 MPa. The HPP treatment showed promising results for week 16 and 20℃ storage. The shelf life of white brined cheese can be extended by keeping the sensorial parameters of the cheese intact which are commercially acceptable.

4.5 Assessment of proteolysis by capillary electrophoresis

Capillary electrophoresis (CE) was carried out for the samples at week 0 and 4. The electrophoresis graph was analyzed by comparing the pattern with Figure 2.3 (CE profile of UF feta cheese). It was found that peaks of p-κ-CN, α_{s1}-CN-8P (8 phosphate groups), β-CN-A1, β-CN-A2 (elution in the same order with minor peaks in between) had a similar pattern in all the samples with many other unidentifiable peaks. In some samples, α_{s1}-CN-9P (9 phosphate groups) and γ-CN were observed. The individual peaks were standardized and were calculated as a % of the total area to give a better representation of the data.

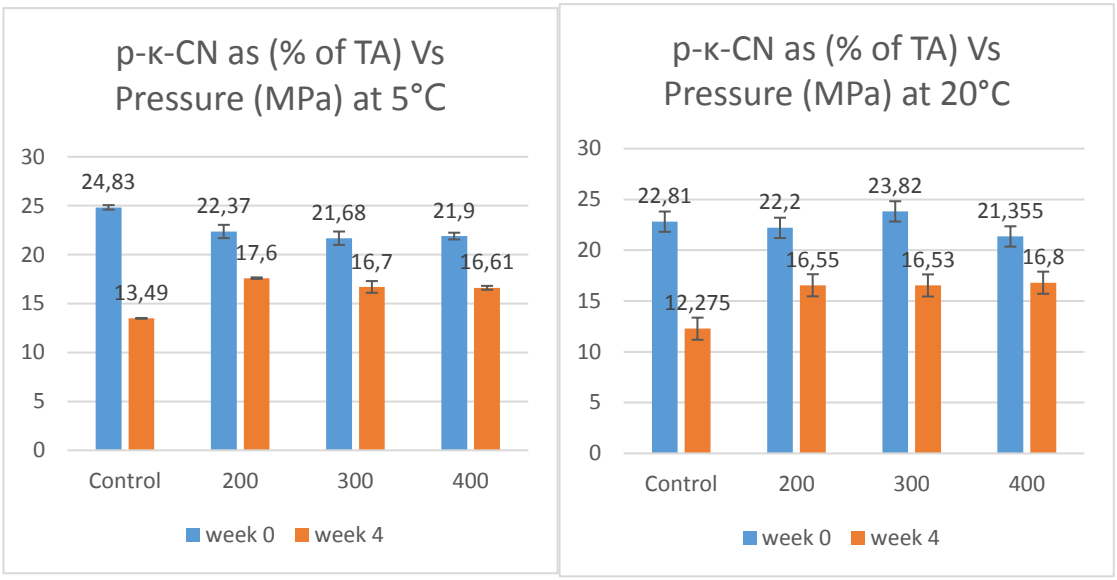

Figure 4.7: Concentration of p-κ-CN as (% of Total Area) Vs Pressure (MPa) at 5°C and 20°C. The error bars represent the standard deviation of three measurements of each sample.

Rennet is responsible to cleave κ-Casein (CN) which results in aggregation of p-κ-CN and release of Glyco-macro-peptide (GMP) in the serum (Walstra et al., 2006). Applying two-way ANOVA analysis (Pressure and storage time) on sample stored at 5°C (Figure 4.7) it was found both pressure and storage time had a significant effect ($P<0.05$).

For week 0, the concentration of p-κ-CN decreased significantly when the pressure was applied. For the pressurized samples, the concentration remained almost the same. After 4 weeks the p-κ-CN concentration of control samples showed high reduction as compared to pressurized samples.

The rennet proteolytic activity is highly resistant to pressure treatment till 800 MPa at 20°C (O'Reilly et al., 2001). Degradation of κ-CN results in more formation of p-κ-CN. While during the ripening p-κ-CN is not hydrolyzed further (Ardö et al., 2017). Although nature

had a strong resemblance to that p-κ-CN from the reference, further validation is required to assess the behavior of data.

For samples stored at 20℃, no significant difference was observed (P>0.05) between the samples for week 0. While for week 4 the trend was similar as compared to the samples stored at 5℃.

After applying three-way ANOVA analysis (Pressure, storage time and storage temperature) no significant difference was observed between the two different storage times. The values of the p-κ-CN as % of total remained quite similar irrespective of the storage temperature in control and pressurized samples suggesting that elevated storage had no effect on p-κ-CN degradation. Elevated temperatures are responsible for the growth of micro-organism which is mainly responsible for further hydrolysis of α_s-and β-CN (Mcsweeney, n.d.).

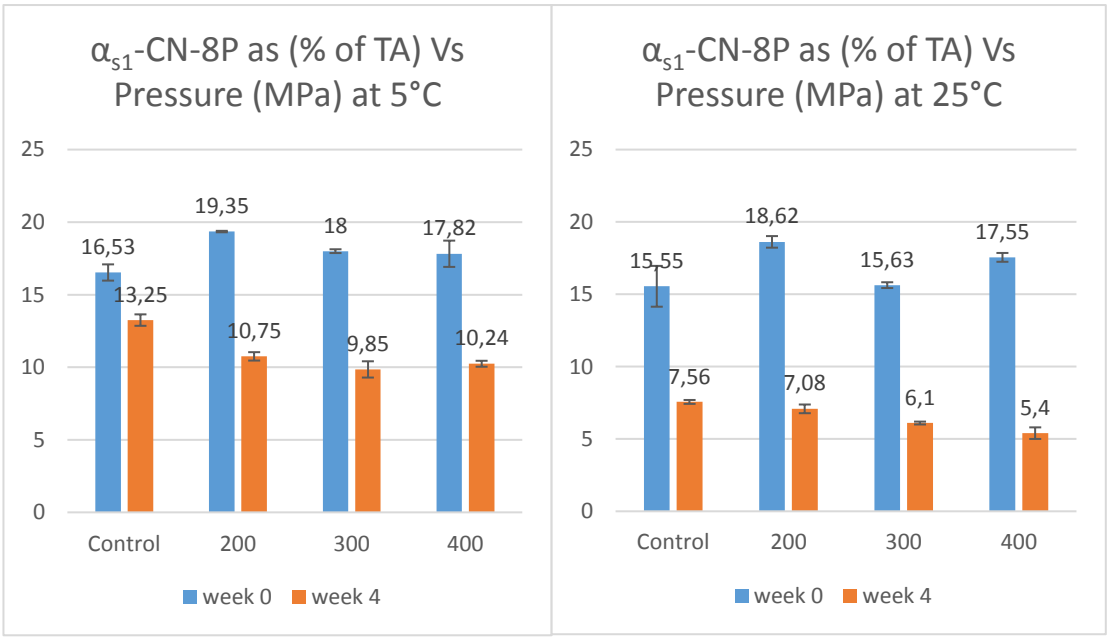

Figure 4.8: Concentration of α_{s1}-CN-8P (% of Total Area) Vs Pressure (MPa) at 5℃ and 20℃. The error bars represent the standard deviation of three measurements of each sample.

α_{s1}-CN is hydrolyzed by chymosin more rapidly as compared to β-CN due to high salt and moisture content of brined cheese (Prasad & Alvarez, 1999). Applying two-way ANOVA analysis (Pressure and storage time) on sample stored at 5℃ (figure 4.8) it was found both pressure and storage time had a significant effect (P<0.05). For week zero the concentration of α_{s1}-CN-8P was significantly lower than the pressurized samples. For the pressurized

samples, the concentration remained almost the same. After 4 weeks the α_{s1}-CN-8P got degraded much faster in pressurized samples as compared to the control. This could mean higher activity from enzymes released from starter LAB for further breakdown (Sousa et al., 2001). Cell lysis of LAB could have caused higher availability of LAB protease for the further breakdown.

For samples stored at 20℃, a similar pattern was observed like 5℃ for both week 0 and 4. After applying three-way ANOVA analysis (Pressure, storage time and storage temperature) there was an interaction between storage time storage temperature between the samples ($P_{stime*stemp}<0.05$). Further hydrolysis of α_{s1}-CN-8P was much more significant in the samples stored at 20℃ as compared to 5℃ in week 4. This suggests higher growth of non-starter LAB which could have led to this accelerated proteolysis of the fraction. Higher storage temperatures have been documented to accelerated proteolysis in cheese (Upadhyay & McSweeney, 2003).

The ratio of α_{s1}-CN-8P and α_{s1}-CN 9P increases with storage time as milk acid phosphatase is responsible to cleave phosphate from casein. The optimum pH for the enzyme is 4 and it is present in the cheese after the heat treatment. Although enzymes from rennet and cathepsin D (indigenous milk protease) also prefers hydrolysis of α_{s1}-CN 9P over α_{s1}-CN 8P (Wium et al., 1998). This shows why the peak α_{s1}-CN 9P was not observed in the result and it was independent of the pressure applied.

Plasmin is highly resistant to pressure treatment between 100-400 MPa (Portfolio, 2008). It is mainly responsible for cleavage of β-CN at three sites with high specificity of lysine (Ardö et al., 2017). When the cheese is pressure under brine it leads to disruption of κ-CN in structure. This leads to release of β-CN into the serum due to higher hydrophobicity (Messens et al., 1998). Plasmin is considered to be less proteolytic as compared to chymosin in brined cheese and its proteolytic activity is enhanced from 200-400 MPa due to higher availability of substrate. Plasmin activity is reduced only at pressure treatment of above 600 MPa for 30 min at 20℃ (Balasubramaniam et al., 2016). While chymosin acts on β-CN during the early stages of maturation when the salt concentration is low in white brined cheese (Sousa et al., 2001). Although β-CN-A1 differs from β-CN-A2 only at 69 positions of amino acid, they hydrolyzed differently by the enzymes. Both the fractions play an important role in the cheese yield, where β-CN-A1 improves rennet coagulation (Massella et al., 2017).

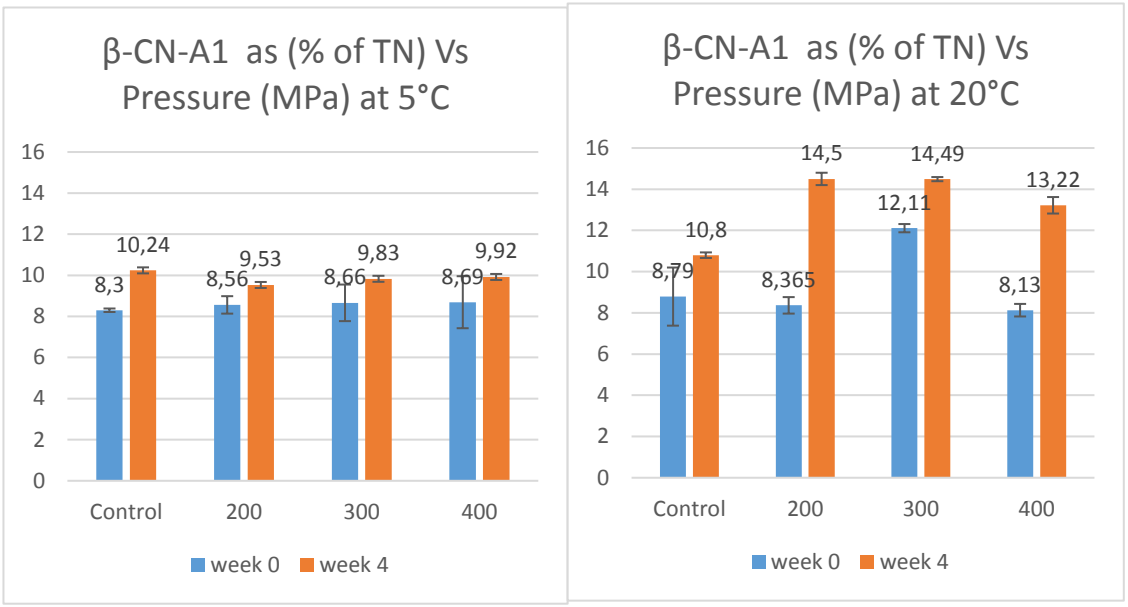

Figure 4.9 - Concentration of β-CN-A1 as (% of Total Area) Vs Pressure (MPa) at 5℃ and 20℃. The error bars represent the standard deviation of three measurements of each sample.

For week 0, No significant difference was found for the samples stored at week 5℃. This also shows that plasmin activity was resistant to the pressure treatment. Three-way ANOVA suggested storage temperature had no significant difference for hydrolysis of the sample. The high concentration of sample 300 MPa/20℃ could be due overlapping of two peaks which must have resulted in higher peak area as compared to the other 7 samples.

After 4 weeks, of storage still, there was no significant difference found in the samples, stored at 5℃. The increase in the peak area was not expected. Samples stored at 20℃ also showed the similar behavior (statistically no difference). As all the casein is suspended in citrate buffer the higher concentration of a particular fraction is not possible. The following could be the reason for this odd behavior: Almost 85-76% of the total casein fraction was represented by p-κ-CN, α_{s1}-CN-8P, β-CN-A1, β-CN-A2 when compared with the total during week 0. After week 4, p-κ-CN, α_{s1}-CN-8P, β-CN-A2 has shown hydrolysis (Figure 4.7, 4.8 and 4.10). The β-CN-A1 fraction was fairly less hydrolyzed as compared to others which must have resulted in higher percentage when expressed in terms of total area. The peak was identified by observing the pattern, it could be that β-CN-A1 was present in the week 0 and it was completely hydrolyzed, the peak that was observed in week 4 could be the degraded casein with similar optical properties to that of β-CN-A1. Further analysis of the casein fraction from HPLC is required.

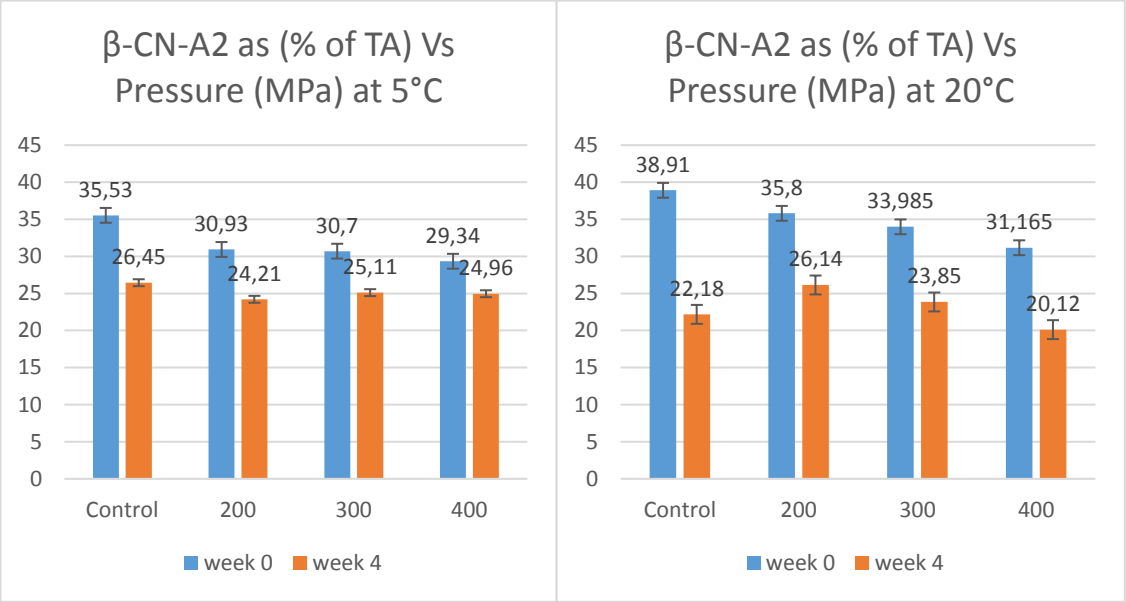

Figure 4.10: Concentration of β-CN-A2 as (% of Total Area) Vs Pressure (MPa) at 5℃ and 20℃. The error bars represent the standard deviation of three measurements of each sample.

For β-CN-A2, which eluted almost in the last peak and have the highest percentage area to total area in the graph. For week 0, pressure treatment had a significant effect on degradation of the fraction for both storage temperatures. Higher availability of the substrate can be the reason for the same. Samples at 400 MPa showed far accelerated degradation as compared to the control in both the storage temperatures. The degradation of β-CN-A2 was less as compared to α_{s1}-CN (Figure 4.8) which was as per expectation.

After 4 weeks, samples showed no significant difference ($P>0.05$) in the concentration of β-CN-A2 at 5℃. Loss of β-CN in the serum during the storage could also be responsible for lower hydrolysis as compared to the control. There was a significant difference in the samples stored at 20℃. The hydrolysis of the control samples was accelerated due to the high storage temperature. On the other significant changes in the β-CN-A2 could attribute to the loss of β-CN in the serum.

Overall storage temperature had a high influence on the degradation of β-CN which can lead to accelerated ripening in the cheese. In case of pressure treated samples, the loss of β-CN in serum phase needs to further investigation to understand better the hydrolysis of the particular fraction.

4.6 Assessment of Lipolysis

Milk lipase, rennet (PGE) and microflora determine the extent of lipolysis in cheese. The activity of milk lipase is reduced due to the heat treatment. Commercially available calf and bovine rennets are normally free from lipolytic activity (Georgala et al., 2005). The cheese sample consists of added lipase to give the characteristic flavor of the feta cheese. The value of DOA content in feta cheese made from 60% traditional rennet and 40% commercial rennet was 4.18 (meq-KOH/100g of fat) on day 3 which increased to 7.02 (meq-KOH/100g of fat) on day 60 and remained constant till day 120 (Georgala et al., 2005). Samples stored at 20°C showed better fat separation as compared to the samples stored at 5°C.

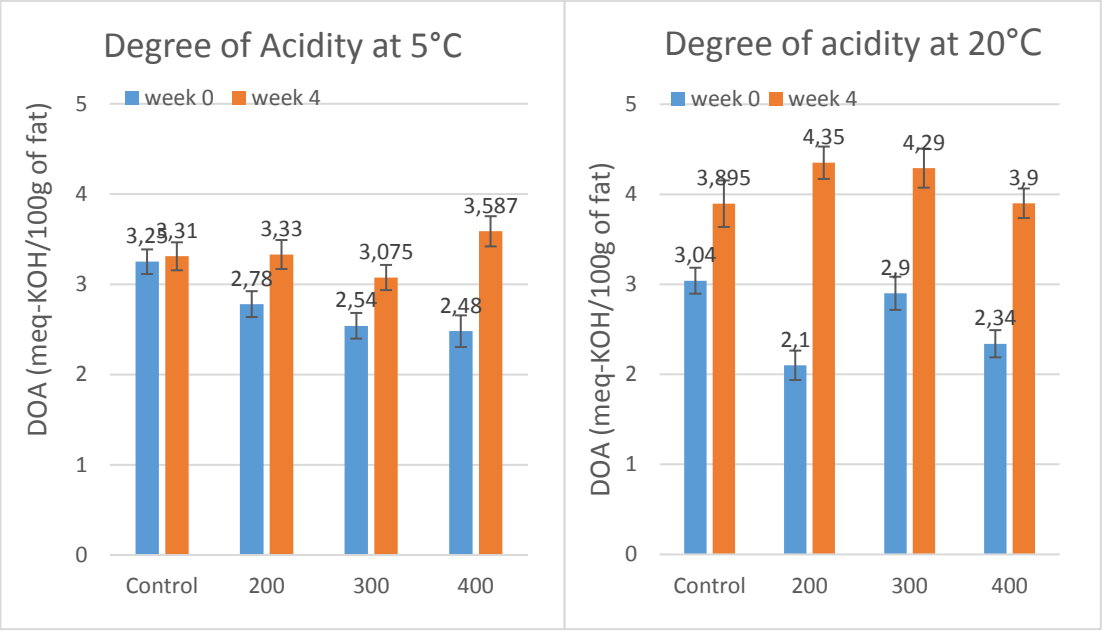

Figure 4.11: Degree of acidity (DOA) (meq-KOH/100 g of fat) Vs samples stored at ***5°C and 20°C*** *for week 0 and 4. The error bars represent the standard deviation of three measurements of each sample.*

All the samples showed significantly lower DOA values as expected showing low lipolytic activity in the cheese. Two way ANOVA analysis showed pressure treatment had significant effects ($P_{pressure}<0.05$) on DOA values of cheese samples. For week 0 at 5°C, there was appreciable depreciation in DOA values as compared to the control samples. The values decreased from 3.25 ±0.136 to 2.48 ±0.175 (meq-KOH/100 g of fat). This could be attributed to deactivation of microflora from the cheese samples which resulted in lower free fatty acids (FFA) in the pressurized samples. Lipase has shown high resistance when it comes to deactivation from high pressure. No inactivation was observed at 600

MPa/15min/20℃ in milk system (Balasubramaniam et al., 2016). While in another study it was reported that lipase has higher activity at 200 MPa for 15 min and less to no activity in 400-600 MPa range (Chen et al., 2017). This was not observed for any of the 200 MPa treated samples.

After a month of storage of the cheese, samples showed a significant difference ($P<0.05$) in DOA of pressurized samples. This means the FFA content in the pressurized samples increased during the storage at 5℃. In the confocal images, it was found that untreated brined cheese has the irregular shape of fat globules in the protein matrix with large empty spaces in between. When the samples were pressurized above 400 the fat globules were more uniform and smaller in size. The protein matrix was denser due to with less empty spaces in the pressurized samples above 200 MPa (Koca et al., 2011). The smaller size of fat globules means more surface area for the enzymes and since the matrix is more closely packed, the substrate (fat) is easily available for the enzymes for degradation.

Two way ANOVA analysis showed pressure treatment and duration of storage had significant effects ($P <0.05$) on DOA values of cheese samples stored at 25℃. 300 MPa sample was the exception which showed higher FFA content this could be due to poor sampling (oxidation of fat during the test). In case of Iranian white brined it was found that ripening time and storage temperature affected lipolysis both linearly and quadratically (Alizadeh et al., 2006). This resulted in a high increase in the FFA content around 4 (meq-KOH/100 g of fat). This resulted in 50-100% increase in the content of FFA in the samples stored 20℃ after a month. The sensorial results also showed detection of lipase taste in the samples stored at 20℃ in week 4.

In case of three-way ANOVA analysis (Pressure, storage time and temperature) interaction was found between the storage temperature and time. Understanding of lipase reaction during different time and temperature has been reported to be complex (Georgala et al., 2005; Karami, 2017).

Further analyzing the profile of FFA in the cheese can give a deeper understanding of the extent of lipolysis in the cheese samples (Georgala et al., 2005).

5 Conclusion

In this study effect of pressure treatment, storage time and the temperature were evaluated for brined cheese for week 0, 2, 4, 8 and 16.

In case of Total aerobic mesophilic bacteria, 3 log reduction was observed for samples treated at 400 MPa as compared to 2 log reduction for 200 MPa treatment for 5 min. This reduction was recovered in 16 weeks of storage at 5°C, as the CFU/g did not differ significantly ($P>0.05$) irrespective of the pressure treatment. Also, the technology proved very effective to prevent the growth of yeast and molds when treated at 400 MPa for 5 min, as compared to the control irrespective of storage temperature. In case of storage at 20°C after 16 weeks, of storage control samples had late package blowing, unlike pressurized samples. Thus HPP treatment can be used to significantly reduce the microbial population to produce safe products with commercial acceptance.

HPP treatment resulted in a significant difference ($P<0.04$) between the pH values of the control and the pressurized samples at 5° and 20°C storage. The difference was diminished with storage time. Moisture content in the cheese samples stored at 20°C was different statistically due to the accelerated moisture migration from the brine until week 8.

Pressurized samples showed significantly lower hardness as compared to the control till week 2 of storage when stored at 5°C.

In terms of sensorial parameters (Appearance, Taste/Smell, Consistency, Structure, and Color) samples treated at 400 MPa at 5°C received a perfect score (except for week 8) as compared to the control. This shows that HPP treatment can enhance the sensorial properties of the cheese.

HPP treated samples showed significant degradation of α_{s1}-CN-8P in 4 weeks for both the storage temperatures. While β-CN-A2 degradation was not affected by the pressure but by the storage temperature. In case of lipolysis, pressure treatment increased Degree of Acidity significantly after a month of storage. This effect was more pronounced for samples stored at 20°C. This can show that HPP can accelerate proteolysis in white brined cheese samples.

6 Perspective

High-Pressure treatment of white brined cheese made cows is lacked literature and research. Effect of the technology on proteolysis, lipolysis, and glycolysis needs to more research attention. Also, the effect of HPP on calcium-casein complex during the storage must be evaluated to give a better interpretation of its effect on the cheese. Overall the added storage temperature parameter led to complexity in data interpretation due to the acceleration of the different process in cheese. The literature on the effect of HPP on cheese block stored in brine is very rare. Therefore data was analyzed and interpret depending the on the effect oh HPP on similar cheese matrix.

In case of microbiology, the accelerated growth of Non-starter LAB resulted in unexpected results. Thus a complete microflora analysis of the cheese would have given a better understanding of the changes happening in the cheese during the storage at 20°C. There is lack of literature for the effect of HPP on the growth of yeast and molds in white brined cheese varieties.

Due to complex interactions of different parameters involved in the cheese texture, analysis of the data was difficult. Evaluating microstructure of the cheese samples during the storage could have led to better interpretation and understanding of the data.

In terms of proteolysis, p-κ-CN and β-CN-A1 showed unexpected results. Analysis of peptide fragment using HPLC can make the interpretation of the data easier. Also, the loss of water-soluble protein fraction in serum needs to be investigated for white brine cheese varieties. Lipolysis can be further investigated by evaluation of the complete free fatty acid profile of the cheese. Both lipolysis and proteolysis needs to be investigated for longer duration of storage (>4 weeks) for the cheese varieties.

Also, different enzymes (milk, rennet and microflora) in the cheese can be analyzed to give overall perspective of its effect on different reactions taking place in the cheese during the storage. Similarly moisture migration between cheese and brine can be evaluated to understand the mechanism and its effect on cheese.

7 Reference

Alizadeh, M., Hamedi, M., & Khosroshahi, A. (2006). Modeling of proteolysis and lipolysis in Iranian white brine cheese. *Food Chemistry*, *97*(2), 294–301. https://doi.org/10.1016/j.foodchem.2005.05.009

Anifantakis, E. M., & Moatsou, G. (2007). Feta and Other Balkan Cheeses. *Brined Cheeses*, 43–76. https://doi.org/10.1002/9780470995860.ch2

Ardö, Y., Magboul, A. A. A., & Upadhyay, V. K. (2017). Chapter 18 – Biochemistry of Cheese Ripening: Proteolysis. In *Cheese* (pp. 445–482). https://doi.org/10.1016/B978-0-12-417012-4.00018-1

Avila, M., Gomez-Torres, N., Delgado, D., Gaya, P., & Garde, S. (2016). Application of high pressure processing for controlling Clostridium tyrobutyricum and late blowing defect on semi-hard cheese. *Food Microbiology*, *60*, 165–173. https://doi.org/10.1016/j.fm.2016.07.008

Ávila, M., Gómez-Torres, N., Delgado, D., Gaya, P., & Garde, S. (2017). Effect of high-pressure treatments on proteolysis, volatile compounds, texture, colour, and sensory characteristics of semi-hard raw ewe milk cheese. *Food Research International*, *100*(April), 595–602. https://doi.org/10.1016/j.foodres.2017.07.043

Balasubramaniam, V. M., Barbosa-Cánovas, G. V., & Lelieveld, H. L. M. (2016). *High pressure processing of food*. https://doi.org/10.1007/978-1-4939-3234-4

Bylund, G. (2015). *Tetra Pak processing handbook. Dairy Industries International* (Vol. 80).

Cadesky, L., Walkling-Ribeiro, M., Kriner, K. T., Karwe, M. V, & Moraru, C. I. (2017). Structural changes induced by high-pressure processing in micellar casein and milk protein concentrates. *J. Dairy Sci*, *100*(9), 7055–7070. https://doi.org/10.3168/jds.2016-12072

Chawla, R., Patil, G. R., & Singh, A. K. (2011). High hydrostatic pressure technology in dairy processing: A review. *Journal of Food Science and Technology*, *48*(3), 260–268. https://doi.org/10.1007/s13197-010-0180-4

Chen, G., Miao, M., Jiang, B., Jin, J., Campanella, O. H., & Feng, B. (2017). Effects of high hydrostatic pressure on lipase from Rhizopus chinensis: I. Conformational changes.

Innovative Food Science and Emerging Technologies, *41*, 267–276. https://doi.org/10.1016/j.ifset.2017.03.016

Chopde, S. S., Deshmukh, M. A., & Kalyankar, S. D. (2014). High pressure technology for cheese processing- a review. *Asian J. Dairy & Food Research*, *33*(4), 239–245. https://doi.org/10.5958/0976-0563.2014.00610.1

Costabel, L. M., Bergamini, C., Vaudagna, S. R., Cuatrin, A. L., Audero, G., & Hynes, E. (2016). Effect of high-pressure treatment on hard cheese proteolysis. *Journal of Dairy Science*, *99*(6), 4220–4232. https://doi.org/10.3168/jds.2015-9907

Delgado, F. J., González-Crespo, J., Cava, R., & Ramírez, R. (2012). Changes in microbiology, proteolysis, texture and sensory characteristics of raw goat milk cheeses treated by high-pressure at different stages of maturation. *LWT - Food Science and Technology*, *48*(2), 268–275. https://doi.org/10.1016/j.lwt.2012.03.025

Ei-salam, M. H. A. (2004). *Cheese Varieties Ripened in Brine*. *Cheese* (Fourth Edi, Vol. 2). Elsevier Ltd. https://doi.org/10.1016/B978-0-12-417012-4/00039-9

Electrophoresis, C., & Food, F. O. R. (1998). 1.1 Basic Elements, 575–612.

Evert-Arriagada, K., Hernández-Herrero, M. M., Guamis, B., & Trujillo, A. J. (2014). Commercial application of high-pressure processing for increasing starter-free fresh cheese shelf-life. *LWT - Food Science and Technology*, *55*(2), 498–505. https://doi.org/10.1016/j.lwt.2013.10.030

Evert-Arriagada, K., Hernández-Herrero, M. M., Juan, B., Guamis, B., & Trujillo, A. J. (2012). Effect of high pressure on fresh cheese shelf-life. *Journal of Food Engineering*, *110*(2), 248–253. https://doi.org/10.1016/j.jfoodeng.2011.05.011

Evrendilek, G. A., Koca, N., Harper, J. W., & Balasubramaniam, V. M. (2008). High-pressure processing of Turkish white cheese for microbial inactivation. *Journal of Food Protection*, *71*(1), 102–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/18236669

Fox, P. F., & McSweeney, P. L. H. (2004). *Cheese: An Overview*. *Cheese: Chemistry, Physics, and Microbiology* (Vol. 1). Elsevier Ltd. https://doi.org/10.1016/S1874-558X(04)80060-5

Georgala, A., Moschopoulou, E., Aktypis, A., Massouras, T., Zoidou, E., Kandarakis, I., & Anifantakis, E. (2005). Evolution of lipolysis during the ripening of traditional Feta

cheese. *Food Chemistry*, *93*(1), 73–80. https://doi.org/10.1016/j.foodchem.2004.09.007

Giannoglou, M., Karra, Z., Platakou, E., Katsaros, G., Moatsou, G., & Taoukis, P. (2016). Effect of high pressure treatment applied on starter culture or on semi-ripened cheese in the quality and ripening of cheese in brine. *Innovative Food Science and Emerging Technologies*, *38*, 312–320. https://doi.org/10.1016/j.ifset.2016.07.024

Gingras, R. (2012). Creating the Future of Arla, page no. 21–24.

Huppertz, T. (2010). *High pressure processing of milk. Improving the Safety and Quality of Milk* (Vol. 1). Woodhead Publishing Limited. https://doi.org/10.1533/9781845699420.4.373

Johnson, M. (2003). Dairy Pipeline. *Dairy Pipeline: A Technical Resource for Dairy Manufacturers*, *15*(2), 1–12.

Karami, M. (2017). Enhancing the lipolysis of feta-type cheese made from ultrafiltered cow's milk. *LWT - Food Science and Technology*, *80*, 386–393. https://doi.org/10.1016/j.lwt.2017.03.001

Kilara, A., & Vaghela, M. N. (2018). *Whey proteins*. *Proteins in Food Processing* (Second Edi). Elsevier Ltd. https://doi.org/10.1016/B978-0-08-100722-8.00005-X

Koca, N., Balasubramaniam, V. M., & Harper, W. J. (2011). High-Pressure Effects on the Microstructure, Texture, and Color of White-Brined Cheese. *Journal of Food Science*, *76*(5), 399–404. https://doi.org/10.1111/j.1750-3841.2011.02201.x

Lawrence, R. C., Creamer, L. K., & Gilles, J. (1987). Texture Development During Cheese Ripening. *Journal of Dairy Science*, *70*(8), 1748–1760. https://doi.org/10.3168/jds.S0022-0302(87)80207-2

Maniou, D., Tsala, A., Moschopoulou, E., Giannoglou, M., Taoukis, P., & Moatsou, G. (2013). Effect of high-pressure-treated starter on ripening of Feta cheese. *Dairy Science and Technology*, *93*(1), 11–20. https://doi.org/10.1007/s13594-012-0060-y

Mann, E. (1996). Feta cheese. *Dairy Industries International*.

Martínez-Rodríguez, Y., Acosta-Muñiz, C., Olivas, G. I., Guerrero-Beltrán, J., Rodrigo-Aliaga, D., & Sepúlveda, D. R. (2012). High Hydrostatic Pressure Processing of Cheese. *Comprehensive Reviews in Food Science and Food Safety*, *11*(4), 399–416. https://doi.org/10.1111/j.1541-4337.2012.00192.x

Massella, E., Piva, S., Giacometti, F., Liuzzo, G., Zambrini, A. V., & Serraino, A. (2017). Evaluation of bovine beta casein polymorphism in two dairy farms located in northern Italy. *Italian Journal of Food Safety*, *6*(3). https://doi.org/10.4081/ijfs.2017.6904

McSweeney, P. L. H. (2011). *Biochemistry of cheese ripening (2011). International Journal of Dairy Technology* (Fourth Edi, Vol. 1). Elsevier Ltd. https://doi.org/10.1111/j.1471-0307.2004.00147.x

Mcsweeney, P. L. H. (n.d.). The microbiology of cheese ripening 54, 117–132. https://doi.org/10.1016/B978-1-84569-060-1.50010-7

Mcsweeney, P. L. H. (2007). *Cheese manufacture and ripening and their influence on cheese flavour*. *Improving the Flavour of Cheese*. Woodhead Publishing Limited. https://doi.org/10.1533/9781845693053.1

Messens, W., Dewettinck, K., Camp, J. Van, & Huyghebaert, A. (1998). High Pressure Brining of Gouda Cheese and its Effect on the Cheese Serum. *LWT - Food Science and Technology*, *31*(6), 552–558. https://doi.org/10.1006/fstl.1998.0414

Miralles, B., Rothbauer, V., Manso, M. A., Amigo, L., Krause, I., & Ramos, M. (2001). Improved method for the simultaneous determination of whey proteins, caseins and para-kappa-casein in milk and dairy products by capillary electrophoresis. *Journal of Chromatography. A*, *915*, 225–230.

Moatsou, G., & Govaris, A. (2011). White brined cheeses: A diachronic exploitation of small ruminants milk in Greece. *Small Ruminant Research*, *101*(1–3), 113–121. https://doi.org/10.1016/j.smallrumres.2011.09.031

Moschopoulou, E., Anisa, T., Katsaros, G., Taoukis, P., & Moatsou, G. (2010). Application of high-pressure treatment on ovine brined cheese: Effect on composition and microflora throughout ripening. *Innovative Food Science and Emerging Technologies*, *11*(4), 543–550. https://doi.org/10.1016/j.ifset.2010.07.001

O'Reilly, C. E., Kelly, A. L., Murphy, P. M., & Beresford, T. P. (2001). High pressure treatment: Applications in cheese manufacture and ripening. *Trends in Food Science and Technology*, *12*(2), 51–59. https://doi.org/10.1016/S0924-2244(01)00060-7

Okpala, C. O. R., Piggott, J. R., & Schaschke, C. J. (2009). Effects of high-pressure processing (HPP) on the microbiological, physico-chemical and sensory properties of fresh cheeses: A review. *African Journal of Biotechnology*, *8*(25), 7391–7398.

https://doi.org/10.4314/ajb.v8i25.

Okpala, C. O. R., Piggott, J. R., & Schaschke, C. J. (2010). Influence of high-pressure processing (HPP) on physico-chemical properties of fresh cheese. *Innovative Food Science and Emerging Technologies*, *11*(1), 61–67. https://doi.org/10.1016/j.ifset.2009.10.003

Otte, J., Zakora, M., Kristiansen, K. R., & Qvist, K. B. (1997). Analysis of bovine caseins and primary hydrolysis products in cheese by capillary zone electrophoresis. *Le Lait*, *77*(2), 241–257. https://doi.org/10.1051/lait:1997217

Patel, H. a, & Huppertz, T. (2014). *Effects of high-pressure processing on structure and interactions of milk proteins*. *Milk Proteins* (Second Edi). Elsevier Inc. https://doi.org/10.1016/B978-0-12-405171-3/00008-8

Portfolio, T. (2008). Application of High Pressure Processing for Extending the Shelf-life of Fresh Lactic Curd Cheese. *Engineering and Technology*, (March).

Prasad, N., & Alvarez, V. B. (1999). Effect of Salt and Chymosin on the Physico-Chemical Properties of Feta Cheese During Ripening. *Journal of Dairy Science*, *82*(6), 1061–1067. https://doi.org/10.3168/jds.S0022-0302(99)75327-0

R.K. Robinson and Tamime, A. Y. (1996). *Feta and Related Cheeses*. *Feta and Related Cheeses*. https://doi.org/10.1533/9781845698225.70

Reps, A., Kolakowski, P., & Dajnowiec, F. (1998). The effect of high pressure on microorganisms and enzymes of ripening cheeses. *High Pressure Food Science Bioscience and Chemistry*, 265–270. https://doi.org/10.1533/9781845698379.4.265

Sousa, M. J., Ardö, Y., & McSweeney, P. L. H. (2001). Advances in the study of proteolysis during cheese ripening. In *International Dairy Journal* (Vol. 11, pp. 327–345). https://doi.org/10.1016/S0958-6946(01)00062-0

Terpou, A., Bekatorou, A., Bosnea, L., Kanellaki, M., Ganatsios, V., & Koutinas, A. A. (2018). Wheat bran as prebiotic cell immobilisation carrier for industrial functional Feta-type cheese making: Chemical, microbial and sensory evaluation. *Biocatalysis and Agricultural Biotechnology*, *13*(November 2017), 75–83. https://doi.org/10.1016/j.bcab.2017.11.010

Tewari, G. (2007). *High-Pressure Processing of Foods*. *Advances in Thermal and Non-Thermal Food Preservation* (Second Edi). Elsevier Ltd.

https://doi.org/10.1002/9780470277898.ch12

Thierry, A., Collins, Y. F., Abeijón Mukdsi, M. C., McSweeney, P. L. H., Wilkinson, M. G., & Spinnler, H. E. (2017). *Chapter 17 - Lipolysis and Metabolism of Fatty Acids in Cheese BT - Cheese (Fourth edition). Cheese* (Fourth Edi). Elsevier Ltd. https://doi.org/https://doi.org/10.1016/B978-0-12-417012-4.00017-X

Upadhyay, V. K., & McSweeney, P. L. H. (2003). *Acceleration of cheese ripening*. *Dairy Processing: Improving Quality*. Woodhead Publishing Limited. https://doi.org/10.1533/9781855737075.3.419

Walstra, P., Wouters, J. T. M., & Geurts, T. J. (2006). *Dairy Science and Technology*. *Facilities* (Vol. 4). https://doi.org/9780824727635

Wilkinson, M. G. (2007). *Lipolysis and cheese flavour development*. *Improving the Flavour of Cheese*. Woodhead Publishing Limited. https://doi.org/10.1533/9781845693053.1.102

Wium, H., Kristiansen, K. R., & Qvist, K. B. (1998). Proteolysis and its role in relation to texture of Feta cheese made from ultrafiltered milk with different amounts of rennet. *Journal of Dairy Research*, *65*(4), 665–674. https://doi.org/10.1017/S0022029998003100

Özer, B. (2014). CHEESE | Microflora of White-Brined Cheeses. *Encyclopedia of Food Microbiology*, *1*, 402–408. https://doi.org/10.1016/B978-0-12-384730-0.00062-8

Appendix A - Microbial Analysis

CFU determination for all the samples: The number of colonies were counted for two suitable dilutions (20-200 colonies). Calculate CFU/gm was calculated as a weighted mean according to the following equation:

$$Weigthed\ mean\ (CFU) = \frac{c_1 + c_2}{n_1 + (n_2/10)} \times \frac{1}{V} \times \frac{1}{d_1}$$

Figure A.1: Weighted mean (CFU) equation. Where C_1 = total count for dilution d_1 (the lowest dilution); C_2 = total count for the following dilution. V = volume inoculated (0.1 ml for spread plating).

Appendix B - Proteolysis by Capillary Electrophoresis

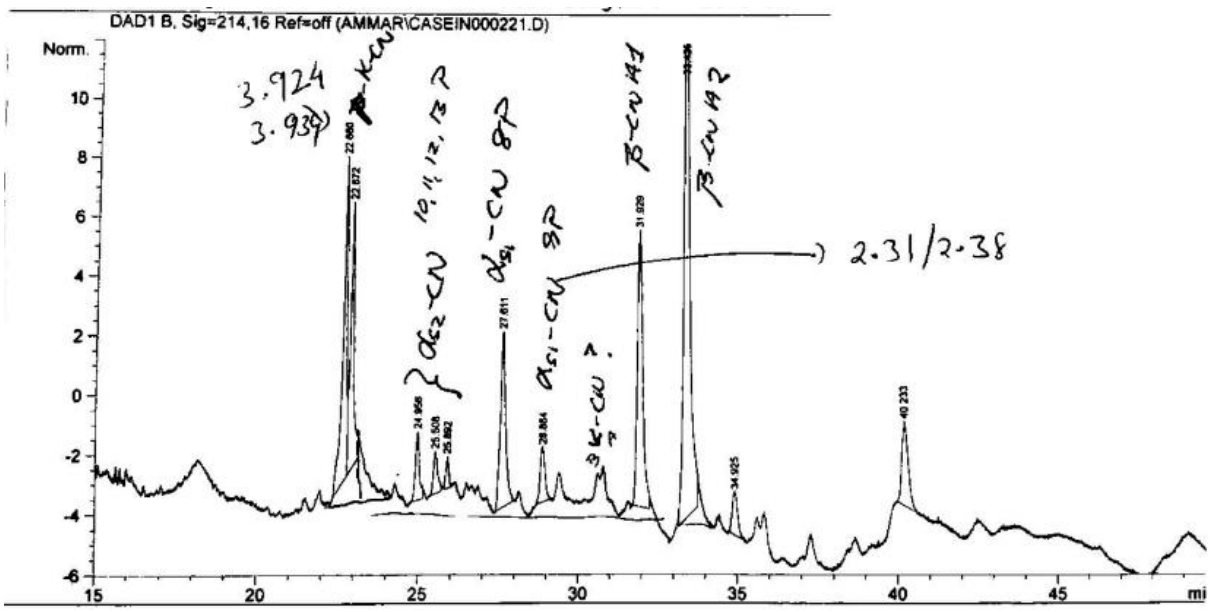

Figure B.1 - Capillary electrophoresis graph for Control sample stored at 20°C.

The first large peak looks like p-κ-CN. The immediate next peak could from degraded protein products which got eluted really quickly. This was followed by three small peaks peak of α_{s2}-CN, with different phosphate groups. One big and one short peak after this was believed to be α_{s1}-CN-8P and α_{s1}-CN-9P respectively. Then lastly one medium sized peak followed by a large peak represents β-CN-A1 and β-CN-A2 variant.

Appendix C - Sensory Analysis

All the analysis was done at Arla Krusa dairy.

Week 0 - rated by Kim Bøgh, Jesper Dalum, Carsten Andersen on Thursday 28-09-2017.

Week 2 - Second shipment rated by Carsten Andersen on Friday 06-10-2017.

Week 4 – Rated by Kim Bøgh and Carsten Andersen on Thursday 26-10-2017.

Week 8 - The samples were delivered to their workshop where it remained for 1 week without cooling, so the November samples have not been tested.

Week 16 – Rated by Carsten Andersen on Friday 02-02-2018.