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Alkaline phosphatase activity in Finnish hard cheeses and milk products

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Tiivistelmä — Referat — Abstract

Alkaline phosphatase (ALP) is an endogenous enzyme found in milk, which is inactivated at higher temperatures than vegetative bacteria and is thus used as an indicator of a successful pasteurisation. The ability of ALP to reactivate allows it to be found in milk products that are claimed to be pasteurised. The aim of this Master's thesis was to understand the reactivation behaviour of ALP in order to ascertain whether high levels found in milk products are correlated to a normal reactivation property of the enzyme or other possible reasons, such as a failed pasteurisation or contamination. This work also aimed to define the mean ALP activities found in specific commercial milk products and their deviation from the acceptable levels. Another scope was to determine the freeze stability of ALP to define its appropriateness for post-stored analysis. Lastly, the examination of ALP location in milk fat membrane globules was examined to interpret the variation of enzyme activity levels in products of different fat content.

The experimental part of the Master's thesis was divided into three parts. The first part included the record of ALP activities of different commercial Finnish milk products which are analysed in different groups according to their fat content and product type. The second part concentrated on the heat-treatment of milk samples at different time-temperature relationships and followed the reactivation behaviour of ALP. The total micro-flora was taken into consideration in order to observe any relation between the increased ALP activities and microbial growth. ALP activities were measured by a fluorimetric method, a quick three minute method which has the advantage of being more accurate compared to colourimetric methods. The third part examined the fraction in which ALP activities are found in milk after separation and its freeze stability when stored at -79^{o}C.

Commercial cheeses showed a high ALP activity in Emmental thermised cheeses and an activity less than 10 mU/g in other cheese types and pasteurised cheeses. In commercial milks, UHT treated and those closer to expiration date, high ALP activities were found, while pasteurised milks had low activities below the higher acceptable levels. The reactivation property of milk samples that were heat-treated in ALP was not related with the microbial growth and was quicker when the milk samples were heat-treated at higher temperatures. After the separation of cream from whole milk samples, ALP activity was found in the skim milk part. In conclusion, ALP activities did not decrease significantly following freeze storage for a few days showing its stable freeze properties.

Avainsanat — Nyckelord — Keywords
Alkaline phosphatase, pasteurisation, thermisation, UHT treatment, reactivation, cheeses, milks, fluorimetric method

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PREFACE

I had the honor to be an Erasmus Mundus Food of Life (EMFOL) student and fruitful my Master program of Food of Life in two different universities. For the first two study periods (2011-2012), I attended lectures in the Swedish University of Agricultural Sciences (SLU). This background equipped me with great knowledge and experience due to the international environment of studies. The second year of Master (2012-2013) took place at the University of Helsinki at the Department of Food and Environmental Sciences. The Master's thesis was carried out in the Finnish Food Safety Authority (Elintarviketurvallisuusvirasto, Evira) in the period of January-May 2013.

I am thankful to Prof. Alatossava Tapani, Professor of Dairy Technology in the University of Helsinki who supervised me and gave me the great opportunity to carry out my master thesis in Evira. I am also grateful to M.Sc. Ritvanen Tiina chemistry researcher who kindly supervised me on behalf of the Chemistry and Toxicology Research Unit of Evira. Both supervisors guided me successfully in every step of my thesis and through all the interesting discussions and meetings that we had I felt more secure and self-confident to continue my work.

I want also to thank Dr. Tiina Putkonen Senior Researcher and Prof. Kimmo Peltonen, Head of the Chemistry and Toxicology Research Unit of Evira who made it possible for me to be part of their research group of Evira.

A lot of thanks to Kristiina Kuitunen who explained me the principles of my study method, gave me important advices for how to work in a lab and encouraged me to improve my Finnish language skills. I would also like to thank Mira Kankare who assisted me at the microbiological part of my study and was always willing to help me. And as the work environment is very important thanks to Soili, Tiina and KETO family who made me feel happy to go for work.

My deepest thanks to my parents Cecilia and Georgios, for their endless love and support.

Finally, I want to give special thanks to Markku Rainakari for giving me a special meaning for everything I do in this life.

Helsinki, May 2013

Aliki I. Ninios

LIST OF ABBREVIATIONS

AI	Advanced Instruments
ALP	Alkaline Phosphatase
AST	Aspartate Aminotransferase
B-ALP	Bone Alkaline Phosphatase
EDTA	Ethylene Diamine Tetraacetic Acid
EU-RL	European Union Reference Laboratories
Evira	Elintarviketurvallisuusvirasto/ Finnish Food Safety Authority
FDA	Food and Drug Administration
GCAP	Germ Cell Alkaline Phosphatase
GH	Growth hormone
HTST	High temperature short time
HYLA	Hydrolyzed lactose milk
IU/L	International units per liter
LDH	Lactate Dehydrogenase
LTLT	Low temperature long time
MFGM	Milk Fat Globule Membrane
NSAP	Non Specific Alkaline Phosphatase
PAbs	Polyclonal antibodies
PEF	Final Pulsed Electric Field
PLAP	Placental Alkaline Phosphatase
SCM	Sub Clinical Mastitis
TNAP	Tissue Non Specific Alkaline Phosphatase
UHT	Ultra-high temperature processing milk

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1 INTRODUCTION

Milk and dairy products are a rich source of energy and nutrients for a number of microorganisms, which can potentially cause health problems to the consumers. Heat-treatments are applied to prevent or eliminate the undesirable causes of the microorganisms. Each microorganism and enzyme has different temperature thresholds. Pasteurisation is applied to kill all the undesired microbes except for the bacteria spores. The endogenous in milk enzyme alkaline phosphatase (ALP), is inactivated in higher temperature than vegetative bacteria and its loss of activity after pasteurisation is used as an indicator of a successful pasteurisation since the 1930s (Aschaffenburg and Mullen 1949; Payne and Wilbey 2009).

Alkaline phosphatases are cell surface membrane enzymes commonly found in all organisms from prokaryotes to eukaryotes (Coleman & Gettins 1983; Fishman 1990).There is three tissue-specific ALP isoenzymes the placental (PLAP), the intestinal, the germ cell (GCAP) and the nonspecific (NSAP). The NSAP is of high interest to dairy science because it is found in significant amounts in the membrane of the milk fat globules. It needs an alkaline environment to hydrolysis phosphoric monoesters and requires for its maximum activity the presence of zinc and magnesium (Linden et al.1977; Le Du et al. 2001).

An EU project had as an objective to measure ALP levels of milk products in different European countries and state an acceptable mean enzyme activity in mU/l. The Chemistry and Toxicology Research Unit of Evira (Finnish Food Safety Authority) determined the ALP in Finnish bovine milk cheeses and found high levels of ALP activity up to 28 mU/g in Emmental cheese coming from pasteurised milk and high levels of ALP in thermised Emmental, Gouda and Havarti cheese. Evira was interested to further the analysis of ALP levels in cheese and other milk products.

ALP values of different milk products vary depending on different factors like the fat content and the heat-treatment that is applied. ALP activity that is usually expected from specific products ranges between specific values. A possible deviation of an expected ALP value may be both the unsuccessful pasteurisation and the cause of post-contamination. According to Knight and Fryer (1989) many bacterial strains produce ALP with higher heat stability than the bovine milk ALP which may lead to a possible false positive ALP

test. The property that makes ALP mostly interesting is the reactivation of the enzyme after its inactivation. Pasteurised milk products or milk products stored for longer period (UHT milks) in stores have a relatively high ALP levels which increases by days and it is claim to be the cause of the enzyme's reactivation.

The scope of this master thesis project was to understand better the inactivation-reactivation behavior of ALP and its relation with microbial growth. This property would give answers to whether high levels found in milk products are correlated to a normal reactivation property of the enzyme or other possible reasons as a failed pasteurisation or contamination. In this work the mean ALP activity levels of some Finnish commercial products like cheeses, pasteurised milks, UHT milks and milk drinks were measured and observed for any deviation from the acceptable levels allowed from EU. For instance, the legal maximum activity limit of ALP test for successfully pasteurised cow milk is defined by the European Union reference Laboratory (EU-RL 2011) as 350 mU/l.

The experimental part of the master thesis was mainly divided into three parts. The one part includes the record of ALP activities from different Finnish products which are selected from markets and analyzed in different groups according to their fat content and product type. The second part concentrates on the time-temperature relationship and follows the reactivation behavior of ALP. Total microbial flora is taken into consideration in order to observe any relation between the increased ALP activities and microbes' growth. The freezing stability was studied for the appropriateness of sample to be used for analysis after being freezed. While the affiliation of ALP with the cream fraction gives clarifies the relation of ALP with milk fat globules.

2 LITERATURE REVIEW

2.1 Alkaline phosphatases (ALPs)

2.1.1 The origin of ALPs

Phosphatases consist of an ample and complex group of enzymes that exist in many organisms. They are found in extracellular fluids and cells and their role is to catalyze the hydrolysis of esters of phosphoric acid. Depending on the kind of the hydrolytic reaction or substrate's chemical nature, phosphatases are divided into four groups. Some enzymes consist the group of phosphomonoesterases which have or lack substrate-specificity. The classification of the substrate-unspecific enzymes is based on their optimum pH (Dean 2002). The enzymes that have their optimum pH at 9 are the glycoproteins alkaline phosphatases (Butterworth and Moss 1966; Moss et al. 1966; Dean 2002).

ALPs were first identified by Suzuki et al. (1907) and they as well as phosphoglycerate mutases, and arysulfatases are all metalloenzymes which belong to the same superfamily. It is assumed that they have similar catalytic core fold to that of nucleotide pyrophosphatases/phosphodiesterases (Gijsbers et al. 2001). ALPs are commonly found in all organisms from prokaryotes to eukaryotes and the catalytic mechanism among different organisms is claimed to differentiate (Coleman and Gettins 1983; Martinez et al. 1992). Using both the techniques of subcellular fractionation and electron microscopic histo-chemistry it was elucidated by Lin et al. (1975) that ALP in HeLa cells is mainly located in plasma membrane. ALP found in animal tissues is said to be linked to the insoluble cellular particles while the soluble ALP of *Escherichia coli* origin is a placed in the periplasmic space within the cell wall and cell cytoplasmic membrane ((Kabat, 1941; Hers et al. 1951; Martinez et al. 1992). Other intracellular sites that ALP is found in lower amounts are the nuclear membranes, the Golgi apparatus and the endoplasmic reticulum (Hugon and Borgers, 1996; Sasaki and Fishman 1973). These cell surface membrane enzymes consists of a big family of dimeric enzymes which have as sugar moiety sialic acid (Fishman 1990; Le Du et al. 2001; Rankin et al. 2010). The Figure 1 depicts the dimeric mammalian and bacterial ALP which are claimed to have a similar structure (Igunnu et al. 2011). The seperation of ALPs' monomers means the loose of its activity (Hoylaerts et al.1998).

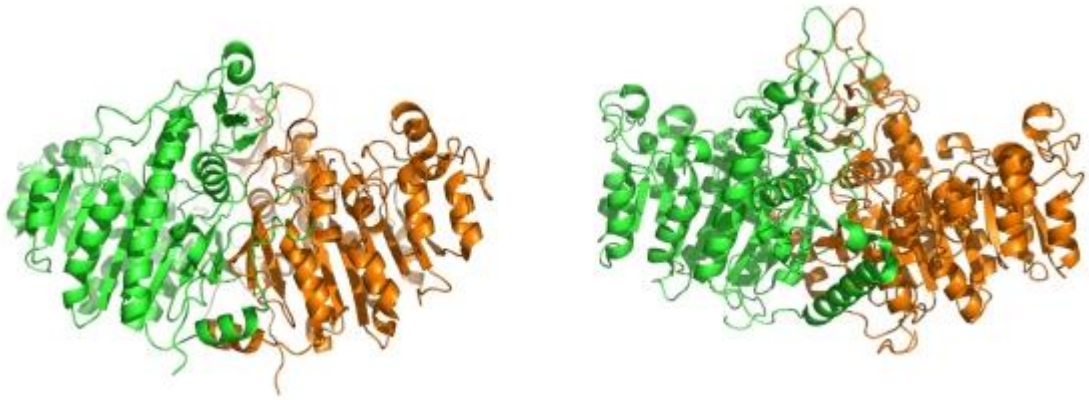

Figure 1. Dimeric ALP of (left) *Escherichia coli* and (right) human placenta **(**Igunnu et al. 2011).

ALP is claimed for taking part in protein synthesis in cell (Bradfied 1946 and Gold & Gould 1951) and being responsible for nucleotide and nucleoprotein metabolism (Brachet and Jeener 1946; Dempsey and Wislocki 1946; Jeener 1947). Further, ALP is showed to have a functional association with RNA and probably participates in the control of the growth and synthesis of DNA (Gavosto and Pileri 1958; Rubini 1963). ALP activates the hydrolysis of phosphomonoesters, R-O-PO3 with little known about the origin of the 'R' group. Serine phosphate formation at the active site is part of the activation mechanism and reacts at alkaline pH with water to liberate from the ALP inorganic phosphate (Holtz and Kantrowitz 1999).

2.2 Physiology of ALP

There are four genes for ALPs in humans (Whyte 1994): three tissue-specific ALP isoenzymes which are 90–98% homologous and the nonspecific ALP (NSAP) which is 50% identical with the others. The tissue-specific are the placental (PLAP), the intestinal and the germ cell (GCAP) which is located in testis, thymus and lung. The localization of ALP in the lung is at type II alveolar epithelial cells in lamellar bodies and at the plasma membrane (Le Du et al. 2001; Sánchez and Samaniego 2002). The genes of these three isoenzymes occupy vicinal positions on chromosome 2 while the tissue unspecific ALP gene is located on chromosome 1 of human genome (Fishman 1990). The chromosome-2 ALP genes are proved to encode the C-terminus a phosphatidyl inositol glycan tail which is used to bind and attach to membranes (Low and Zilversmit 1980; Fishman 1990).The NSAP can be found in bone, liver, kidney and blood cells and has as a part of its natural substrates pyridoxal 5′-phosphate and phosphoethanolamine (Coburn et al. 1998; Le Du et

al. 2001; Rankin et al. 2010). The intestinal specific ALP is claimed to be five times smaller than the human NSAP and the *Escherichia coli*'s ALP has 30% identity with the human PLAP (Schwartz and Lipmann 1961; Moss et al. 1986; Le Du et al. 2001). The isoenzymes of ALP in bone and liver are different chemically immunochemically and electrophoretically from the intestinal ALP (Schlamowitz and Bodansky 1959; Fishman and Kreisher 1963; Robinson and Pierce 1964; Moss 1965). Additionally there is claimed to be a relation between the blood group substances and the intestinal part of serum ALP (Arfors et al. 1963; Bamford et al. 1965). The normal level of ALP activity in human blood serum is 44 to 147 IU/L (international units per liter) with expected variations coming from the laboratory practicalities, the gender, and age with children and pregnant women having normally occurred growing raises (Eastman and Blxler 1977; Pratt 2010).

Activity of ALPs diverges in variant tissues hence their activity in serum is commonly used in clinical tests to investigate specific diseases (Simopoulos and Jencks 1994). Such diseases are the adult coeliac disease, the hyperparathyroidism, the Paget's disease and particularly disease in liver and bones (Bessey et al. 1946; Hill and Sammons 1967; Harris et al. 1969; Goldstein et al. 1980). In a study accomplished by Sánchez and Samaniego (2002), the levels of ALP were measured and compared among groups of children and adults that suffered or not from lung disease. According to the study results (Table 1), high molecular weight (HMW) ALP was significantly higher in both children (n=32) and adults (n=22) in the diseased condition while the liver ALP was higher only in children suffered from lung disease. The concentration of ALP from lung fluids has been utilized as a damaged marker of type II alveolar epithelial cells (Sánchez and Samaniego 2002).

Table 1. The activities of ALP in human serum (Sánchez and Samaniego 2002).

ALP type	Children		Adults	
	Control (n=15)	Lung disease	Control (n=30)	Lung disease
Total ALP (UI/L)	522 + 142	619+144	190+45	176+51,3
Bone ALP (%)	84,8+5,5	75,9+6,9	30,8+11,2	29,9+11,1
Liver ALP (%)	14,1+5,5	18,1+5,5	66,8+13,9	63,4+11,5
HMW ALP (%)	0,23+0,18	5,6+2,1	2,4+2,2	6,7+3,6
p<0,001	UI/mg * 10^{-3}			

Bone alkaline phosphatase (B-ALP) is found in serum and elsewhere but it is produced barely in bone (Stei et al. 1990; Ohlsson et al. 1993). The growth rate of children was studied and showed to accelerate after growth hormone (GH) therapy. Parallel with this

acceleration the bone alkaline B-ALP also increased. Because of this behaviour total serum B-ALP activity is considered as an important marker for bone formation and success index of GH therapy (Farley and Baylink 1995; Tobiume et al. 1997). Bone formation is stimulated at certain embryonic calvaria concentrations when happens *in vivo* and at this concentrations it was increased when fluoride was used. Additionally, *in vitro* treatment of bone cells sodium fluoride showed to increase both by ALP activity and bone proliferation (Farley et al. 1983). Some clinical interpretations are based on the levels of ALP isoforms hence they need to be fractioned from the total ALP activity (Deftos et al. 1991). ALP has also a very important role in humans addressed to skeletal mineralization (Whyte 2001). Mutations in the tissue non-specific alkaline phosphatase (TNAP) encoding gene cause hypophosphatasia which leads to skeletal deficiencies of varying degrees. The serum levels of ALP get lower than normal hence the bone-forming cells express less NSAP (Whyte 1994 &1996). Hypophosphatasia is related to high pyridoxal 5′-phosphate concentrations in plasma and deficit of ALP activity. The pyridoxine phosphate phosphatase activity in serum is under the responsibility of ALP (Whyte et al. 1985). Hypophosphatasia may lead to some diseases such as ricket, this is a genetic disease related with the resistance of target organ to the action of 1.25-dihydroxyvitamin D3 (Hughes et al. 1988). ALP is found in normal activity levels in serum when rickets is healed but it is found in remarkable amounts from the early stages of rickets. Hence, this deficiency considers ALP as a detection index of rickets. Additionally, NSAP is claimed to regulate the production of vitamin B-6 as the metabolism of the vitamin deviated from the normal one after the inactivation of the tissue NSAP encoding gene (Waymire et al. 1995). Furthermore, ALP has been studied for its role as an indicator for cancer. Elevated level of ALP above normal is recommended in patients with big tumours as a supplementary method besides for conventional hepatic function tests. This is an additional insurance to avoid postoperative hepatic failure (Didolkar et al. 1989).

ALP has also been suggested as one of the markers for the diagnosis of mastitis (Akerstedt et al. 2011). Cows with subclinical mastitis (SCM) showed to have higher mean activities of ALP and lactate dehydrogenase (LDH) but almost no much deviation in aspartate aminotransferase (AST) values compared to milk derived from non-infected udders (Babaei et al. 2007; Matei et al. 2010). In an experimental procedure *Escherichia coli* mastitis was induced to cows in order to investigate the changes in ALP activity. Cows that suffered from moderate to severe mastitis had increased ALP activity of isolated bovine

blood neutrophils which was more notable one week after the infection (Heyneman and Burvenich 1992).

2.2.1 The catalytic mechanism of ALP

The pH at which ALP seems to have its optimum activity depends on the initial concentration of the substrate (Ross et al. 1951). The study by Ross et al. (1951) showed that the lower was the concentration of substrate the lower was the rate of hydrolysis at the optimum pH while a higher proportion of available phosphate was expected when the phosphate released was low. The concentration of substrate is expressed logarithmically and has a linear relation with the optimum pH (Ross et al. 1951). According to Fosset et al. (1974) the pH at which ALP has its highest stability is alkaline, between 7.5 and 9.5, while the optimal pH is between 8 and 10 (Latner et al. 1970; Fosset et al. 1974). The thermostability of ALP in poikilothermic and homoeothermic species show specific differences in treatments above 56°C, while the optimum temperature is uniform to 37°C (Lustig and Kellen 1971). The hydrolysis that ALP causes to p-nitrophenyl phosphate was studied and the determination of hydrolysis rate was presented by a kinetic assay by Dean (2002). According to this study the higher gets the pH the quicker gets the speed of the reaction. ALP seemed to respond to magnesium and zinc ions, when Mg^{2+} concentration increases from 1 to 5 mM, speed of the reaction rises, for the increased concentration of Zn^{2+} stimulates the speed of p-nitrophenyl phosphate hydrolysis by ALP while at higher levels inhibiting it. The products from the hydrolysis reaction inhibit ALP competitively and the L-phenylalanine uncompetitively the ALP (Dean 2002).

ALP is a phosphormonoesterase which has a non-specific catalytic role functioned via a covalent phosphoseryl intermediate (E-P) formation (Kim and Wyckoff 1991). According to the reaction mechanism of human PLAP (E), a Michaelis complex is formed when the enzyme is connected to the substrate 4-nitrophenyl phosphate (NPP). ALP serine residue is phosphorylated and 4-nitrophenolate anion (NP) is removed. One of the reaction paths is followed by hydrolysis of phosphoryl–enzyme and dissociation of enzyme-bound phosphate to inorganic phosphate (Pi). In the second reaction path, a phosphoryl group is transferred to an alcohol receiver (ROH) and is dissociated as shown in Figure 2 (Huang et al. 1998). Additionally to inorganic phosphate, the hydrolysis catalyzed by mammalian or bacterial ALP, releases alcohol (Schwartz and Lipmann 1961). Various alcohols are

claimed to be released during the phosphoryl transfer reaction catalysed by ALP (Kim and Wyckoff 1991). Throughout the reaction mechanism ALP undergoes phosphorylation–dephosphorylation (Fernley et al. 1971).

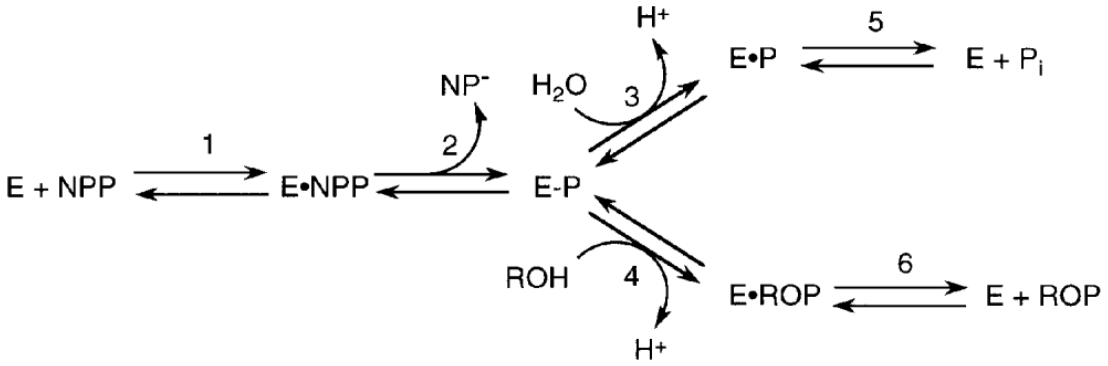

Figure 2. The reaction mechanism of human PLAP includes non-covalent association (•) and covalent chemical bond (-) (Huang et al. 1998).

2.2.2 Function-structure relationship of ALP

The biological function and the role of an enzyme in catalytic processes are closely depended on the structure with divalent ions ensuring its stability (Bortolato et al. 1999). Barman and Gutfreund (1966) studied the stability of ALP and concluded that structural changes are highly pH-dependent. According to Wright and Tramer (1956), the active form of bovine ALP molecule forms complexes with zinc atoms that impart structural integrity and functional properties. The molecule of *Escherichia coli* ALP is around 10nm×5nm×5nm in size and consists of two monomers with 449 amino acids each (Reid and Wilson 1971). Each monomer has an active site with a distant between the active sites close to 3nm (Reid and Wilson 1971). Every active site consists of one phosphorylatable serine and three exclusive binding sites for metals: two for zinc ions and one for magnesium with human ALP having one more metal binding site for calcium ion (Kim and Wyckoff 1991; Llinas et al. 2006). ALP of *Escherichia coli* origin is depicted in Figure 3 and differs from these of mammalian ones in Asp-153 and Lys-328 active site residues (Murphy et al. 1995).

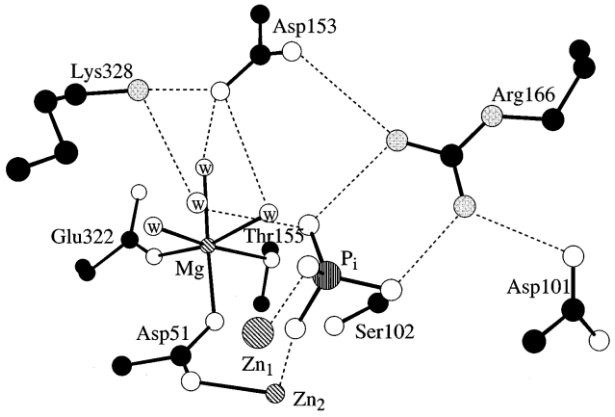

Figure 3. ALP of *Escherichia coli* origin is illustrated with two zinc ions and magnesium ion on its structure and bound phosphate at the active site region. Hydrogen bonds and water are pictured with (⋯) and (W) respectively (Holtz and Kantrowitz 1999).

The presence of zinc and magnesium divalent metals is required for the maximum activity of phosphoric monoesters (Linden et al. 1977). At the active form the stability of the enzyme is indirectly ensured by magnesium which works as a stimulator whereas the two zinc ions are directly involved in catalysis (Simpson et al. 1968; Anderson et al. 1975; Linden et al. 1977; Holtz and Kantrowitz 1999). Two structural and two functional zinc atoms do consist the possible ALP model. It is claimed that the zinc ions required for the activity have altered binding constants for the apo-enzyme (Lazdunsk et al. 1969). The function of the catalysis that is the role of the zinc ions activates the serine and water for the accomplishment of nucleophilic attacks (Fishman and Ghosh 1967; Kim and Wyckoff 1991). The phosphate and the substrate are bound by the help of the one zinc ion. The nucleophilic attack on the phosphate needs the deprotonated form of serine. The latter is stabilized by the second zinc ion when it interacts with active serine's hydroxyl group. This zinc metallo-protein has an enzyme to metal ratio ¼. Even though the zinc can be replaced on its all four bounding sites by manganese and cadmium, the four zinc atoms are protected from EDTA's removal by the inorganic phosphate (Lazdunsk et al. 1969). At the presence of urea thiol reduction and additionally acid treatment are claimed to dissociate the dimer and denature reversibly the ALP (Schlesinger and Levinthal 1963; Levinthal et al. 1962).

There are three among other mechanisms which are claimed to reversibly denature the ALP of *Escherichia coli* origin. Each of the mechanism leads to another form of reversibly inactive protein. Levinthal et al. (1962) studied the mechanism which forms a subunit free of sulfhydryl residues. This is the inactive product after the reduction of thiol in the

presence of 8 M urea. The reactivation was possible when a thiol-containing buffer was present and the reoxidation of the sulfhydryl group was part of the reactions. Another mechanism leads to the formation of a polypeptide chain with intact S-S bridges. This subunit is formed from the enzyme when this is exposed at pH less than 3, temperature up to 90^{o}C or by to guanidine hydrochloride of 6 M (Schlesinger and Levinthal 1963). In the third mechanism EDTA seems to inhibit reversibly the ALP (Garen and Levinthal 1960). This inactivation was studied by Plocke et al. (1962) who came into the conclusion that if the zinc, which is essential for the activity of the enzyme, is blocked or removed, ALP is inactivated. The enzyme was reactivated speedily in the presence of its metal content. Schlesinger and Barrett (1965) indicated that zinc atoms do not play an important role on the dimer form to maintain, as EDTA inactivates the enzyme and o-phenanthroline is a dimer. There might be other chelators that bind and block the metal and inactivate the enzyme. The potential relation between the different forms of ALP is depicted in Figure 4 (Schlesinger and Barrett 1965).

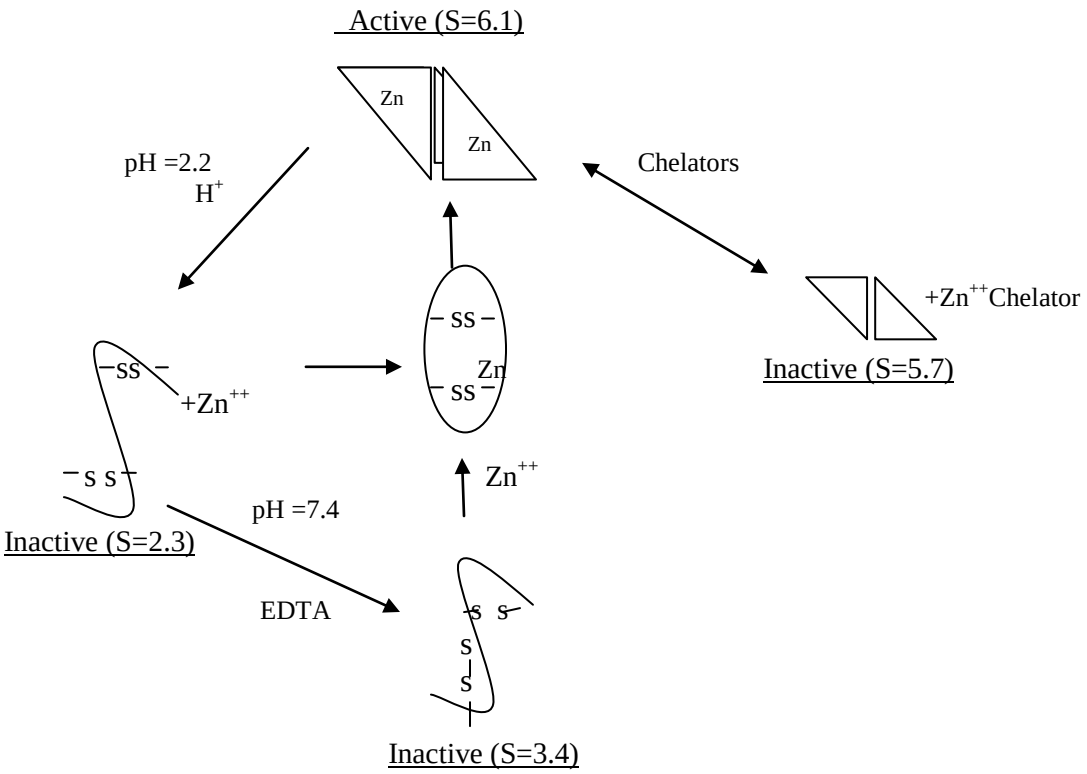

Figure 4. Different forms of ALP of *Escherichia coli* origin (modified from Schlesinger and Barrett 1965).

2.2.3 Inhibition factors of ALP

There are four classes of esterases that may act unspecifically as inhibitors of ALP. Some inhibitors are acoc'-dipyridyl and ethylenediaminetetraacetic acid (EDTA) which are metal-binding agents, zinc and beryllium chlorides that are inhibitory metal salts, amino-binding agents as keten, phenyl isocyanate, nitrous acid and formaldehyde and inorganic phosphate which is a competitive inhibitor (Roche 1950; Roche and Thoai 1950). Morton (1955) studied the inhibition and substrate specificity of calf intestinal mucosa and cow milk ALP. The enzymes seemed to be substrate specific and to hydrolyze speedily all true orthophosphate monoesters, the enolic phosphate, phosphoenolpyruvate, the orthophosphoamide and the phosphocreatine. They did not show any pyrophosphatase activity and did not hydrolyze ADP, ATP, and DPN, diphenyl pyrophosphate, sodium pyrophosphate and sodium hexametaphosphate. At the specific conditions of the assay the milk and intestinal ALP were both fairly inhibited by fluorophosphonates, phosphonates, phosphates, phosphites and a number of organic polyphosphates at concentrations of 2-10 M. The strong inhibition was caused by cysteine and iodine at concentrations of 3-10 mM.

According to Martland's and Robison's (1927) first description, the specific reaction product of ALP, the inorganic phosphate (Pi), inhibits the ALP of calf-intestinal-mucosal, *Escherichia coli* and human-placental (Morton 1955; Ahmed and King 1960; Garen and Levinthal 1960). Though, Pi can be a substrate besides for an inhibitor under specific conditions. Additionally, PPi acts also as ALP's inhibitor (Morton 1955). In conclusion, EDTA inhibits ALP even though ALP is not vulnerable to fluoride ion divalent cation-chelating agents (Dean 2002).

Gasser and Kirschner (1987) indicated that amino acids inhibited the intestinal ALP that was extracted by butanol. While the single substrate ALP was inhibited by phenylalanine un-competitively, the Pi acted as a competitive inhibitor and the regulation of extracellular ALP activity was claimed to be the role of inorganic phosphate (McComb et al. 1979; Stenesh 1993; Coburn et al. 1998). Ghosh and Fishman (1966) studied the inhibitory role of L-phenylalanine, on the rat intestinal ALP. The inhibition degree showed to be pH-dependent with a range from 0 to 66 % and pH between 7.8 and 10.4. The inhibition had a peak at pH 8.7 for β-glycerophosphate and 9.2 for phenylphosphate. Independently of the presence of the inhibitor, V_{max} participated as a function of pH. At the presence of

substrates β-glycerophosphate and phenylphosphate and pH of 8.8 and 9.8 respectively, rat intestinal ALP displayed its optimum activity with the existence of the L-phenylalanine which did not act as an inhibitor (Ghosh and Fishman 1966). While the inhibition of intestinal ALP by L-phenylalanine is time-independent, the inhibition by amino acids is time-dependent and accomplishes its supreme activity after one hour of pre-incubation with ALP. A dissociable ALP-amino acid complex formed at an allosteric zinc site is the mechanism primarily speculated to cause the inhibition of ALP by amino acids. The Zn^{2+} formation constant of amino acid sets one by one the degree of inhibition and dialysis or the addition of exogenous Zn^{2+} cause the reverse of inhibition. On the one hand, sodium was not used in the butanol extractions causing the miss of sidedness of the intact tissue. On the other hand, its presence was necessary for effectiveness of the inhibition of ALP by amino acid. The inhibitory site was speculated to be intracellular since the amino acid uptake is sodium-dependent and comes from the intestine. The intestinal ALP is suggested to connect the catalytic site with the apical membrane. The allosteric inhibitory site is claimed to be accessible from the cytoplasm and the catalytic site from the lumen (Gasser and Kirschner 1987).

2.3 Endogenous alkaline phosphatase in milk

2.3.1 Properties of milk ALP

ALP is one among the sixty enzymes found in raw bovine milk which has one reactive serine hydroxyl group in each molecule (Schwartz 1963; Schlimme et al. 1997). ALP and acid phospho-monoesterases are the main phosphatases found in milk (Kelly and Fox 2006; Silanikove et al. 2006). Normal cow milk has much lower ALP activity compared to this found in intestine and kidney. The Q value of the enzyme is around 20, isoelectric point between pH 5.4 to 6.0 and molecular mass of 187 kDa (Morton 1953; Vega-Warner et al. 1999).

Localisation of milk ALP

ALP is seated in the mammal tissue of lactating animal at the peri-alveolar network and is claimed to be harshly bound to lipoprotein like insoluble particles. Particularly 30-40% of the enzyme is absorbed to the milk fat globule membrane (MFGM) /butterfat and after

cream separation ALP is claimed to be concentrated to this phase (Kay and Graham 1933; Hansson et al. 1946; Morton 1953; Gruünfeld 1964). In the study of Zittle and Della Monica (1950), ALP activity was remarkably found in the skim milk after removing the fat globules. In skim milk ALP exists in microsomes which come from the milk fat globule membrane (Zittle and Della Monica 1950; Morton 1953, 1954). During the milk storage or after variety of treatments such as homogenization, agitation and pumping the MFGM breaks down and forms vesicles with diameter of 100 nm after being diffused into the milk serum (Morton 1954; Zittle et al. 1956). When getting butter after churning cream or eluding by distilled water ALP is liberated into the buttermilk hence the aqueous phase and its activity is found to be deficient in the butterfat. ALP bounded with the lipoprotein particles precipitated when organic solvents, acids and salts cause precipitation of casein. The latter aggregates casein and ALP is blocked into the protein precipitate with a result the loss of its activity (Kay and Graham 1933; Morton 1953). Study carried out by Painter and Bradley (1997) agrees with previous studies which relate high ALP activity to milk of high fat content (Table 2).

Table 2. The mean ALP values measured by fluorimetric method. Milks were heat-treated at low temperature long time (LTLT) and high temperature short time (HTST) **(**Painter and Bradley 1997).

	ALP (mU/l)	
Milk products	LTLT	HTST
Whole fat	81.8 ± 4.8	169.7 ± 12.3
2% lowfat	66.4 ± 5.9	145.2 ± 9.3
1% lowfat,	56.4 ± 2.1	98.6 ± 8.9
Skim milk	39.1 ± 3.9	72.5 ± 4.2
Half & half	35.0 ± 1.2	38.4 ± 4.6
Chocolate-flavored milks	91.3 ± 7.7	157.3 ± 6.5

Factors affecting ALP levels

The levels of bovine ALP is said to be influenced by the amount of produced milk, animal breed and stage of lactation (Haab and Smith 1956; Murthy et al. 1992). ALP level of Jersey milk varies depending on the season (Murthy et al. 1976). In sheep milk of industrial origin the activity of ALP showed a more than 2-fold increase in its activity between January and June, a 4-fold increase in January and February and until the end of lactation it stayed constant (Chavvari et al. 1998). ALP activity in cow milk was measured to be 2 to 3-fold lower than in sheep milk which rises during the lactation (Scintu et al. 2000). According to Hodošček et al. (2012), the fat content of cow milk is 3.8 g/100g and in sheep milk 6 g/100g. Because of the higher fat concent in sheep milk, ALP activity was also higher in sheep cheese than cheese made from cow milk. In both goat and cow's milk

there was found an inverse relationship between ALP activity and pyridoxal phosphate concentration. In human's milk the relationship was not like that while the levels of ALP and pyridoxal phosphate secreted from the mammary gland were low. The bovine milk had comparatively lower levels of pyridoxal phosphate even if it had similar total vitamin B-6 concentration with caprine milk. An important part of vitamin B-6 found in cow, goat and pig milk is secreted in the form of pyridoxal phosphate. Depending on activity of ALP pyridoxal phosphate is hydrolyzed back to pyridoxal (Coburn et al. 1992). ALP activity in bovine milk differs from humans with the former being 40-fold higher than the later (Heyndrick 1962). ALP in human's milk is transferred during breastfeeding into milk and from mother's blood into colostrum and this transfer may cause the deviant of ALP activity between mature milk and colostrum. These two differ in nutrients' concentrations and ALP activity possibly in order to offer specific necessities to the nourished infant. During the first month of lactation, ALP activity decreases when colostrum transforms to mature milk while inorganic phosphates increases (Bjelakovic et al. 2009). Karmarker and Ramakrishnan (1959) studied the levels of ALP in 60 women during lactation. An increase of ALP activity was observed when women intake a specific dietary fat content per day. The fat content was estimated from previous studies related to the association of ALP to the fat concentration and was up to 72 g per day (Stewart et al. 1958). Additionally, a three to four week supplementation with protein lead to raised ALP levels (Belavady 1960). Vitamin and protein supplementation did not have any effect on the assimilation, metabolism and digestion of fat that are part of ALP's action (Karmarkar et al. 1963).

2.3.2 Factors causing the inactivation/denaturation ALP

The effect of temperature on specific microorganisms and their enzymes' inactivation has been intensively studied with various heat-treatments applied (Figure 5). Thermisation (60-69^{o}C for 20 sec) is considered as a mild treatment that mostly eliminates the psychrotrophs, whose enzymes are very heat-resistant (Walstra et al. 2006). Low temperature long time pasteurisation (LTLT) and high temperature short time (HTST) pasteurisation are applied at 63^{o}C for 30 min and 72-75^{o}C for 15-20 sec respectively. At pasteurisation temperatures ALP is inactivated (Aschaffenburg and Mullen 1949). A similar bacterial reduction relative to ALP decrease which covers the demands for a successful pasteurisation is expected (Rankin et al. 2010). Because of these properties ALP is used as an indicator of a successful pasteurisation since the 1930s (Aschaffenburg and

Mullen 1949; Payne and Wilbey 2009). The legal maximum activity of ALP considered as pasteurised cow milk is defined by the regulation 1664/2006 as 350mU/l (EU-RL 2011). At ultra-high temperature treatment (UHT, 135-140^{o}C for few seconds) all vegetative microorganisms are killed and most of the enzymes are inactivated. ALP cannot be used as an indicator for a successful UHT treatment in this case, because only a low percentage of ALP is inactivated (Chandan and Kilara 2011). Further, according to Advanced Instruments, high fat dairy products which undergone UHT treatment showed a profound increase in ALP activity during storage (AI 2003). At sterilization (115-120^{o}C for 20-30 min) all microbes and spore forms are killed and also many milk enzymes are inactivated (Walstra et al. 2006). The pathogen *Mycobacterium tuberculosis* had been the most heat-resistant bacteria in raw milk and showed to be killed in lower heat-treatment than ALP, as shown in Figure 5 (Fox and Kelly 2006). For the guarantee of the elimination of *Mycobacterium avium paratuberculosis* industries extend the holding times during pasteurisation (Marshall 2002).

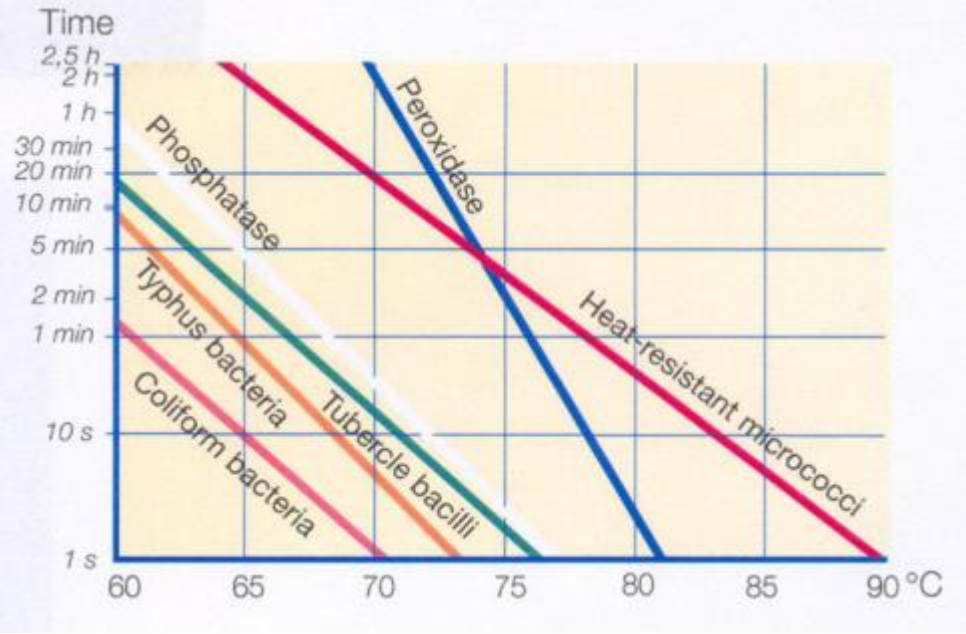

Figure 5. The inactivation curve of milk enzymes and bacteria at heat-time relationships (Walstra et al. 2006).

Higher milk fat content is claimed to lead to higher ALP level after heat-treatment as the fat-globule membrane protects the enzyme (Painter and Bradley 1997). The kinetics of inactivation of ALP in different fat content milks was studied by Claeys et al. (2002). The kinetics was affiliated in whole milk or semi-skimmed and skimmed milk even though ALP activity in skimmed milk was below the other milks. As a conclusion, the pasteurised

milk's ALP test results did not show to be significantly affected by the fat content (Claeys et al. 2002).

The factors studied for causing ALP inactivation, besides for heat-treatment are: pulsed electric field (PEF), hydrostatic pressure (HHP) and gamma irradiation. Shamsi et al. (2008) studied the inactivation of ALP under the effect of final field PEF treatment. The temperatures used were 15°C and 60°C and the field intensities 25 to 37 kV cm^{-1}. ALP of microbial flora in raw milk and ALP enzyme showed to be inactivated as the combined effect between the field intensity and temperature. The inactivation of ALP was at least twice less at 15°C than at 60°C. HHP is said to inactivate enzymes and vegetative bacteria (Ludikhuyze et al. 2000). The effect of different pressures on ALP was studied in three different mediums (Kouassi et al. 2007). The mediums used were buffer, fat-free milk and 2% fat milk and the pressures were between 206 and 620 MPa. According to the kinetic data, after 6 min of pressure treatments ALP was not affected and the medium in which the enzyme was prepared did not play any role. ALP activity was significantly reduced after 12 min at 620 MPa (Kouassi et al. 2007). Another study from Rademacher and Hinrichs (2006) measured the inactivation of the indigenous ALP in milk under high pressure treatment in a laboratory multi-vessel pressure unit. The temperature used was 5 to 40^{o}C and the pressure between 400 to 800 MPa. After severe pressure, ALP was reactivated at low activity values while moderate pressure did not cause any significant inactivation. Reactivation of ALP was observed at all temperatures applied to high pressure treatment while there was no reactivation when the milk was stored in cold. Micro-organisms found in milk as well as γ-glutamyltransferase and phosphohexoseisomerase enzymes are less pressure resistance than ALP. The result of ionizing radiation on dairy cows' udders was studied for its effect on the activity of some enzymes. The exposure of Co^{60} gamma irradiation with dose levels between 750 and 1.800 R and threshold from 500 to750 R caused an increase in ALP activity. In order to have enzyme inhibition in fresh milk a variation of dose levels was applied (Luick and Mazrimas 1966). Even though, ALP is known to be vulnerable to ionizing radiation fat seems to protect it from radiation's effects (Tsugo and Hayashi 1961; Umemoto and Sato 1961; Glew 1962). ALP is connected to microsomes of phospholipid particles at the milk fat globule membrane (Richardson et al. 1964).

2.4 Reactivation properties of ALP

ALP is an enzyme that should be inactivated after a successful pasteurisation. Nevertheless a high ALP activity of pasteurised milk products can be found and may indicate a possible raw milk post-pasteurisation contamination or biochemical reactivation (Harding 1991; Rankin et al. 2010). Generally, a milk product which is rich in fat has a higher initial ALP hence a higher reactivation after heat-treated (Murthy et al. 1976). Goat's and cow's milk have lower fat content than ewe's milk. Hence the temperature of heat-treatment which is needed for the successful pasteurisation of the ewe milk and the acceptable maximal ALP limits in ewe's pasteurised milk are more significant (EU-RL 2011).

Schlesinger and Barrett (1965) studied the reversible dissociation of ALP originating from *Escherichia coli*. Purified ALP was inactivated reversibly with dilute acid. ALP dissociated being in acid environment of pH 3.0 and according to kinetic studies it segregates into subunits. When the active ALP has a molecular sedimentation of 6.1 the subunits have around 2.3 at pH of 2.0 (see Figure 4). The sub-units coming from acid treatment re-bunch rapidly in buffers with zinc ions and low ionic strength with temperature being a crucial parameter (Schlesinger and Barrett 1965). ALP of pig kidney was also studied for its reversible inactivation at low pH. The enzyme was inactivated partly after acid treatment at 0°C. After being incubated in alkaline or neutral buffer at 30°C ALP was reactivated. The strength of acid treatment influenced the degree of the reactivation. For instance, a very small reactivation was observed after treatment in pH values below 3. Additionally, high temperature and mechanisms of subunit interactions seemed to speed up the reactivation (Butterworth 1968).

The reactivation of milk ALP after heat-treatment was studied earlier by Wright and Tramer (1956). According to this study, ALP was boiled for 45 sec and highly reactivated. After ALP being incubated at 37^{o}C its activity was reactivated up to 10-30%. Metal ions are claimed to play part in the reactivation of bovine ALP after been inactivated by heat-treatment (Wright and Tramer 1956). When Hg, Cd and Zn found in milk in low concentrations, they acted as inhibitors of ALP's reactivation (Lyster and Aschaffenburg 1962). At the study accomplished by Murthy et al. (1976) liquid Jersey milk products were heated to a range between 87.8 and 121.1^{o}C for a time period less than 1sec in order to measure the ALP reactivation. The heat-treatment was continuous and took place in a two-

phase slug-flow heat-exchanger. The activity of ALP was affected at a different degree depending on the temperature. The higher reactivation occurred when the milk was incubated at 34^{o}C after heated at 104.4^{o}C while homogenization showed to decrease reactivation rate when applied before heating (Murthy et al. 1976).

2.5 Additional sources of ALP activity

Besides for the native ALP found in milk, there are other sources which may contribute to the positive test of ALP. The positive test might be an indicator of improperly pasteurised milk, the contamination of it with raw milk, microbes added for the purposes of the manufacture or other ingredients interfering with the physiology of the test.

In milk products like cheese and butter, bacteria are used as part of the manufacture (Walstra et al. 2006). It is claimed that many bacterial strains produce ALP with higher heat stability than the bovine milk ALP. This may lead to a possible false positive ALP test (Knight and Fryer 1989). Microbes like *Bacillus anthracis*, *cereus* and *megaterium*, *Micrococcus sadonesis* and *Saccharomyces cerevisiae* produce heat-stable and –labile ALP (Dobozy and Hammer 1969; Gorman and Hu 1969; Glew and Heath 1971). Pratt-Lowe et al. (1987) recommended a re-pasteurisation after an unexpected positive ALP test. After the repasteurisation an insisting positive result can be ALP of microbial origin. There are specific types of cheeses that show a positive ALP result. The microorganisms that are used for the cheese manufacture seemed to be responsible for the production of it. Some of the cheese types that show this characteristic are Swiss cheeses, Camembert, Hispanic-style and blue-veined cheeses such as Danish blue, French Roquefort, Italian Gorgonzola and Israeli Gallyl (Ziobro 2001; Rosenthal et al. 1996). In Switzerland, ALP activity was measured in hard, semi-hard and soft Swiss cheeses. The revised method ISO 11816-2 was used for cheese sampling and fluorometric method ISO 11816-2 / IDF 155-2 for analysis. Hard cheeses like Emmental, Gruyère originating from raw milk had all ALP activity levels above 10 mU/g with levels ranging from 670 to 3589 mU/g. Raw semi hard cheeses have ALP levels from 194 to 4373, thermised from 36 to 1396 and pasteurised from 0.7 to 4 mU/g. Pasteurised soft cheeses have ALP levels from 0.7 to 8.2 (Egger et al. 2011). Analysing Swiss cheese originating from raw, pasteurised and micro-filtrated milk the amount of facultative heterofermentative bacteria was found in higher levels in raw milk. Among these bacteria are lactobacilli, enterococci, micrococci and propionibacteria

(Grappin and Beuvier 1997). Rosenthal et al. (1996) used the Scharer rapid phosphatase test to estimate the levels of ALP in a variety of blue-veined cheeses and found that ALP levels increased with storage time, which was related to the amount of blue mold growth. This result explains that the positive results of these old ripened cheeses are probably due to the presence of the *Penicillium roquefort*.

US Food and Drug Administration (FDA) has set a maximum level of ALP activity allowed in cheeses coming from pasteurised milk (21 CFR part 133). As shown in Table 3, dairy products that are made from pasteurised bovine milk have a great ALP level above which the products are considered as adulterated. This is mentioned in the section 402(a) (4) of the Act (21 U.S.C. 342 (a) (4)). The maximum level of ALP activity found in brick, semisoft and semisoft part-skim cheeses was 20 micrograms phenol equivalents per gram while Limburger and other cheeses had 16 and 12 micrograms phenol equivalents per gram respectively (FDA 2009).

Table 3. Maximum acceptable levels of ALP activity (μeq/g) in different cheeses coming from pasteurised milk (FDA 2009).

Dairy product	**ALP (μeq/g)**
Brick, semisoft, semisoft part-skim cheeses	20μg/ g
Limburger cheese	16μg/g
Other cheeses	12μg/ g
Dairy products other than cheese	>=2.0g

The EU-RL suggests as an unsettled ALP activity limit of pasteurised cheeses a level of less than 10 mU/g. In a study accomplished on Slovenian cheeses ALP activity of cheeses made from pasteurised cow milk was below 10 mU/g of cheese while the activity was 411 and 4076 mU/g in cheeses made from thermised and raw milk, respectively (Hodošček 2012).

Copper (Cu) is used in the manufacture of Finnish Emmental cheese. The effects of Cu supplement were studied and cheeses were produced with or without a protective culture *Lactobacillus rhamnosus* Lc705 (facultative heterofermentative strain) and copper supplement. According to sensory and chemical analyses, copper seems to play a significant role in the regulation of biochemical and physiological activities of bacteria. The level of primary proteolysis increased and secondary proteolysis slowed after the addition of copper to cheese-milk (Mato Rodriguez et al. 2011).

Cheeses with higher pH, moisture and lower salt contents are better substrate for bacteria to grow on comparing to long-ripened and cooked cheeses. Cheeses are made from pasteurised milk in order to avoid health risks. Although in Italy, Switzerland and France a significant amount of ripened cheeses up to 700,000 tons per year are made from raw milk (Grappin and Beuvier 1997).

2.6 ALP tests

Some well-known ALP methods are the EC method, Aschaffenberg and Mullen (A&M), Charm PasLite system and Fluorophos (Table 4). Phosphatase tests are of high immense of importance for the prevention from dangerous pathogenic bacteria such *as Listeria mono-cytogenes*, *Campylobacter jejuni* and *Salmonella Dublin* (Center for Disease Control 1984 and 1985; Hayes et al. 1986).

Table 4. Brief representation of the basic aspects of ALP test methods (VAM 2002).

Test method	Substrate	Reaction time	ALP accepted activity [a]
EC	Phenyl phosphate	1 hour	4µ/ml
A &M	p-nitrophenyl phosphate	2 hours	10µ/ml
Fluorophos	Non-fluorescent aromatic monophosphoric ester	3 minutes	500mU/l
Charm PasLite	Phosphate quenched luminescent chemical	3 minutes	350mU/l

a: Accepted maximal activity, interpreted as negative

EC method

In EC method disodium phenyl-phosphate is incubated with milk. After an hour of incubation time the released phenol reacts with dibromo quinonechlorimide and produces a bluish product. Dibromoindophenol is evaluated by colourimeter at 610 nm (VAM 2002).

A&M

In this method a comparator disc is used to measure visually the yellow p-nitrophenol which is the product of the hydrolysis of p-nitrophenyl phosphate by ALP (VAM 2002).

Charm PasLite system

In Charm PasLite system a luminescent phosphorylated molecule is the substrate for the reaction with ALP. The luminescence is retarded from the phosphate radicle and after incubation the reaction is terminated by the addition of EDTA buffer at pH of 7.5. Luminometer is used to measure the luminescence at 540 nm (VAM 2002).

Fluorophos system

While the colourimetric tests are based on the interpretation of colour to verify the success of pasteurisation, fluorimetric method uses fluorescence to measure the amount of compound liberated. Since May 2007, fluorimetric method or fluorophos assay replaces the colourimetric methods and represents the official EU reference method for ALP determination of heat-treated milk (EU Commission 2007). Fluorimetric method is acknowledged by International Dairy Federation (IDF155 2006), CEN European Standards Organization, AOAC, NCIMS/FDA and the International Standards Organization (ISO). ISO standard 11816-1|IDF 155-1:2006, defines the estimation of ALP by fluorimetric method (ALP, EC 3.1.3.1) in pasteurised flavored milks and milks of non-fat or different fat content. Fluorimetric method is adapted for milks coming from cows, goats and sheep and can determine high ALP levels of diluted raw or heat-treated milk over 2000 mU/l. International accepted protocols are required in order to validate the use of other methods except for fluorimetric method (EU Commission 2007). This is a sensitive, quick, simple to carry out and comparatively accurate method compared to older ones (Table 5).

Table 5. Properties of fluorimetric method (VAM 2002).

Properties	Qualifications
Test time	3 minutes
Sample size	75 μL
Sample capacity	Single sample
Sensitivity	0.003% raw milk
Reagent stability	2 years from date of manufacture at 1°C
Optics	90° optical fixed filter
Drift	Less than 3 FLU/hour
Memory back-up	Lithium cell, 5 years
Power	Fluorometer 150 Watts; Heating block 30 Watts
Memory backup	Integral lithium cell; 5 years min in absence of power
Dimensions	H 15cm x W 40cm x D 30cm
Weight	11kg

A continuous Fluorimetric direct kinetic assay is used to measure ALP activity of the samples in a three minute period from which the first minute is used for the equilibrium and the last two minutes for the estimation of ALP activity. ALP is calculated as milliunits per litre (mU/l). ‘’A unit of ALP activity is the sample of enzyme solution that catalyses the transformation of 1 micromole of substrate per minute’’ (ISO 2006). A fluorescent product called Fluoroyellow® is the result of hydrolysis of a non-fluorescent aromatic

monophosphoric ester at 38°C. The hydrolysis takes place in a buffered milk sample (VAM 2002).

According to fluorimetric method, ALP activity is considered as negative when the reading on the digital display is ‘’<10 mU/l’’ (ISO and IDF 2010). The average ALP value of most pasteurised milks is less than 50 mU/l, while the maximum acceptable activity of pasteurised milk has been defined as 350 mU/l (ISO 2006).

2.7 Certain factors interfering ALP tests in milk and milk products

ALP can be measured by different tests which work with different mechanisms. Many of them have shown unreliable results because of the nature of their mechanism or because of the interference of some ingredients. Ice cream is a milk product mixture, consisted of a variety of ingredients. Some of these have shown to affect the outcome of the pasteurisation, hence the result of ALP test: Ice creams were pasteurised for 30 min at 61, 62 and 63^{o}C and tested with Gilcreas and Davis test and the New York field test (Caulfield and Martin 1938). ALP test was positive in both tests when the mixtures of ice cream contained sugar but showed a negative result when sugar was not included. As a conclusion, sugar seemed to play a protective role to the ALP (Caulfield and Martin 1938). Salt seem to play a similar role as sugar: Salt used in butter showed to interfere with the enzyme and to contribute the reactivation of ALP (Rosenthal et al. 1996). According to the AOAC method, butters made of pasteurised creams and the ones being salted had a negative and low or no ALP activity respectively. The butters coming from under-pasteurised creams and the high salted ones had high ALP activities. The highly salted butters showed a doubled increase in ALP activity, after being stored for 12 months, without a clear incidence for the microbial origin or reactivation of ALP (Karmas and Kleyn 1990). In order to avoid the problem of phosphatase-reactivation, Freeman et al. (1968) suggested buttering plants a pasteurisation condition with higher heat time and lower temperature and storage temperature of less than 1^{o}C.

The presence of antibiotics in milk was studied for its effect on the ALP test. Penicillin and oxytetracycline gave a false result of unpasteurized milk when measured by colourimetric method, Gilcreas and Davis test. The reason was the formation of blue colour by

antibiotics in the presence of the reagent used in the colourimetric method. Moreover, erythromycin, neomycin and streptomycin showed an inhibition to colour formation (Manolkidis and Alichanidis et al. 1970). Also adding pesticides like phosphamidon and 0,0-dimethyl-2,2-dichlorovinyl phosphate gave a false positive ALP result when a phenol release test was used (Kumar et al. 1973). Vega-Warner et al. (1999) referred to an immunological way that has been used to estimate ALP activity left out the intervention of antibiotics, pesticides and microbial ALP. This method is depended on the provoked produce of polyclonal antibodies (PAbs) by bovine milk ALP.

3 EXPERIMENTAL RESEARCH

3.1 Aims

This Master's thesis scope was to understand the inactivation-reactivation behaviour of ALP in order to ascertain whether high levels found in milk products are correlated to a normal reactivation property of the enzyme or other possible reasons, such as a failed pasteurisation or contamination. The foremost aims of this thesis can be abbreviated into:

- Definition of the mean ALP activities found in specific commercial milk products and their deviation from the acceptable levels
- Study the inactivation-reactivation behavior of ALP after heat-treatments
- Examination of ALP location
- Determination of the freeze stability of ALP and qualify ALP's appropriateness for post-stored analysis

3.2 Materials and methods

3.2.1 Commercial products

One of the scopes of this work was to record ALP activities from various dairy products. This would give a picture of the mean values found in specific products and could be used as a comparable data. For this purpose commercial products were used for both cheese and milk analysis. Products that are claimed to undergone heat-treatments at a manufacture scale could be a reliable element.

Commercial cheeses

Commercial Finnish hard cheeses collected from markets and used for the analysis of ALP activities are shown in Table 6. The cheeses were made from milk that was heat-treated in pasteurisation conditions or lower temperatures. Information about the heat-treatment implied to milk was provided by the label found on the package. The

Table 6. Data of commercial Finnish hard cheeses included in this study.

Type of cheese	Information	Weight (g)	Fat %	Heat-treatment	Code
Emmental					
Emmental sinileima/Blåstämplad	Mild & aromatic, I 2, M 3	350	30	T	FI 60137 EY
Emmental punaleima/Rödstämplad 17%	Strong & robust, I 4	350	17	P	FI 60137 EY
Emmental punaleima/Rödstämplad	strong & robust , I 4	350	30	T	FI 60137 EY
Musta leima Emmental	Long M, bit, strong	245	31	T	FI 60203 EY
Sinileima Emmental Kuusamosta	M 3	600	30	T	FI 60403 EC
Suomalainen mieto Emmental koko perheelle 17%		600	17	P	FI 60403 EC
Mustaleima Emmental Kuusamosta	M 9	300	31	T	FI 60403 EC
Suomalainen Emmental Kuusamosta, 17%		300	17	P	FI 60403 EC
Emmental Mustaleima, aito, hienostunut maku/ Svartstämplad, s	LF, LS, M 9	257	29	T	FI 60507 EY
Kevyt Emmental 15%, Mustaleima, aito, hienpostunut maku/	LF, LS	280	15	T	FI 60507 EY
Polar					
Polar Valio täyteläinen	PF, I 3	350	28	P	FI 60203 EY
Polar Valio, kevyt 5%	PF, I 3	300	5	P	FI 60203 EY
Polar Valio, 10%, Sydänystävä	PF , I 3	300	10	P	FI 60203 EY
Polar Valio, 15%, Alkuperäinen	PF, I 3	350	15	P	FI 60203 EY
Edam					
Edam Valio		350	24	P	FI 60220 EY
Edam ValioTäyteläinen		350	28	P	FI 60220 EY
Edam Valio kevyt 9%		350	9	P	FI 60220 EY
Edam Valio 17%		350	17	P	FI 60220 EY
Gouda					
Gouda Salaneuvos,	distinct taste I 3	350	28	P	FI 60203 EY
Gouda Salaneuvos 17%,	distinct taste , I 3	350	17	P	FI 60203 EY
Gouda Musta Salaneuvos,	mild-strong, M 8	300	30	P	FI 60203 EY
Kermajuusto					
Valio Ritari	strong cream cheese , mild - strong	300	32	P	FI 60203 EY

LF: Lactose-free
PF: Preservative –free
M: Matured for months
I: Intensity out of 5
LS: low-sodium

cheeses were divided in five groups in order to establish a calibration curve for each group. The division criteria were the fat content and the cheese type (ISO 11816/ IDF 155-2 2010). The code gives information about the product and the country it was packed.

Commercial milks

ALP activity of commercial milks (Table 7) was measured by the fluorophos method in order to estimate the mean ALP levels of the products sold in markets. It was questioned whether ALP activity found in the products after being stored in the markets represents the level expected according to specific heat-treatment indicated in the product package. The milk products were milks and milk drinks, all full-fat in order to use the same calibration curve during the analysis. The milks differed in heat-treatment, which was recorded on the package of each product. The products were carefully agitated before the analysis in order to prepare a representative sample.

Table 7. Data for commercial milks included in this study.

Product	Information	Fat %	Heat-treatment	Code	Expiration date
Arla, Ingman	Full-fat, LL	3.5	UHT	FI 60503	09.05.13, 09:30
Valio	HYLA	3.5	UHT	FI 60143 EY	30.04.13, 30.01.13, 06:27
Rainbow	LL	3.5	UHT	FI 60245	06.05.13, 13:04
Rainbow	LL	3.5	UHT	FI 60245 EU	09.04.13, 20:10
Valio	HYLA	3.5	UHT	FI 60143 EY	11.12.12-11.03.13
Rainbow	Milk drink, LF	3	HP	FI 60246	20.02-13.03, 19:41
Valio	Milk drink, LF	3	HP	FI 60321, EY	22.02-10.03, 19:00
Arla, Ingman		3.5	P	FI 60141 EC	02.03-10.03, 16:30
Valio Hilja	Old-time, non-homogenized	4.1-4.4	P	FI 60412 EY	01.0310.03, 03:27

LL: Low-Lactose
LF: Lactose-free

3.2.2 Milks used for different heat-treatments

Lorry milk

Full-fat lorry milk picked by Valio lorry was received from the Viikki pilot dairy plant of the Division of Food Technology (Department of Food and environmental, Sciences). Samples that were used for the thesis were lorry raw milk and pasteurized milk. The heat-treatment was made in the pilot plant by B.Sc. Jyri Rekonen, in order to reach a treatment of manufacture scale for short time. The pasteurisation applied at 75^{o}C for 15sec. There were two different samples of lorry milk received from the lorry, the sample A and B. The pasteurized milks originated from separate raw milk batches.

Farm milk

Full-fat raw milk samples were provided by University Viikki Research Farm and were heat-treated in a water bath at the Chemical and Toxicological Research Unit of Evira. The milks samples C, D, E, F and G originated from different raw milk and were heat-treated at different low temperature-long time (LTLT) and high temperature short time (HTST) conditions. Plastic conical tubes with capacity of 50 ml were filled up to 25 ml with raw milk and were placed in the water bath (Kottermann GWB) with the water covering 3/5 of the tube. The water bath reached a specific temperature and the tubes were removed from it at a specific time. The final temperature reached was checked by a thermometer inserted in a milk sample.

All the raw and heat-treated milk samples (Table 8) were collected and stored for a series of days in cold at 4^{o}C and in some cases at room temperature at around 24^{o}C. The total microbial flora and ALP activities were measured regularly during the storage. ALP levels were analyzed at the chemical and toxicological department of Evira, while the total microbial flora at the section for food microbiology, both at Evira's headquarters in Viikki, Helsinki.

Table 8. Raw lorry and farm milks and the heat-treatments applied at specific temperature and time.

Raw samples	Heat-treatments
Lorry milks	
A	75°C, 15sec
B	75°C, 15sec
Farm milks	
C	58°C, 30 min
D	60°C & 63°C, 30 min
E	59 °C, 1h
F	61 °C, 1h
G	74.5°C, 1h

Unhomogenized full-fat milk

Full-fat unhomogenized pasteurised milk (Valio Hiljamaito, traditional milk) of 500 ml was added in a flask and left to cream over night at 4°C. The cream was removed with spoon and diluted into a 500 ml phosphate buffer. The buffer of NaCl 0.1 M and 0.1 M Na_2HPO_4 was made to have pH 6.7 in order to be close to that of milk (pH around 6.6). The pH was regulated by adding to the buffer NaH_2PO_4.

3.2.3 Fluorimetric method for determination of ALP activity

Fluorophos® instrument (Figure 6) was used for the determination of ALP in both cheese and milk. Fluorimetric is a method applicable to milk, soft cheeses, semi-hard and hard cheeses, except for blue veined cheeses. It is based on the production of a high fluorescent product after the hydrolysis of a non-fluorescent aromatic monophosphoric ester substrate, 2'-[2-benzothiazolyl]-6'-hydroxybenzothiazole in the presence of ALP in the sample (Marshall 2002).

Apparatus and reagents used in fluorimetric method

- Filter fluorimeter/Fluorophos® instrument

  Allow the excitation at a wavelength of 440 nm and emission between 540 nm and 560 nm. It is able to operate at 38°C ± 1°C because of its cuvette holder that is thermostatically controlled.

- Cuvettes

  Non fluorescent glasses of 75mm length and diameter of 12mm.
- Incubator block
- Vortex mixer
- Centrifuge*
- Grinding device (Moulinette S, Moulinex)*
- Working substrate

  It is a mixture of 144 mg of Fluorophos® substrate and 240 ml diethanolamine (DEA) buffer solution. It is test for its suitability every day before used.
- Working calibration solution

  Calibration solution A (0 μmol/l of Fluoroyellow®), B (17.24 x 10^{-3} μmol/l of Fluoroyellow®) and C (34.48 x 10^{-3} μmol/l of Fluoroyellow®). Calibration was made by adding with a pipette 2ml of each of the control sets A, B and C in cuvettes and then to the heating block for 20 minutes at 38^{o}C. In each of the cuvettes 75 μL of the controlled sample was added, mixed and measured at the Fluorophos® instrument to take the calibration curve.
- Daily instrument control solution

  34.48 x 10^{-3} μmol/l of Fluoroyellow®
- PhosphaCheck Pasteurisation Controls (part number FLA260)

  PhosphaCheck negative, positive and normal control 3 mL each, ensure that the performance of ALP test and reagents are reliable.
- Fluorophos® Cheese extraction buffer*

  DEA buffer (pH 8.0) with magnesium and Triton X-100.

Fluorophos® and Fluoroyellow® reagents were available from Advanced Instruments Inc. USA.

(*): used for commercial cheese samples, not for determination of milk ALP activity

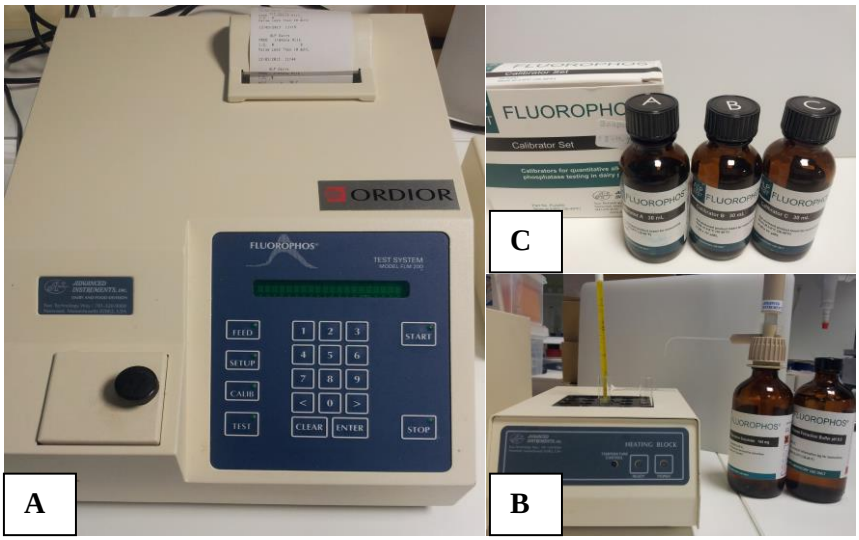

Figure 6. Fluorophos® instrument (A), incubator block with Fluorophos® working substrate (B) and working calibration solutions (C).

Analysis of cheese samples

The procedure is specified of ISO 11816 / IDF 155. For the sampling recommended in ISO 707/ IDF 50[1] part of the rind (0.5cm) of the hard cheeses was removed. From the part remained after rind cut, 1 cm was cut and blended and a cheese portion of 0.3-0.5g was weighted into a 10 ml glass beaker (Figure 7). 5 ml of cheese extraction was added to the beakers and stayed for 15 min. After the cheese samples stayed with the extraction, they were homogenized with Ultra-turax and transferred to 25 ml volumetric flasks. The homogenizer in the beakers was rinsed 3 times with 5 ml extraction. The missing 5 ml were added to fill the 25ml and the flask was shaken gently.

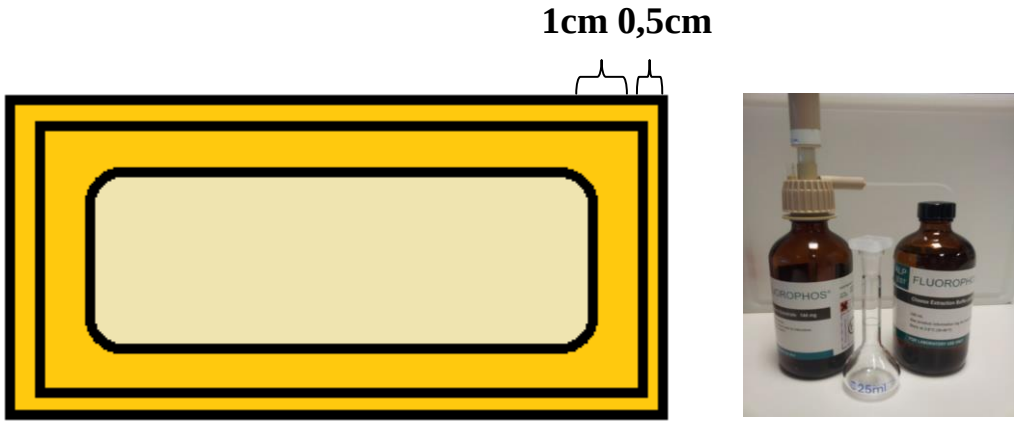

Figure 7. The rind (0.5cm) was removed and 1cm was used as a representative sample (left). Buffer extraction was used after cheese homogenization in order to get the ALP diluted in the buffer (right).

The content of flasks was introduced in centrifuge tubes for fat seperation. The centrifugation took place in Labofuge 400R, at 4^{o}C for 10 min and 2300rpm. The cheeses were categorized in five groups: Gouda and Edam low and high fat cheeses, Emmental and Polar low and high fat and very low fat cheeses. For the calibration of each cheese category, was used a pasteurised representative with fat content closer to that of the average of its group. After calibration, 2 ml of phosphorous substrate was placed in cuvettes to the incubator block for 20 minutes at 38^{o}C and 75 μL extract of each cheese type or milk was placed and measured in Fluorophos® instrument.

The reading does not give directly ALP activity in mU/g but it is calculated by the following equation:

$$ALP = \frac{ALP_1 \times 25 \times F_2}{1000 \times m}$$

ALP: numerical value of ALP activity of the test sample (mU/g)
ALP_1: numerical value of ALP of the supernatant (mU/l)
f_2: numerical value of the dilution factor
m: mass in grams of the test portion in the 25 ml one-mark volumetric flask

Milk sample analysis

The sampling was made according to ISO 707/IDF 50. Samples, whose analytical range of assay was > 7000 mU/l, had to be diluted with ALP-free milk. In order to prepare ALP-free diluter, milk was boiled up to 95^{o}C for 10 min and tested for its ALP activity which had to be <10mU/l. The ALP activity recorded from the fluorimetric filter is given in mU/l. The channel A/D test for 4% milk fat was selected in order to get the representative calibration curve for our samples which were all whole-fat milks or milk drinks.

3.2.4 Calculation of total micro-flora

The total microbial flora of the milk samples were measured according to the ISO 4833:2003 and the instructions of Evira 3420/2.

Equipments, tools, culture media, and reagents used:

- Peptone dilution and stomacher homogenization

Peptone dilution or PEPSU 408 is a dilution of 0.1% Peptone Bacteriological (Oxoid LP0037) and 0.8% of NaCl (Merck 1.06404) with a pH of 7.0 ± 0.2. PEPSU was made following the guideline of LAB 7504/2 (Evira) and was used to dilute the milk samples to a 10^{-1} dilution. PEPSU was stored in cold at 4^{o}C and was left to warm up to room temperature before its use. In order to get the dilution of 10^{-1}, a portion of 10ml of milk sample was added in a sterile Stomacher-400 bag by a 10 ml capacity plastic sterile pipette (Cellstar/greiner bio-one). Stomacher homogenization was used to mix the milk sample with 90 ml PEPSU. The mixture time was 1 min.

- Dilushaker II and Dilucup MRD 0.9 ml

Dilucup (LabRobot Products AB-Sweden) is a buffered sterile solution with 0.85% NaCl, 0.1% peptone and pure analytical grade water which is adjusted to pH of 7,0 ± 0,2 according to EN ISO 6887-1:1999. A portion of 1 ml from the 10^{-1} milk dilution was added in a Dilucup of 9 ml, with a pipette of 1000 μl, in order to get a dilution of 10^{-2}. The dilution continued until the 10^{-4} dilution was made. After Dilucups were placed on Dilushaker II and were shaken for couple of seconds.

- Plates and plate count agar (PCA)

The method used is horizontal enumeration of microorganisms, colony-count technique at 30°C, based on the ISO 4833:2003. According to LAB 7626/1 (Evira) instructions, PCA with pH of 7.0 ± 0.2 was made of 5.0 g casein-peptone, 2.5 g yeast extract, 1.0 g glucose. An amount of 14.0 g agar was sterilized in autoclave at 121°C for 15 min. PCA was in solid form when 200 ml stored in bottles at 4°C. Before PCA's use, bottles were boiled for 30 min, according to the practice LAB 702 (Evira), and left to the heat incubator at 45^{o}Cfor 30 min. With a 1000μl pipette, 1ml of diluted and undiluted milks samples were added in the plates and a portion of approximately 25-30 ml of PCA were added in each plate. After the addition of the PCA the plates were shaken according to the guidance of LAB 702 (Evira) and left to cool down.

- Incubator

Plates were left in the incubator at 30^{o}C for 72 hours.

- Equation of total microbial flora

According to the guidance of LAB 703/3 (Evira):

$$N = \frac{C_1 + C_2 + \ldots + C_n}{n_1 x\ V_1 + n_2 x\ V_2 \ldots + n_3 x\ V_n}$$

$C_1 + C_2 + .. + C_n$= the number of colonies calculated from each plate

n_1 = the calculated number of colonies from the first dilution's parallel samples

n_2 = the calculated number of colonies from the second dilution's parallel samples

V_1 = the first colony count corresponding to the sample volume (the original sample)

V_2 =the second colony count corresponding to the sample volume (the original sample)

3.3 Results

3.3.1 ALP activities in commercial products

ALP activities in commercial Finnish hard cheeses

Commercial brands of Finnish hard cheeses and their estimated ALP values are shown in the Table 9. All pasteurised cheeses, independent the cheese type, had ALP values less than 10 mU/l and were thus considered as negative in ALP. The mean ALP values of Emmental cheeses made from thermised milk ranged from <10 to 1403 mU/g. The thermised cheeses that had ALP activity 22 and below 10 mU/l probably were incorrectly considered as thermised. The information about the heat-treatment giving on the package might have not been accurate and the milk was treated at temperatures close to pasteurisation. The lowest ALP activity had Emmental cheese with 15 % fat content while Emmental cheese with fat content 31 % and maturity period 9 months had the highest ALP values. High fat content and processes during cheese maturity seemed to contribute to high ALP levels.

Table 9. ALP levels of Finnish hard cheeses made from pasteurised (P) or thermised milk (T).

Groups of cheeses	Fat (%)	Heat-treatment	ALP (mU/g)	Total ALP[a]
Gouda type/medium fat				
Edam Valio	24	P	<10	-
Edam Valio 17%	17	P	<10	-
Gouda Salaneuvos 17%	17	P	<10	-
Gouda type/High fat				
Gouda Salaneuvos	28	P	<10	-
Gouda Musta Salaneuvos	30	P	<10	-
Edam ValioTäyteläinen	28	P	<10	-
Emmental type/high fat				
Emmental sinileima/Blåstämplad, matured for 3 months	30	T	<10	-
Emmental punaleima/Rödstämplad	30	T	22	+
Musta leima Emmental	31	T	<10	-
Sinileima Emmental Kuusamosta, matured for 3months	30	T	680	+
Emmental Mustaleima, aito, hienostunut maku/ Svartstämplad	29	T	397/186	+
Mustaleima Emmental Kuusamosta, matured for 9months	31	T	715/1403	+
Polar Valio täyteläinen	28	P	<10	-
Valio Ritari	32	P	<10	-
Emmental type/medium fat				
Emmental punaleima/Rödstämplad 17%	17	P	<10	-
Suomalainen mieto Emmental koko perheelle 17 %	17	P	<10	-
Suomalainen Emmental Kuusamosta, 17%	17	P	<10	-
Kevyt Emmental 15 %, Mustaleima, aito, hienpostunut maku	15	*T	124	+
Polar Valio, 15%, Alkuperäinen	15	P	<10	-
Low fat				
Edam Valio kevyt 9%	9	P	<10	-
Polar Valio, kevyt 5%	5	P	<10	-
Polar Valio, 10%, Sydänystävä	10	P	<10	-

a: ALP result is considered as negative when found less than 10mU /g
(-): negative ALP result
(+): positive ALP result
Heat-treatment: data from the package
* Heat-treatment: data from the producer

ALP activities of commercial milks

ALP activities of commercial milks that were treated at different heat-treatments are shown in Table 10. High pasteurised milks had negative ALP values and pasteurised milks mean values ranging from 22.5 to 30.8 mU/l. All the pasteurised and high pasteurised milks were well below the acceptable ALP value. Both treatments gave ALP levels below 350 mU/l, which is the maximum legal activity for milks characterized as pasteurised (EU-RL 2011). The ALP values of UHT were high and some exceeded the 350 mU/l. The UHT milk that had the highest activity was the one stored for longer period. The UHT milks were sterile from microbes with zero total microbial flora which we expect from the definition of UHT milk (Walstra et al. 2006). Hence the reason for ALP activity raise during extend storage period was not the microbial but ALP reactivation.

Table 10. ALP activity of full-fat milk products found on the markets. Values of ALP below10 mU/l are considered as negative.

Commercial milk product	Stored (days)	Days to expire	Heat-treatment*	ALP (mean± SD)
Valio, HYLA	83	5	UHT	1607±1.7
Arla, Ingman , low-lactose	-	64	UHT	500,15±8.25
Rainbow, lactose-free	-	36	UHT	349±25.6
Rainbow , low-lactose	-	61	UHT	141,6±0.0
Valio, HYLA	35	55	UHT	123±5.3
Rainbow, milk drink, lactose-free	14	7	HP	<10
Valio, lactose-free, milk drink	16	4	HP	<10
Arla, Ingman	4	4	P	30.8±2.1
Valio Hiljamaito, traditional, unhomogenized	5	4	P	22.5±0.0

*****P: Pasteurised
*HP: High pasteurisation
* UHT: Ultra high temperature
Milks and milk drinks: 3-3.5 %fat
Vanhanajan, unhomogenised: 4.1-4.4% fat
(-): not measured

3.3.2 Inactivation of ALP

Raw milk pasteurized in a continuous way by heat-exchanger

The samples A and B were two different lorry milks both pasteurised at 75°C for 15 sec. The inactivation of ALP activity (mU/l) and the reduction of total microbial flora (cfu/ml) were recorded after the pasteurisation of both samples.

ALP values before and after samples A and B were pasteurised are depicted in the Figure 8. The activities of both samples reduced significantly after the treatment from 100 thousands in the raw milks to decades in the pasteurised. For sample A the reduction was up to 99.99% and for sample B up to 99.98%.

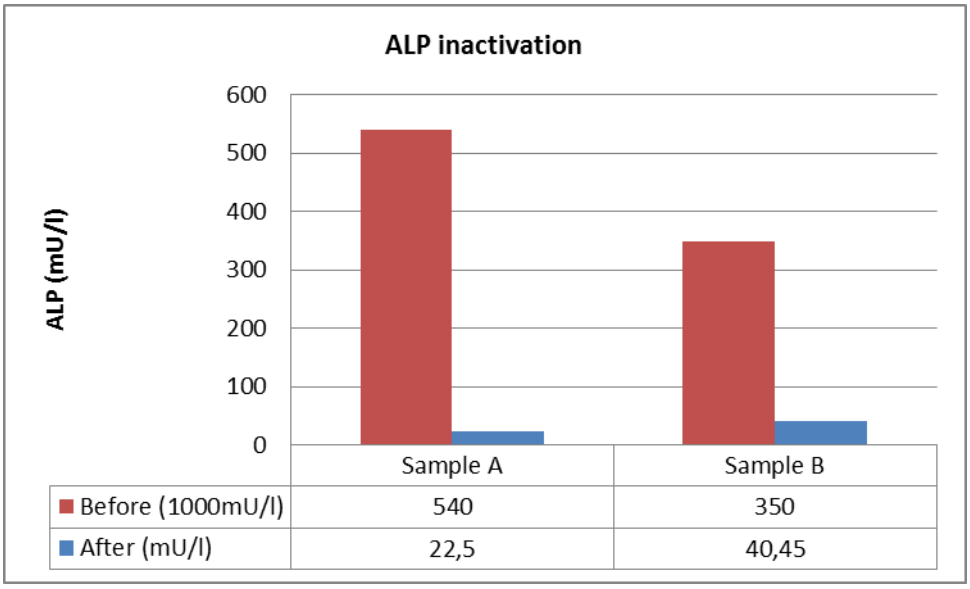

Figure 8. ALP values of sample A and B before (left column) and after (right column) been pasteurized at 75°C for 15 sec.

The total microbial flora of raw milks had values of 10 thousands and reduced to hundreds after pasteurisation. The reduction of total microbial flora was up to 97% and 98% for the samples A and B respectively (Figure 9).

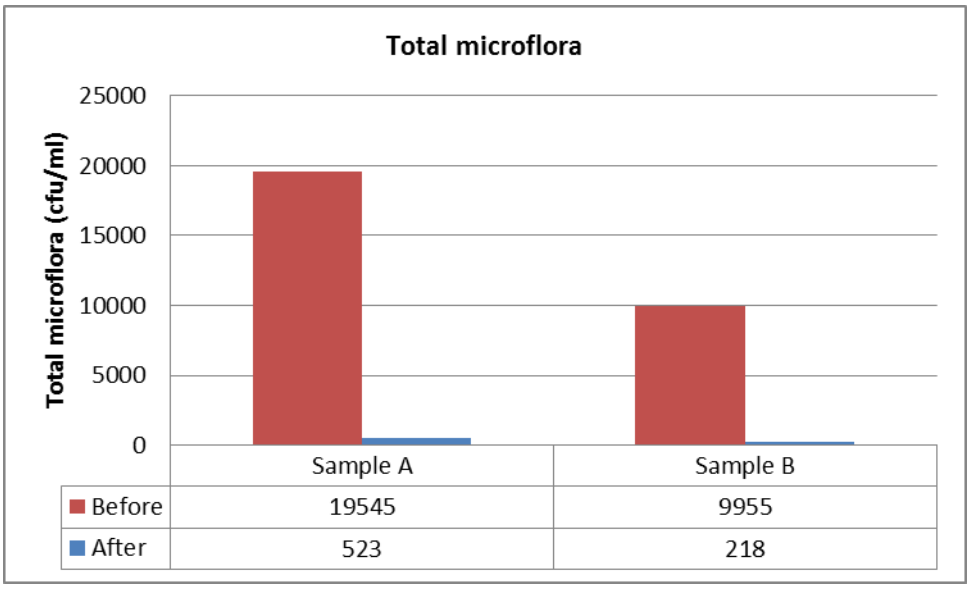

Figure 9. Total microbial flora of samples A and B, before (left column) and after (right column) pasteurisation at 75°C for 15 sec.

LTLT treatment in water bath

The samples C and D were different raw farm milks. Both samples were heat-treated in a water bath at different temperatures for 30min. The inactivation of ALP activity (mU/l) and the reduction of total microbial flora (cfu/ml) were recorded after the treatment of both samples.

Raw milk sample C was treated at 58°C for 30 min. The comparison of ALP activities before and after the treatment showed a decrease of ALP (1000 mU/l) activity up to 61.87 % and a simultaneous reduction of total microbial flora (1000 cfu/ml) up to 39 % (Figure 10).

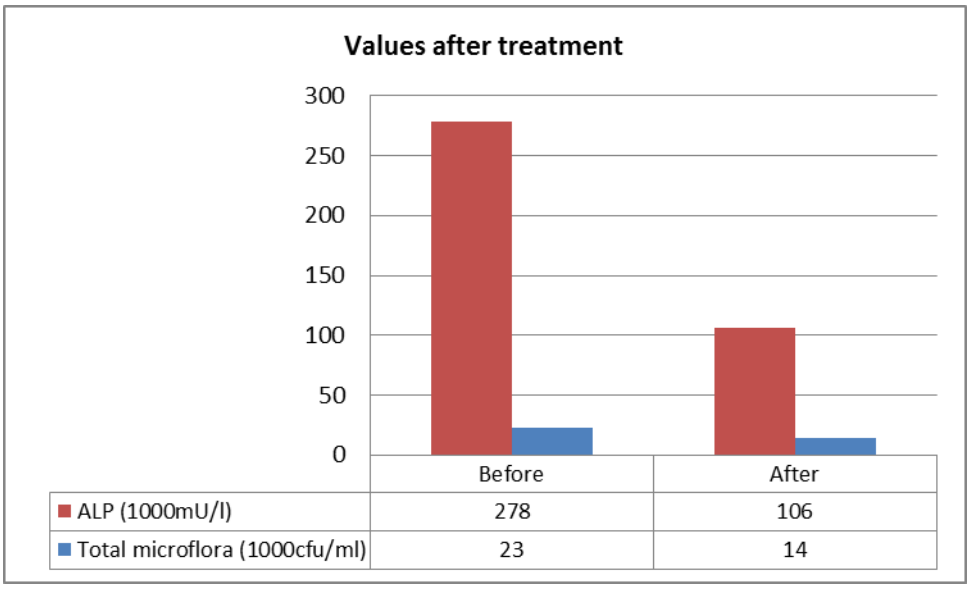

Figure 10. ALP values (left column) and total microbial flora (right column) before and after the heat-treatment of raw sample C at 58°C for 30 min.

The sample D with initial ALP activity x mU/l and initial total microbial flora y cfu/ml was treated at 60°C and 63°C for 30 min. The initial ALP levels and total microbial flora were decreased after both heat-treatments with higher drops performed in higher temperatures. As shown in Figure 11, the ‘’x’’ ALP activity of sample D reduced 60.6 % and ‘’y’’ total microbial flora 55 % more when the temperature was raised from 60°C to 63°C.

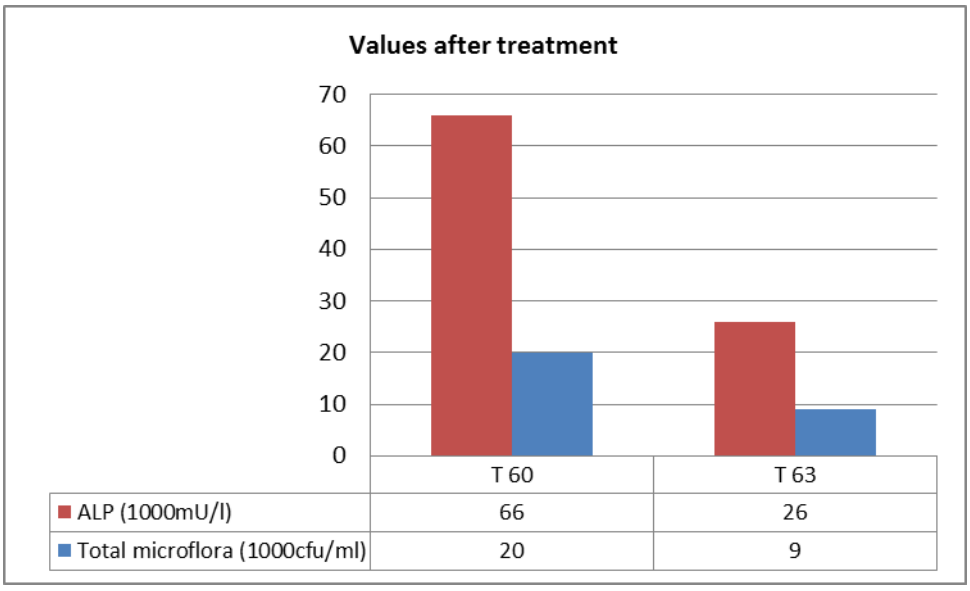

Figure 11. ALP activity and total microbial flora reduction after sample D was treated at 63°C and 60°C for 30 min.

Different farm raw milk samples E, F and G were heat-treated at 59, 61 and 74.5°C for 1h respectively. The raw and treated samples were analyzed for their ALP and total microbial flora before stored in cold at 4°C. ALP activities decreased up to 86, 99.93 and 99.99%, for samples treated at 59, 61 and 74.5°C temperatures respectively (Figure 12).

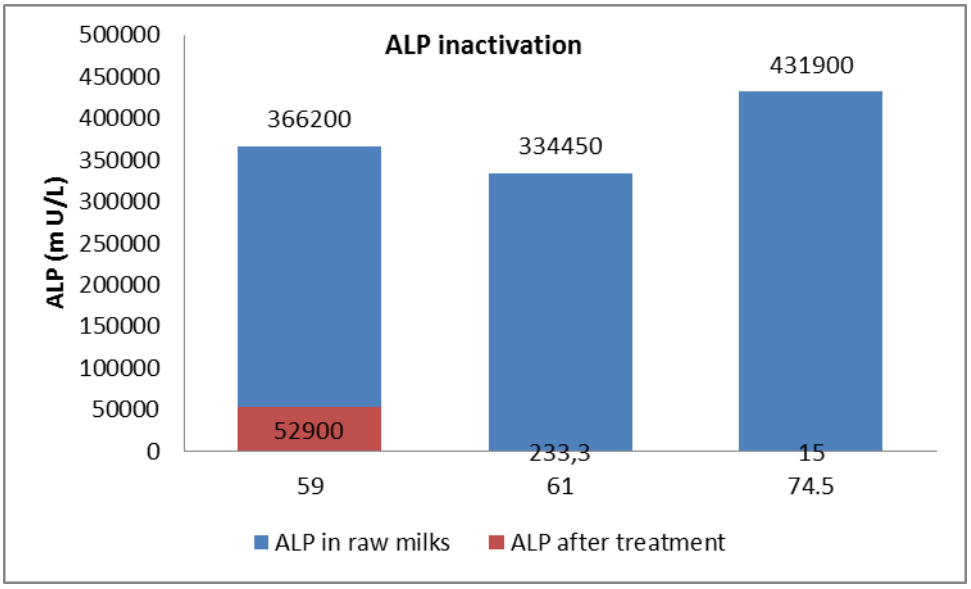

Figure 12. ALP values before and after samples E, F and G were heat- treated at 59, 61 and 74.5°C for 1h respectively.

As depicted in Figure 13, total microbial flora of raw milk samples E, G and F reduced up to 98.98, 99 and 95.6 % after been treated at 59, 74.5 and 61°C for 1h respectively.

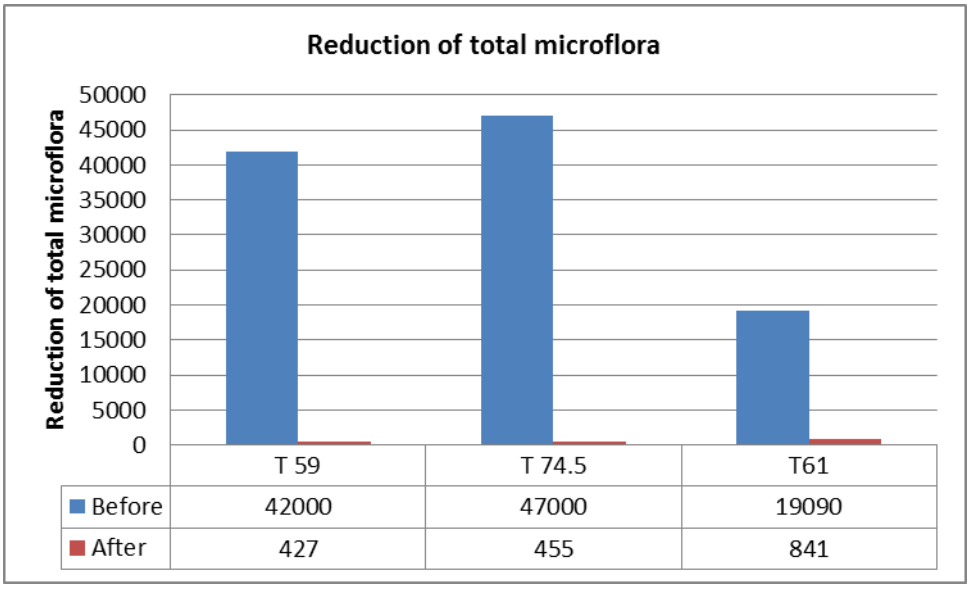

Figure 13. Total microbial flora of samples E, G and F before (left column) and after been heat-treated at 59, 74.5 and 61°C for 1h respectively (right column).

3.3.3 ALP reactivation

Raw milk pasteurized in a continuous way by heat-exchanger

The raw samples A and B and their pasteurised portion (75^{o}C for 15 sec) were both stored in cold at 4^{o}C after the heat-treatment. ALP activity and total microbial flora were recorded by time in days, with day zero being the first day that raw milk and pasteurised milk were tested before their storage at 4^{o}C.

ALP activity levels of pasteurised milks A and B during storage are shown in Figure 14. In the general picture, the mean enzyme activities for both samples remained practically stable for the first 6 days of storage. After 6 days the values of sample B increased 25%.

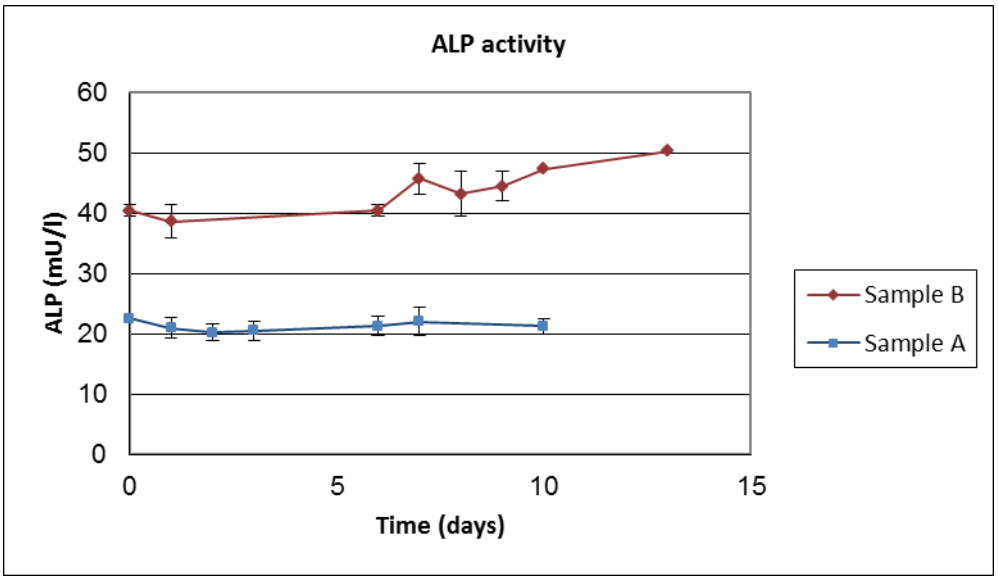

Figure 14. The mean ALP values in pasteurised milk samples A and B during storage in cold for 10 and 13 days respectively.

ALP activity increased during cold storage of raw milk sample A. The increase was more profound after 6 storage days indicating a similar ALP reactivation behavior with that of the pasteurised samples (Figure 14). ALP levels increased up to 3% from day 6 to day 10 in storage (Figure 15).

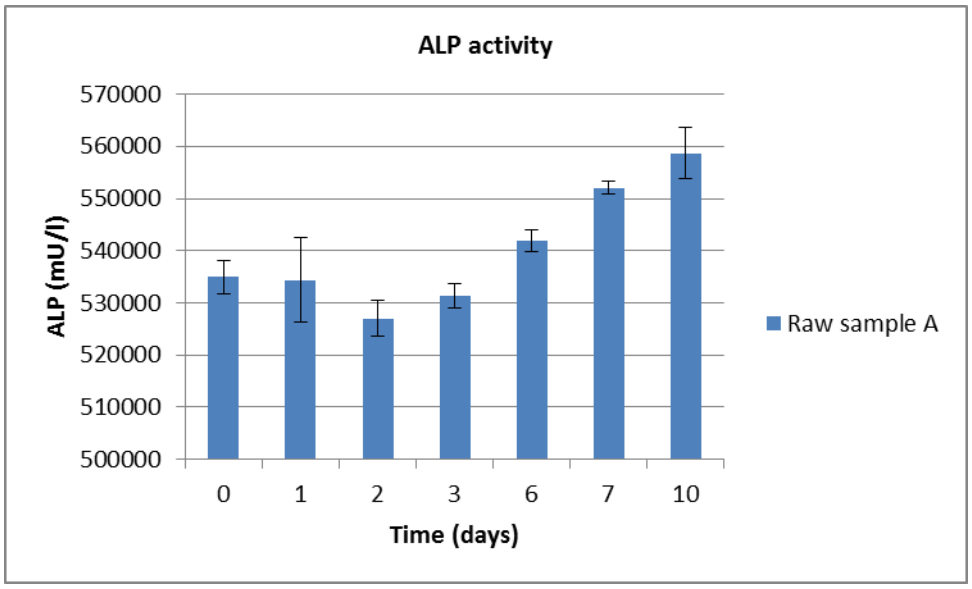

Figure 15. ALP values of raw milk sample A stored in cold for 10 days.

The microbial flora of raw milk A increased significantly after 1 day of storage (Figure 16) while the initial microbial flora of the pasteurised milk was 523cfu/ml and stayed practically stable during the 10 days of storage.

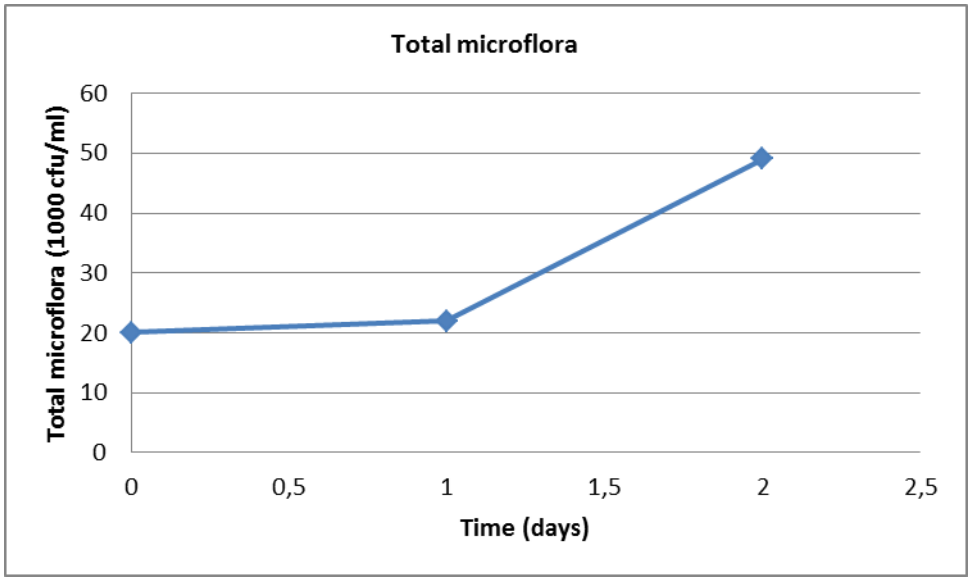

Figure 16. Total microbial flora of raw sample A stored in cold for 2 days.

LTLT treatments in water bath

The raw and heat-treated samples C and D were stored in cold at 4°C. ALP activity and total microbial flora were recorded by time in days with day 0 being the day of the treatment and the day that samples were transported to cold.

The treated sample C (58°C for 30 min), had mean ALP activity 106×10^3mU/l which remained stable during 4 days of storage, while ALP increased for both treated samples D (60°C and 63°C for 30 min) during the first day of storage at 4°C. When the sample D was treated at 63°C, ALP activity showed a higher reactivation than the same sample treated at 60°C (Table 11).

Table 11. ALP activities of raw sample D after been treated at 60°C and 63°C for 30 min and stored at 4°C for 2 days.

Time (days)	ALP (mU/l)	
	63°C	60°C
1	26000	66000
2	29000	70000
Increase (%)	11.54	6

Raw milk samples E, F and G and their heat-treated samples for 1h at 59, 61 and 74.5°C temperatures respectively were tested for their ALP activity and total microbial flora progress during storage in cold at 4°C.

ALP activity values, of treated samples E and F stored in cold are depicted in Figure 17. Both treated samples showed an increase of ALP levels after 6 days of storage. Sample F treated at 61°C had overall a higher and quicker reactivation than sample E treated at 59°C. ALP of treated sample F increased up to 588% while treated sample E up to 6.4% during 9 days of storage in cold.

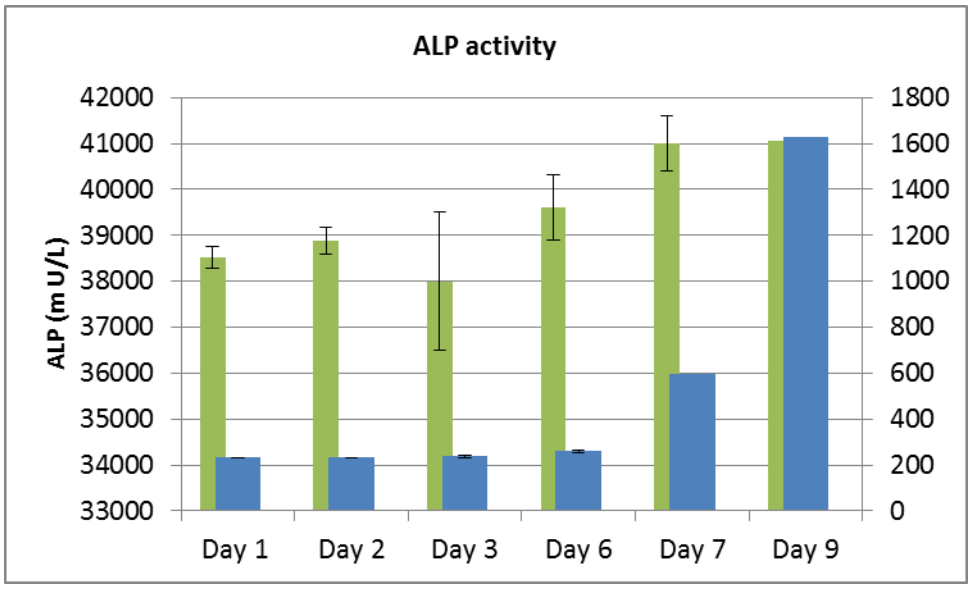

Figure 17. ALP reactivation of raw sample E (left column) and F (right column) after treated at 59 and 61°C respectively and stored 9 days in cold.

The progress of total microbial flora of treated samples E and F was followed during 7 storage days in cold and is depicted in Figure 18. The sample E treated at 59°C had an earlier and higher increase of total microbial flora than sample F treated at 61°C. The increase in total microbial flora (see Figure 18) and the ALP reactivation (see Figure 17) did not increase simultaneously.

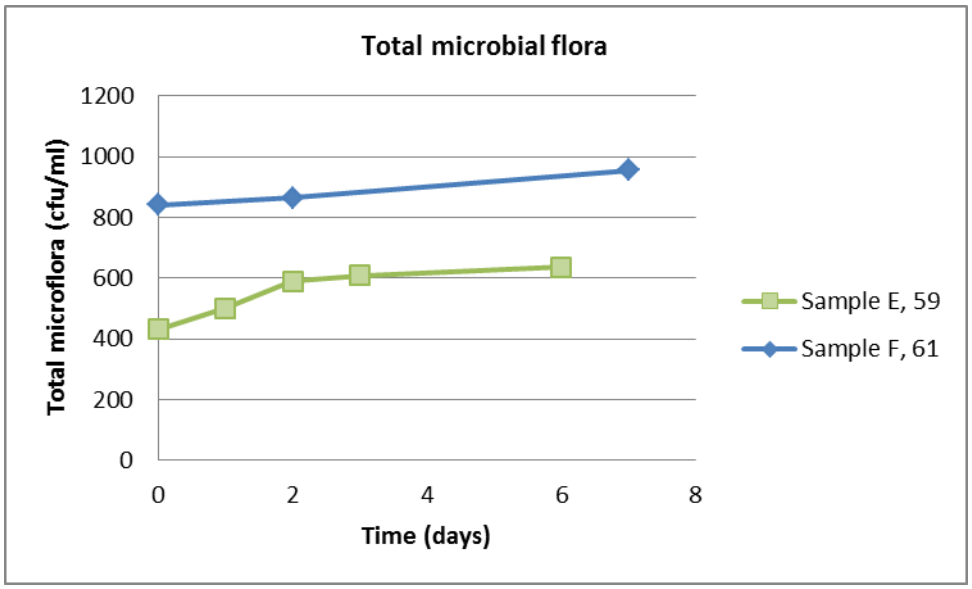

Figure 18. Total microbial flora of raw sample E and F after treated at 59 and 61°C respectively and stored in cold for 7 days.

Comparing ALP values of treated sample G heated at 74.5°C (see Figure19) to the values in Figure 17 it can be seen that ALP values of sample G increased more and quicker already during the first days of storage. The initial ALP after treatment was 15 mU/l and increased up to 12.5% during the first 2 days of storage. The reactivation (R) of ALP thus was higher and quicker at the samples treated at higher temperatures: $R_{74.5}>R_{61}>R_{59}$.

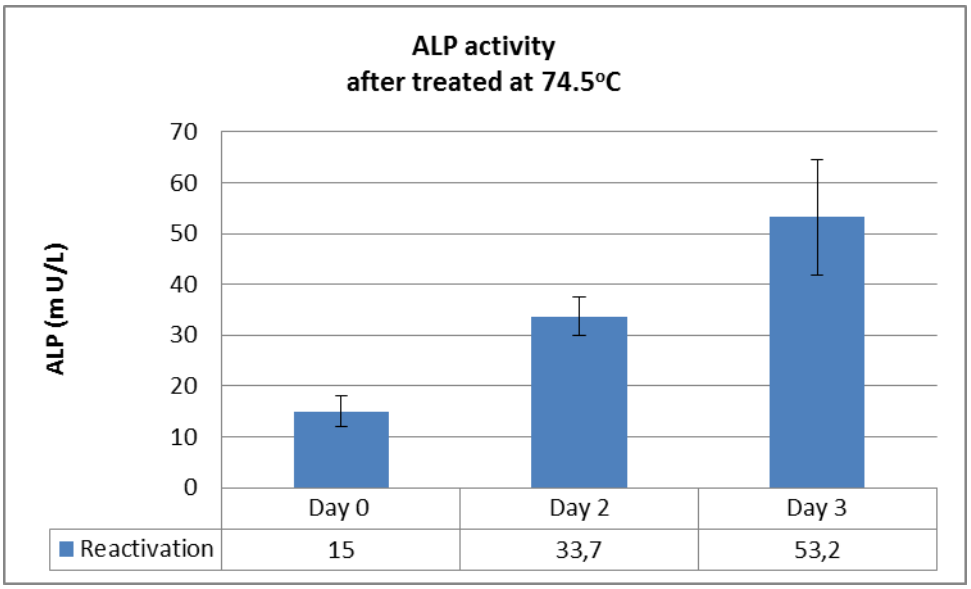

Figure 19. ALP reactivation of sample G after been treated at 74.5°C for 1h and stored in cold for 3days.

HTST treatment in water bath

Milk was added in a flask of 50ml and inserted in a water bath when it was warmed up to 95°C. The incubation time was 5min. The initial ALP activity after heat-treatment was 10mU/L and increased rapidly during the first 2 days of storage. The rest days in storage ALP activity increased slower and slighter. ALP activity increased up to 153% from day 0 to day 8 and 140% of the increase happened during the first 2 days of storage (Figure 20).

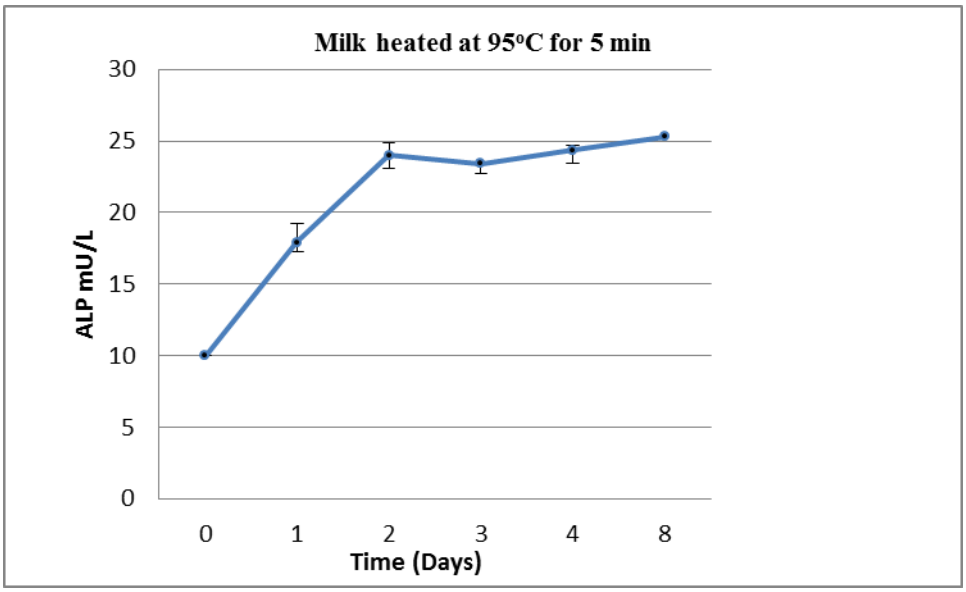

Figure 20. ALP values of milk sample heat-treated in water-bath at 95°C for 5min and stored in cold for 8 days.

3.3.4 ALP reactivation-storage temperature

The milk sample F was heat-treated in a water bath at 61°C for 1h and divided into two portions. The two portions were separately stored in bottles in cold, at 4°C and at room temperature, at 24°C. ALP activities of the two treated milk samples F during storage are depicted in Figure 21.

When the sample was stored at room temperature the increase of ALP level was quicker and higher than when stored in cold. During the first day of storage, ALP increased 5.3% and 0.96% when stored at room temperature and in cold respectively.

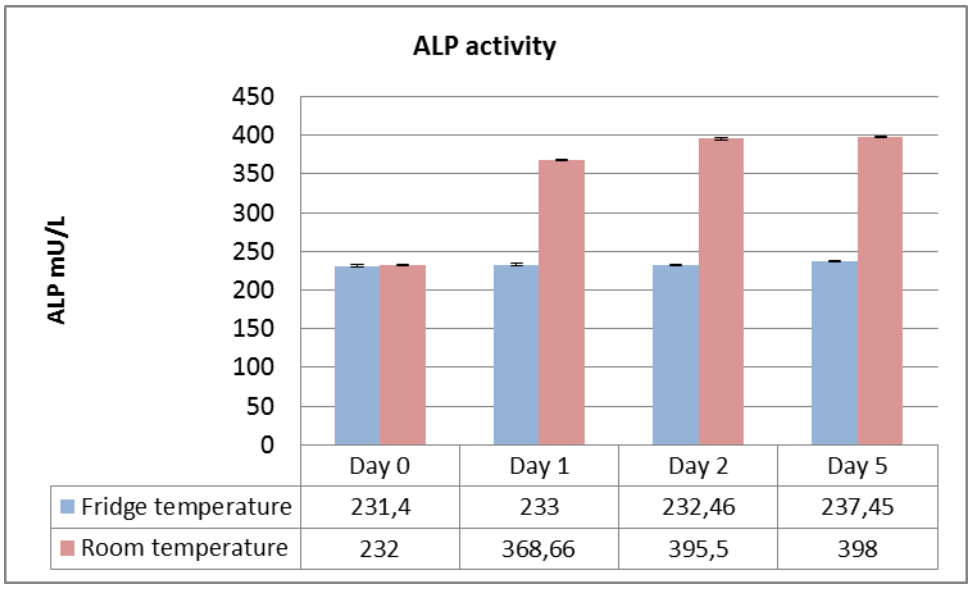

Figure 21. ALP values of milk sample F treated at 61°C for 1h and stored in cold (left column) and room temperature (right column).

Total microbial curves are presented in Figure 22. The microbial increase was 121% after 1day of storage at 24°C with a simultaneous sharp increase in ALP levels (see Figure 21). During the second day of storage the total microbial flora increased 503% with a slighter raise in ALP levels (see Figure 21).

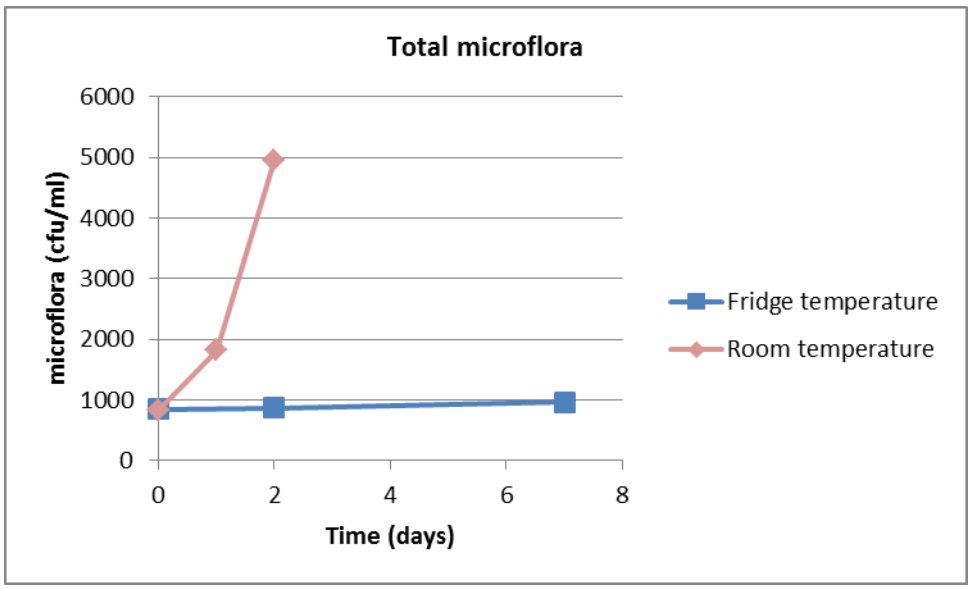

Figure 22. Total microbial flora of sample F treated at 61°C for 1h and stored at fridge and room temperature.

Samples E and G heated at 59^{o}C and 74.5^{o}C for 1h respectively were followed for their ALP values during storage at 4^{o}C and 24^{o}C. The values of ALP activity during storage at 24^{o}C fluctuated a lot day by day giving unfaithful results. The samples had to be well agitated before the analysis and small pieces were present the last storage days. This feature did not allow the continuing of the analysis as the results would not be reliable any more.

3.3.5 ALP's activity in separated milk

Un-homogenised full-fat milk of 500ml volume was let to rest and cream. The separated cream was diluted in phosphate buffer with volume equal to this of full-fat milk before separation. ALP activity measured in diluted cream was less than 10mU/l while the activity in skim milk was 19.5mU/l.

Table 12. ALP activity of unhomogenised whole milk before and after separation.

ALP (mU/l)		
	After seperation	
Whole milk	Cream	Skim milk
22,5	<10	19,5

3.3.6 Freezing stability of ALP

The freezing stability of ALP was tested in order to estimate its appropriateness for post-storing analysis. ALP activities of milk samples were estimated before and after milk samples were frozen at -79^{o}C (Table 15). The samples were first treated under different time-temperature relationships and after stored in the freezer for a different time period. The highest ALP activity drop was performed from samples treated at74.5^{o}C and 61^{o}C with a concurrent long storage life up to 2 months. The sample treated at the lowest temperature at 59^{o}C, for 3 weeks had the lowest ALP drop. The strength of sample treatment and the storage time seemed to influence the percentage of ALP decrease during freeze storage.

Table 13. ALP activity in mU/l before and after being freezed at -79°C for a period of days. Sample is represented by a letter and number. The letter represents the specific heat-treatment applied and the number the days that the sample was stored at 4°C after the treatment. Values calculated in mean ± SD.

Sample	Days in freezer	Before freezing	After freezing	Mean ALP decrease (%)
A7/UT	4	552100±2300	578100±7600	-4.70
A1	8	21±2.3	19.3±0.0	+8.10
A0	9	22.5±0.0	20.4±3.2	+9.30
UT0	14	537150±5230	567500±1100	+5.65
B7	20	41000±600	40900±700	+0.24
C7	25	593.5±4.9	197±19.4	+66.8
A0	49	40.45±0.95	36±0.0	+11.00
UT0	49	348900±6700	308000±3200	+11.72
D2/UT	62	369130±3672	317650±9150	+13.94
D1/UT	63	421450±6400	318400±10600	+32.36
D0	64	15±1.75	<10	>+33.33

A: 75°C for 15seconds
B: 59°C for 1hour
C: 61°C for 1hour
D: 74,5°C for 1hour
UT: Untreated

3.4 Discussion

3.4.1 ALP activities of commercial Finnish hard cheeses

Cheeses made from pasteurised milk had all values <10mU/g, which supplement the specifications of EU-RL (2011) and agree with the claim that ALP is inactivated after pasteurisation (Aschaffenburg and Mullen 1949). The following thermised cheeses had positive alkaline phosphatase values: Mustaleima Emmental 29%, light Emmental 15%, Emmental Mustaleima from Kuusamo 31% and Sinileima Emmental 30%. According to Aschaffenburg and Mullen (1949), ALP is inactivated after pasteurisation; hence milder than pasteurisation temperatures, like thermisation, do not inactivate completely the ALP. The ALP values of cheeses made from thermised milk ranged from 124 to 1403 mU/g (Table 9). These results were similar with the study results in Swiss and Slovenian thermised cheeses with mean enzyme values between 36 to 1396 mU/g and 411 mU/g respectively (EU-RL 2011; Hodošček 2012). The Emmental cheese that had the lowest enzyme activity (124 mU/g) had fat content 15 %, while the cheese with the highest enzyme activity (1403 mU/g) had fat content 31 %. These results came with agreement with the study of Painter and Bradley (1997) who claimed that higher milk fat content leads to higher ALP level after heat-treatment as the fat-globule membrane protects the enzyme. The cheese that had the highest enzyme activity was also the cheese with the longest maturity period which may play a role to this high value. Emmental cheese belongs to Swiss type of cheeses. Ziobro (2001) and Rosenthal et al. (1996) studied this type of cheeses and discovered that a number of microbes that is used for their production, produce ALP of microbial origin which contributes to the total ALP activity. Hence the higher ALP levels in Emmental compared to other cheeses may be due to ALP derived from microbes. During cheese maturity microbes produce enzymes which contribute to specific texture-flavor characteristics. Additionally, Emmental mustaleima Kuusamosta had a long storage time and high ALP values may be ALP reactivation that is claimed to happen during storage (Lyster and Aschaffenburg 1962; Murthy et al. 1976).

3.4.2 ALP activities of commercial milks

All pasteurised and high pasteurised milks, fulfilled the requirements for successful and well pasteurised milk with ALP values less than10 mU/l and 50 mU/l respectively (VAM 2002; ISO and IDF 2010). The UHT milks had high ALP values with some of them exceeding the 350 mU/l which is the legal negative activity limit (ISO 2006; EU-RL 2011). These results compromise with previous studies which claim that ALP is slightly inactivated after UHT treatment (Chandan and Kilara 2011). A possible explanation is the inactivation of ALP inhibitors at higher than pasteurisation temperatures or the formation of factors that contribute to the reformation of the denatured enzyme. Furthermore, the absence of microbial flora in UHT milk indicates that the profound high values of ALP were not related to a possible microbial growth. Hence the high ALP levels were probably barely the reason of the reactivation of the enzyme. The UHT milks that were stored for a longer period had a higher ALP value which is explained by the reactivation of ALP which was performed for longer time (Lyster and Aschaffenburg 1962). Additional factors, that may played a role to high or low ALP levels is the homogenization and enzymes added to hydrolysis the lactose. Murthy et al. (1976) studied the effects of homogenization in milk manufacture and came into the conclusion that it decreases ALP reactivation rate when applied before heating.

3.4.3 ALP inactivation

Raw milk pasteurised in a continuous way by heat-exchanger

Raw milk samples A and B had both ALP activity values of 100 thousands. ALP values after pasteurisation reduced to decades, a reduction up to 99.99% and 99.98% for sample A and B respectively (Figure 8). The total microbial flora of raw sample A and B reduced up to 97% and 98% after pasteurisation respectively (Figure 9). Both covered the EU standards that require a bacteria count for good quality raw milk < 100000cfu/ml and for pasteurised <30000cfu/ml. The results agree with the study of Rankin et al. (2010) who claimed that a similar bacterial reduction relative to ALP decrease covers the demands for successful pasteurisation.

Even though, the total microbial flora reduced more in pasteurised sample B the reduction in ALP was less. Painter and Bradley (1997) indicated that higher milk fat content leads to higher ALP level after heat-treatment as the fat-globule membrane protects the enzyme. A higher fat content may protected ALP and was the reason for a lower reduction of ALP levels in sample B.

LTLT treatment in water bath

Raw milk sample C had ALP activities and total microbial flora counts up to 100 and 10 thousands respectively. After treatment at 58^{o}C for 30 min, ALP values and microbial flora reduced up to 61.87 and 39% respectively (Figure 10). The inactivation of ALP was higher compared to the reduction of total microbial flora. This indicates that the temperature at 58^{o}C was not strong enough to kill the bacteria but it was enough to cause a significant drop in ALP levels.

The treated sample D showed a different percentage decrease of ALP and total microbial flora when treated at 60 and 63^{o}C for 30 min (Figure 12). A higher ALP drop and microbial decrease was observed when higher temperature (63^{o}C) was applied, which indicates the sensitiveness of ALP to temperatures higher than its optimal (38°C).

The raw milk samples E, F and G had initial ALP levels up to 100 thousands. After heat-treatment at 59, 61 and 74.5^{o}C for 1h the values dropped to 10 thousands, hundreds and decades respectively (Figure 12). These results showed a direct relation between ALP value drop and temperature increase. There was a similar effect of temperature to total microbial flora which reduced from 10 thousands to hundreds after all the treatments (Figure 13). The Figure 5 gives the inactivation curve of ALP and bacteria (Walstra et al. 2006)

3.4.4 ALP reactivation

Raw milk pasteurised in a continuous way by heat-exchanger

The pasteurised sample A, as shown in Figure 14, did not show any significant bacteria growth or ALP activity increase during 10 days of storage. This result indicates that

pasteurised milk products keep the qualifications that pasteurisation applies, throughout their storage until the expiration date. The total microbial flora of raw milk A, increased significantly after 1 day of storage at 4^{o}C (Figure 16). This was probably caused by growth of psychrotrophs which benefit from cold temperatures (Walstra et al. 2006). ALP activity did increase slightly just after storage day 6 (Figure 15) indicating an irrelevant relation between microbial growth and raise in ALP values.

ALP activity of milk sample B increased significantly just after day 6 (Figure 14), which may be the result of reactivation. This could be due to the higher fat content of the product which is claimed to protect ALP and contribute to its quicker reactivation (Painter and Bradley 1997). The increase of enzyme activity was profound after milk was stored in cold for a week. This means that for the period that pasteurised milks are stored in the markets ALP levels do not have the time to increase.

LTLT treatment in water bath

ALP activity of treated sample C (58^{o}C for 30 min) did not increase during 4 storage days, while sample D treated at 60^{o}C and 61^{o}C showed a raise in enzyme values by days of storage (see Table 11). Reactivation of ALP was seemed to be benefited from treatments at high temperatures. This is compatible with the results of Murthy et al. (1976) who observed a higher ALP reactivation when milk samples were heated in higher temperatures.

The samples E, F and G were all treated for 1h at 59 61 and 74.5^{o}C respectively. ALP activity and total microbial flora seemed to increase with a different speed during storage depending on the temperature applied during the treatment. As shown in Figure 17, both treated samples E and F showed an increase in ALP activity after 6 days storage in cold. This result is similar with the ALP activity values found in this study for sample A and B (see Figure 14). ALP activity drop for treated sample F (61^{o}C) was higher compared to treated sample E (59^{o}C). Though, ALPS' reactivation for F was higher and quicker reaching an increase up to 588% from day 6 to day 10, while treated sample E increased up to 6.4 % for the same storage period. The sample G had the highest drop in ALP activity after treated at 74.5^{o}C compared to the samples treated at 59 and 61^{o}C but reactivated even more and quicker than the samples treated in lower temperatures (Figure 19). These results

indicate that treatments at higher temperatures benefit the post-treatment reactivation of ALP activity. This observation agrees with the study that claim a profound increase in ALP activity during storage in UHT treated milks (AI 2003).

For sample E treated at 59^{o}C the total microbial flora showed a high and rapid increase after 1 day of storage (see Figure 18) while ALP activity for the same sample did not increase significantly for the first 6 days (see Figure 17). The sample F treated at 61^{o}C did show slower increase in total microbial flora with ALP increasing also after 6 storage days. ALP and total microbial flora for both samples E and F showed an increase (Figure17 and Figure18). Though, the reactivation of ALP was not simultaneous with total microbial flora indicating that ALP increase was irrelevant with microbial growth.

In conclusion, high temperatures contributed to a higher ALP reactivation. Freeman et al. (1968) observed also this phenomenon and suggested to buttering plants a pasteurisation condition with higher heat time and lower temperature and storage temperature of less than 1^{o}C in order to avoid ALP reactivation.

HTST treatment in water bath

The increase of ALP levels of sample treated at 95^{o}C for 5min was profound during the first days of the storage in cold (Figure 20). This rapid increase compared to the heat-treatments in lower temperatures is correlated with previous study. Murthy et al. (1976) studied the reactivation of the enzyme in liquid milk products heated at 87.8 to 121.1^{o}C for less than 1 s. The higher reactivation observed at milks that were heated at 121.1^{o}C. One possible explanation may be the inactivation of ALP inhibitors at treatments stronger than pasteurisation or the formation of factors that participate in the reactivation of ALP.

3.4.5 ALP reactivation-storage temperature

When treated sample F stored for 1 day at 24^{o}C, ALP levels increased 70 % while the increase when stored in cold was 0.8 %. ALP activity of treated sample F, showed a higher and quicker reactivation when stored at room temperature than in cold (Figure 21). The warm temperature seemed to benefit the reactivation of ALP. These outcomes agree with

the study of Wright and Tramer (1956) who observed a reactivation of ALP after been inactivated and incubated at 37^{o}C. Also, Rademacher and Hinrichs (2006) studied the reactivation of ALP after applying pressure-treatment and found that ALP did not reactivated when sample was stored in cold.

Total microbial flora increased significantly at room temperature as expected while it remained almost stable when stored in cold (Figure 22). Psychrotrops are killed during the heat-treatment and the thermophils are benefit from warm temperature (Walstra et al. 2006).

Samples E and G, heated at 59^{o}C and 74.5^{o}C for 1h respectively were followed for their ALP values during storage at 4^{o}C and 24^{o}C. The irregular fluctuation of ALP results at samples stored at 24^{o}C did not give trustful results, which were finally rejected. The milk samples were exposed to a prolonged heat-treatment which probably caused changes in the milk structure and denaturation of whey proteins. ALP was probably masked from some components and thus gave irrelevant ALP values.

3.4.6 ALP's activity in separated milk

After the milk separation, ALP activity was found in higher amounts in the separated milk than in cream (Table 12). This study result agrees with the study of Zittle and Della Monica (1950) that found a remarkable ALP activity in the separated milk after removing the fat globules. According to Morton (1953 and 1954) ALP in skim milk exists in microsomes which come from the milk fat globule membrane (MFGM) with 30-40 % of the enzyme absorbed on it. These results comes into objection to the study of Morton (1953) who claimed that ALP is harshly bound to lipoprotein nature insoluble particles and after cream separation ALP is concentrated to this phase (Kay and Graham 1933; Hansson et al. 1946). It can be concluded, that ALP enzyme is not bound firmly to MFGM and can easily remove to skim milk after agitation or shaking. Another reason may be that the amount of the enzyme bound on MFGM is actually lower than expected.

3.4.7 Freezing stability of ALP

According to the guidance of ISO 707|IDF 50:2008 the milk sample taken for chemical, physical, microbial or sensor analysis has to be representative and the alteration or damage of it during storage should be avoided. The freezing stability of ALP is of high immense of interest in order to estimate its suitability for post-stored analysis. ALP activity decreased during storage days. As shown in Table 13, the decrease of ALP activity was higher when the milk was heat-treated in a high temperature before storage. The samples seem to have a smaller ALP drop when stored for less than 20 days and exposed to a milder temperature treatment before the freeze-storage. The ALP resistance to freezing-damage is probably decreased the more days the sample is exposed to the low temperature while the heat-treatment that is applied to samples makes the enzyme more vulnerable to the damages.

4 CONCLUSIONS

The mean ALP levels of commercial Finnish cheeses were estimated in this study and compared to values from previous studies and their deviation from values considered as acceptable. The cheeses made from pasteurised milk had all enzyme values less than 10 mU/g, which is considered as negative. These negative ALP values are expected after pasteurisation and cover the suggestions of EU-RL (2011). The case that ALP activities did stay negative after maturity period and storage time, indicates that no increase of ALP was enhanced or took place. In this work, ALP values of Emmental cheeses made from thermised milk ranged from 124 to 1403 mU/g. These results came in agreement with study results in Swiss and Slovenian thermised cheeses with mean ALP values between 36 to 1396 mU/g and 411 mU/g respectively. Cheeses with higher fat content and longer maturity period had higher ALP values. Fat, as supported from previous studies, acts as a protector to the enzyme during heat-treatments. Additionally, proteolytic and other physiological changes during maturity seem to benefit ALP high levels which may be produced or reactivated during this period. Deviations of the expected mean ALP values in pasteurised and thermised cheeses may indicate a contamination with microbial ALP or unsuccessful heat-treatment.

Commercial full-fat milks and milk drinks depending on the heat-treatment applied to them showed a different ALP values range. All the high pasteurised milk drinks had negative ALP activities which are expected in successfully pasteurised milks. For pasteurised milks, the values ranged from 22.5 to 30.8 mU/l. These levels were below the 50 mU/l which is the mean acceptable value for well pasteurised milks. Differences in levels may be affected by homogenisation or enzymes added to hydrolysis lactose. UHT milks had significantly high ALP activities ranged from 123 to 1607 mU/l. High ALP levels in this type of cheeses was not of microbial origin as the total microbial flora of UHT milks was tested and confirmed to be sterilised. According to previous studies UHT treatment inactivates only slightly ALP, this is the reason for high levels after the treatment. ALP in UHT milks tends also to reactivate more. This can be explained by a possible inactivation of specific ALP inhibitors at temperatures higher than pasteurisation or the production during the treatment

of factors that benefit the formation of the active ALP. Furthermore, UHT milks are stored for long time which means longer period for reactivation.

All raw milk samples seemed to have mean ALP levels at around 350000 mU/l. The higher was the heat-treatment applied, the higher was the ALP activity drop with a simultaneous reduce of total microbial flora. A week seemed to be the period in which most of the products hold the reduced ALP levels after heat-treatment, before it started to increase significantly. In the general picture of ALP reactivation, there was no clear relation with microbial growth and ALP increase. There was a profound difference between ALP reactivation in samples treated at different temperatures. The higher the temperature in which sample was treated, the higher was the reactivation during storage. This result is explained with the same reasons for high ALP reactivation in UHT treated milks. The storage temperature seems to play role to the reactivation of ALP. The enzyme reactivated quicker and higher when stored at room temperature than in cold.

One of the scopes of this thesis was to study the freeze stability of ALP. This is a property very useful when the samples need to be stored before their analysis. Possible bacteria growths and enzyme reactivations at temperatures in cold may change significantly the initial values. In order to have representative samples for analysis, the milk samples can be stored at very low temperatures (-79^{o}C). The ALP resistance to freezing-damage is probably decreased the more days the sample is exposed to the low temperature, while the heat-treatment that is applied to samples makes the enzyme more vulnerable to the damages. Samples treated at low temperatures could be stored for up to two-three weeks, while samples treated at high temperatures could be stored for one week without a significant drop in ALP values.

ALP is indicated from previous studies to be bound on milk fat membrane globules (MFGM) and to be present in higher levels in milks of high fat content. This property has strengthened the assertion that after milk separation, ALP would be found in the cream part. In this work, ALP was concentrated to skim phase after cream separation. It can be concluded, that ALP enzyme is not bound firmly to MFGM and can easily remove to skim milk after agitation or shaking. Another reason may be that the amount of the enzyme

bound on MFGM is actually lower than expected. An unhomogenous milk sample may give false ALP activities if the sample is taken when the cream part seems to be seperated.

The reactivation property of ALP has been studied but there are still questions to be answered. An interesting topic that could be studied further, are the mechanisms that benefit the ALP reactivation after treatments at high temperatures.

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