UTILIZATION OF COMMERCIAL ADJUNCT CULTURE IN THE MANUFACTURE OF ULTRAFILTRATED WHITE CHEESE AND ITS EFFECTS ON THE CHEESE PROPERTIES

ULTRAFİLTRE BEYAZ PEYNİR ÜRETİMİNDE TİCARİ EK KÜLTÜR KULLANIMI VE BUNUN PEYNİR ÖZELLİKLERİNE ETKİSİ

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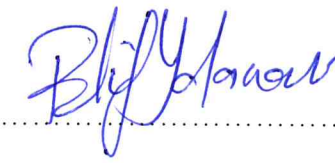
Member ..... 

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SATTAR EGHBALIAN

I dedicate this thesis to my Heavenly father,

Yaghoub EGHBALIAN

ABSTRACT

UTILIZATION OF COMMERCIAL ADJUNCT CULTURE IN THE MANUFACTURE OF ULTRAFILTRATED WHITE CHEESE AND ITS EFFECTS ON THE CHEESE PROPERTIES

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In this study, the effect of the commercial adjunct culture of CR-319 containing *Lactococcus lactis* subsp. *Lactis* and *Lactococcus lactis* subsp. *cremoris* on the microbiological, chemical, biochemical, textural and sensorial properties of the full-fat ultrafiltrated (UF) white cheese was investigated. UF cheese was manufactured by using normal starter culture (control cheese) or by the addition of adjunct culture of CR-319 in addition to starter culture (experimental cheese). Cheese samples were analysed during 120 days of ripening. It was found that addition of adjunct culture influenced all chemical composition, cheese yield, number of lactic acid bacteria, proteolysis, lipolysis, organic acid and sugar levels, volatile compositions, textural and sensorial characteristics of the cheese within different levels.

Biochemical changes were more evident after the 60^{th} day of ripening. Depending on high aminopeptidase activity of CR-319 commercial adjunct culture, secondary proteolysis was found at higher level, especially on the 120^{th} days of ripening. However, primary proteolysis was also affected at experimental cheeses. Those results were supported by urea-PAGE and RP-HPLC. The total free fatty acid level was also affected by adjunct addition. But, its effect was limited ($P > 0.05$). In experimental cheeses, residual lactose

level (~1.54%) was higher than the control cheese while residual galactose level (~0.15%) was lower. It has been found that the adjunct culture containing UF cheese samples had higher level of ketones such as diacetyl, 2,3 pentanedione, acetoin than the control cheeses. Sensorial properties of experimental cheeses were positively affected by adjunct culture and total acceptability was higher than the control cheeses. The use of adjunct culture slightly improved flavour intensity and decreased bitterness. Overall, CR-319 adjunct culture enhanced proteolysis, lipolysis, taste and aroma characteristics which are important for balanced flavour, whilst bitterness was reduced. It leads to partially much more creamy-like texture in UF cheeses. But, those effects were limited until the 60^{th} or the 90^{th} day of ripening.

Keywords: Ultrafiltrated white cheese, adjunct culture, bitterness, proteolysis, lipolysis, organic acid, volatile flavour compounds

ÖZET

ULTRAFİLTRE BEYAZ PEYNİR ÜRETİMİNDE TİCARİ EK KÜLTÜR KULLANIMI VE BUNUN PEYNİR ÖZELLİKLERİNE ETKİSİ

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Bu çalışmada, *Lactococcus lactis* subsp. *lactis* ve *Lactococcus lactis* subsp. *cremoris* içeren CR-319 ticari kültürün tam yağlı ultrafiltre (UF) beyaz peynirin mikrobiyal, kimyasal, biyokimyasal, tekstürel ve duyusal özellikleri üzerine etkisi incelenmiştir. UF peynir starter kültür (kontrol peyniri) veya starter kültüre ilave olarak CR-319 ek kültürü kullanılarak üretilmiştir (deneme peyniri). Peynir örnekleri 120 günlük olgunlaşma süresince analiz edilmiştir. Ek kültür ilavesinin tüm kimyasal bileşimi, peynir verimini, laktik asit bakterilerinin sayısını, proteolizi, lipolizi, organik asit ve şeker miktarlarını, uçucu bileşenleri, tekstürel ve duyusal karakteristikleri değişik düzeylerde etkilediği bulunmuştur.

Biyokimyasal değişiklikler olgunlaşmanın 60. günde belirgin hale gelmiştir. Ticari kültür CR-319'un yüksek aminopeptidaz aktivitesine bağlı olarak, özellikle olgunlaşmanın 120. gününde ikincil proteolizin yüksek seviyede olduğu tespit edilmiştir. Bununla beraber, deneysel peynirlerde birincil proteolizde etkilenmiştir. Bu sonuçlar üre-PAGE ve RP-HPLC ile de desteklenmiştir. Toplam serbest yağ asidi miktarı da ek kültür ilavesinden etkilenmiştir. Fakat bu etki sınırlı düzeyde olmuştur ($P > 0.05$). Deney peynirlerinde kalıntı laktoz seviyesi (~%1.54) kontrol peynirlerinden yüksek bulunurken kalıntı galaktoz

seviyesi (~%0.15) kontrolden düşük bulunmuştur. Ek kültür içeren UF peynir örneklerinin diasetil, 2,3-pentadion, asetoin gibi ketonları daha yüksek düzeylerde içerdiği bulunmuştur. Deneme peynirlerinin duyusal özellikleri ek kültür ilavesi ile pozitif yönde etkilenmiştir ve bu peynirlerde toplam kabul edilebilirlik, kontrol peynirlerine göre daha yüksek düzeyde bulunmuştur. Ek kültür kullanımı aroma intensitesinde kısmi düzelme sağlarken acılaşmayı azaltmıştır. Sonuç olarak, CR-319 ek kültürü dengeli aroma için önemli olan proteolizi, lipolizi, tat ve koku karakteristiklerini iyileştirirken acılaşmayı azaltmıştır. UF peynirlerinde kısmen daha kremimsi bir yapı oluşturmuştur. Ancak, bu etkiler olgunlaşmanın 60. veya 90. gününe kadar sınırlı düzeydedir.

Anahtar kelimeler: Ultrafiltre beyaz peynir, ek kültür, acılık, proteoliz, lipoliz, organik asit, uçucu aroma bileşikleri

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SYMBOLS AND ABBREVIATIONS

Symbols

α	Alpha
β	Beta
γ	Gamma
δ	Delta

Abbreviations

ANOVA	Analysis of Variance
CFU	Colony Forming Unit
FA	Fatty Acids
FAA	Free Amino Acids
FFA	Free Fatty Acids
FID	Flame Ionization Detector
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
GMP	Glycomacropeptide
LAB	Lactic Acid Bacteria
MF	Microfiltration
NF	Nanofiltration
NSLAB	Non-starter Lactic Acid Bacteria
PCA	Principle Component Analysis
RI	Retention Index
RO	Reverse Osmosis
RP-HPLC	Reverse Phase High Pressure Liquid Chromatography
SPME	Solid-Phase Microextraction
SCFA	Short Chain Fatty Acids
TCA	Trichloroacetic Acid
TFA	Trifluoroacetic Acid
TNBC	Trinitrobenzenesulphonic Acid
UF	Ultrafiltration
Urea-PAGE	Urea Polyacrylamide Gel Electrophoresis
WSE	Water Soluble Extract
WSN	Water Soluble Nitrogen

1. INTRODUCTION

Cheese is one of the most consumed fermented milk product in the world. Comparing with the other food products for which stability is the basic principle, cheese has a completely dynamic system which undergoes significant biochemical and microbiological changes during ripening period [1].

The cheese as a word represents the products which can be different with each other in the term of texture, flavour, colour, maturity period, additional ingredient, method, and place of production and etc. Cheese products can be classified as rennet-coagulated cheeses which can be fully or scarcely ripened, acid curd cheese, fresh cheese, and even processed cheese [2].

Ultrafiltrated (UF) white cheese is classified as the rennet-coagulated type and it is one of the most produced and consumed cheese groups in the Mediterranean region [3, 4]. This type of cheese is usually consumed freshly and maximum ripening period of that is not longer than 4 months. However, ripening period of rennet-coagulated cheese can differ about two weeks to more than two years and during this period, microbiological and biochemical changes lead to the development of different characteristic flavour and texture of the cheese varieties.

Biochemical changes in ripening period can be divided into primary and secondary events. Primary events include proteolysis, lipolysis, and metabolism of residual lactose and of lactate and citrate. Secondary events, which are very important due to the development of various volatile compounds, contain metabolism of fatty acids and amino acids [1].

Metabolism of lactose to lactate is the main reason that affords cheese called as a fermented dairy product and this action is getting affected by selected cultures of lactic acid bacteria (LAB) known as starter LAB [1].

LABs comprise a group of Gram-positive, catalase-negative organisms which are mostly non-sporulating facultative anaerobic form, those produces lactic acid as the main end product of lactose fermentation [5].

Mesophilic *Lactococcus* and *Leuconostoc* species, thermophilic *Lactobacillus* species and *Streptococcus thermophilus* are generally used as starter cultures in the cheese manufacture [6].

The main role of the starter culture is the metabolism of lactose and reduce pH of the milk until the isoelectric point (~ pH 4.6) of casein 'principal protein of the milk' and causing coagulation of milk or is the preparation of milk for the action of rennet [7].

Despite the main role of LAB, which is producing lactic acid, they have weakly proteolytic ability and they possess a very wide-ranging proteinase/peptidase system [6], in other word LAB have different role in cheese making: some species lead to fermentation of lactose and decrease pH of the cured as the result, which is known as starter LAB (SLAB), and the other group that have significant role in metabolic activities in the cheese during maturation, that are recognized as non-starter LAB (NSLAB) or adjunct cultures (while they are added deliberately to the cheese milk) [8].

NSLAB plays a complementary role of SLAB due to their proteolytic activity that causes production of smaller molecular weight peptides and free amino acids (FAA) [6].

The main difference between adjunct culture and starter culture is that they are not added to milk to produce lactic acid and acidify the cheese [6].

LAB contains different metabolic activities such as the ability to the development of flavour, texture enhancement and nutritional aspect of the cheese [8].

Depending on the properties of the organisms the outcome of the addition of the adjunct cultures is different [6].

In term of the nutrition and health aspect, selected NSLAB as adjunct cultures could play a probiotic role in several fermented products [9]. Also, some species may produce bioactive peptides and γ-aminobutyric acid and/or may have exist antigenotoxic activities [8].

FAA and small peptides contribute to the flavour development of most cheese varieties [1]. Therefore, NSLAB strains with peptidolytic activity may be considered for use as adjunct culture in cheese-making to enhancement flavour profile of cheese varieties [6].

Adjunct cultures can improve the flavour of reduced/low-fat cheeses and develop aroma compound by increasing the proteolysis and peptidolysis. Also, they can reduce bitterness by some specific aminopeptidase activity [8, 10, 11].

The other flavour enhancement function of some adjunct cultures is their ability to metabolise citrate (Cit+ microorganisms) and produce acetate, diacetyl, acetoin and 2,3-butanediol as the principal flavour compounds [1]. In addition to the enhancement of

cheese texture, it has been reported that some strains have positive effects on the texture of reduced/low-fat cheeses [12-14].

UF is a concentration process of milk proteins. In this technology, cheese-milk is concentrated between 2 to 4 fold. The main advantages of UF are a higher cheese yield due to the incorporation of whey proteins, and the automation in cheese manufacture process which let to continuous cheese production. However, in the manufacture of UF cheeses, all the coagulant (rennet) is retained in the curd which may cause bitterness. Chr. Hansen (Denmark) has developed attenuated *L. lactis* cultures with high aminopeptidase activity that enhances the cheese flavour and suppresses unwanted flavours like sour, bitter and flat (the CR^{TM} cultures).

The purpose of this study is the utilisation of CR-319 (non-acid forming or very low acidification rate culture and contains *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* strains having high aminopeptidase activity) as a commercial adjunct culture in the manufacture of UF white cheese to produce non-bitter, creamy and sensorial quality enhanced cheese and to investigate the microbiological, chemical, biochemical, textural and sensorial effect of CR-319 during the ripening period.

2. LITERATURE REVIEW

2.1 Introduction

The human body needs good nutrition and sufficient diet for growth and development during childhood and adult life. Milk and its derivative products (dairy products) play the main role in human diet and nutrition throughout the life [15].

Animals were domesticated for livestock approx. 8000-10000 years ago throughout the Agricultural Revolution and it has played a significant role in the development of human civilizations. Domestication of sheeps and goats were earlier than cattle, however, cattle have become the principal dairy animal associated with the milk [15, 16].

Cattle milk has been used for the production of various dairy products such as yoghurt, cream, butter, kefir, and cheese. Also with the progress of technology, many other dairy products are enabled to produce. The principal chemical component of milk includes protein, fat, carbohydrate (lactose), minerals, organic acid and water. The amount of this components could change by numerous factors such as the difference between species, seasonal differences, stage of lactation and health of the animal [2].

Lactose is characteristic carbohydrate of the milk and composed of glucose and galactose. Metabolism of lactose has a significant role in the most dairy products like yoghurt and cheese [2].

Triacylglycerols are the main fat content of the milk. Milk's triacylglycerols vary in chain length (2 to 20 carbon atoms) and in saturation (0 to 4 double bonds). Free fatty acids (FFA), monoglycerides, diglycerides, phospholipids, and cholesterol are also the other lipid content of the milk [2].

Normal Bovine milk contains approximately 3.5% protein which 80% of that consists of casein. Caseins present in micelle form and consist of α_{s1}-, α_{s2}-, β-, and κ-casein [2, 16]. Destabilization of casein by acid or enzyme is the basic and essential factor of the conversion of milk to some dairy products like yoghurt and cheese. Another type of proteins that exist in milk is serum proteins such as β-lactoglobulin and α-lactoalbumin. There is also numerous minor proteins in milk, including approximately 60 indigenous enzymes [16].

K, Na, Ca, Mg, Cl and phosphates constitute the milk predominant mineral substances. Milk also contains organic acids, ionised salts –primarily citrate- and vitamins in trace amounts [2].

2.2 Cheese Definition

Cheese, generally, is the name of the dairy product that is basically obtained by coagulating the pasteurized or unpasteurized milk with an enzyme or/and acid addition. It becomes consumable after/without ripening period [17].

It is believed that cheese evolved some 8000 years ago in 'Fertile Crescent' district, which now is covering southern Turkey to the Mediterranean coast. More than 1000 cheese varieties are estimated to be exists in the world. Some references listed more than 400 varieties while some itemised 510 varieties. Eventually, Jim Path (University of Wisconsin) has collected a list of 1400 cheese varieties [18].

Nowadays more than 2000 cheese varieties are known which accounts for approximately 35% of total milk production [19].

According to the Turkish Food Codex, definition of cheese is "the milk based product which is obtained as coagulating of milk by using a suitable coagulant material and separation of whey from curd or coagulating of milk after removing the milk permeate, in different hardness and the fat content, with brine or dry salted or without salting, with using starter culture or without using that, with cooking curd or without cooking, consumed before or after ripening and showing the characteristic features of own varieties [20].

White cheese (Beyaz peynir in Turkish) is the most consumed cheese type in Turkey and constitutes approximately 70% of the Turkey's cheese production [20, 21]. Turkish white cheese is classified as enzymatically coagulated cheeses that mesophilic lactic acid culture is used as its starter culture and matured under certain conditions. It is also classified as the semi-soft type and has slightly acidic and salty taste [22].

Nowadays, besides traditional methods, Turkish white cheese is produced with ultrafiltered milk. Milk is concentrated in this method by using of membrane technology.

2.3 Membrane Technology

The idea of membrane applications dates back some 300 years ago [23]. In the 18th century, Abbe´ Nolet discovered a permeable property of pig's bladder and he used the word osmosis to define the separation of ethanol and water through a diaphragm that he

was made with pig's bladder. Many other researchers had continued these studies and with the beginning of the 20th century, membrane technology moved to a new phase [23, 24].

Using membrane processes were first presented as a chemical and biomedical analytical application in laboratories and then expanded very rapidly into industrialized methods and has a very significant commercial and technical effect on industrial products and processes [25-31].

Today, membrane technology has wide usage in various operations, such as concentrations, purifications, molecular fractionation, separation, etc. Desalination of seawater, concentration, purification, or fractionation of macromolecular mixtures in the food and drug industries and design and production of artificial organs and drug delivery devices are other fields for membrane technology utilisation [23, 32].

2.3.1 Principles of membrane filtration

The membrane can be described as an incomplete barrier (semi-permeable) between two solutions which allows passing some components under limited conditions through the barrier and with this dedicated transport, separation action takes place [33].

In membrane process, feed (fluid) was separated into two distinct streams. The stream that passes through the membrane is known as "permeates" and the retained stream is known as "retentate" [34]. If the purpose of the operation is concentration, the retentate will generally be the product stream [35]. Separation action takes place when a driving force such as concentration difference, pressure difference, temperature gradient or electrical potential gradient is applied to the components in the feed (Figure 2.1).

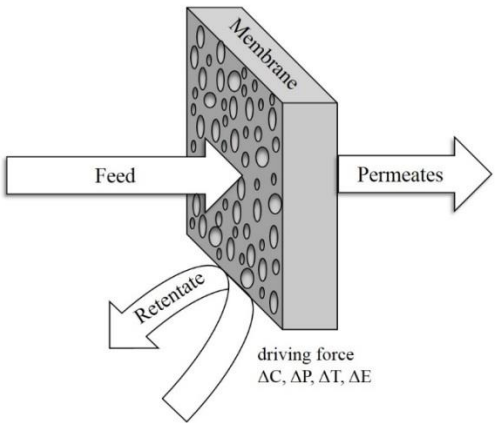

Figure 2.1. Schematic feature of membrane process, where the ΔC: concentration difference; ΔP: pressure difference; ΔT: temperature gradient; ΔE: electrical potential gradient, lead to separation of the feed stream to permeate and retentate streams [35].

Depending on scientific and technological developments, membrane separation techniques, (i.e. reverse osmosis “RO”, nanofiltration “NF”, ultrafiltration “UF” and microfiltration “MF”) has found a wide range of applications in industrial scale.

2.3.2 Utilisation of membrane technology in dairy industry

Utilisation of membrane technologies can be an economical and suitable alternative system in dairy industry so that the operation of many important systems such as evaporation systems, centrifugation, bactofugation, etc, can be replaced with membrane processing system [24].

Membranes with different pore size and properties are commonly used in dairy industry are given below.

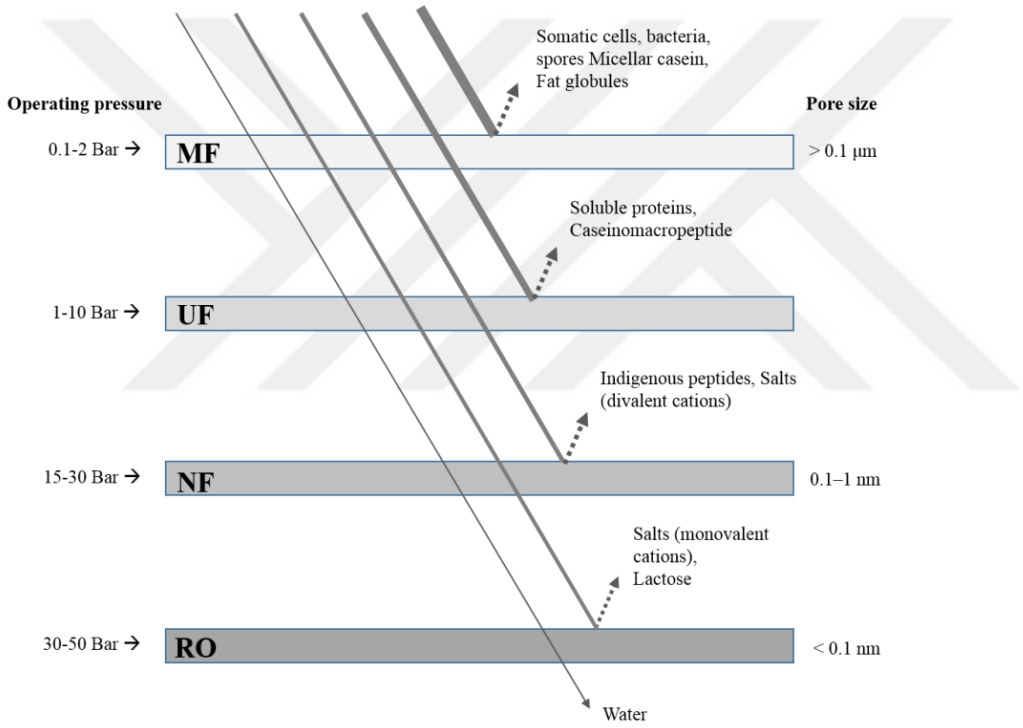

Figure 2.2. Schematic filtration feature of milk component by MF: Microfiltration; UF: Ultrafiltration; NF: Nanofiltration, RO: Reverse osmosis and their pore sizes and operation pressures [24].

2.3.2.1 Microfiltration (MF)

MF is one of the membrane separation techniques with a pore size of 0.05-10 µm and is operating at low pressure (< 2 bars). MF is usually used to separate of substance with molecular weights of >200 kDa [36]. MF in the dairy industry are used in the separation of somatic cells, bacteria, fat globules and large casein micelles from milk and 98-99% of the bacteria in skim milk can be removed with this system. [24, 37, 38].

2.3.2.2 Nanofiltration (NF)

Pore size of NF membrane is about 0.1–2 nm and is used for separation of substances with a low molecular weight between 200-1000 Da like inorganic salts and glucose. NF is a medium to high pressure-driven membrane filtration process and for separation with this method; 10-30 bar pressure is applied. 40-90% of monovalent and 5-20% of polyvalent ions can pass through the NF membrane. In the dairy industry, it is widely used in partial demineralization [24, 38].

2.3.2.3 Reverse Osmosis (RO)

RO is a high pressure-driven membrane separation process and required pressure for this system is 25-80 bars. This membrane system just allows water molecules (<150 Da) to pass through the membrane and in the dairy industry is used for concentration of milk and whey [36].

2.3.2.4 Ultrafiltration (UF)

UF is a medium pressure-driven membrane separation process and the pressure required for this method is 1-10 bars. UF membranes have pore size about 1– 50 nm and allow passing components in the range of 1-200 kDa. Most dissolved and some of the non-dissolved substances can pass through the membrane but large molecules are rejected. The result of applying the UF to the milk is a retentate that consists of proteins, fat and colloidal minerals which bound to casein micelles and permeate contains non-protein nitrogen compounds, lactose, water and water-soluble vitamins and minerals [24, 36]. Milk composition that concentrated approximately 3× by UF and RO membranes are given in Table 2.1.

Table 2.1. Milk component values with concentration (3×) by RO (reverse osmosis) and UF (ultrafiltration) membranes

Components (%)	Milk	RO	UF
Total solids content	12.2	36.6	28.0
Fat	3.5	10.5	15.5
Total protein	3.2	9.6	9.5
Lactose	4.8	14.4	4.1
Ash	0.7	2.1	1.3

2.4 UF Cheese

UF technology is applied for production of various types of the cheeses, like Cheddar, Camembert, Roquefort, Quarg, Ricotta, Mozzarella and also the brined type cheeses (e.g. feta cheese) [4,11, 24, 27, 28, 37, 38]. The concentration level of the milk by UF method can differ depending on cheese variety, for instance, in hard cheeses such as Cheddar, milk concentrated 1.6 to 1.7 fold while in Cottage cheese this value is 1.2 to 1.7. In Feta type cheeses, milk concentrated 3 fold and total dry matter of milk is adjusted to 25-28% [37].

UF white cheese is one of the high consumption cheese types in the Mediterranean countries. The full-fat milk is generally used for the production of this type of cheese manufacture [3]. Using of UF technology has many advantages. Increase in the cheese yield and plant capacity, reduction of production cost can be achieved by this method. In comparison with the traditional method, cheese varieties those are produced by using UF milk, have differences in terms of the manufacturing method and also in ripening and texture quality [38].

A remarkable characteristic of UF cheese is whey proteins which are incorporated in cheese curd and the amount of this incorporation depends on the type of the cheese and level of UF concentration [39]. During concentration of milk by UF membrane, mineral salts bound to casein micelles (Ca, Mg, P) are also concentrated in the same percentage as proteins and leads to increase in buffering capacity of the retentate. Increasing in buffering capacity effects the acid production of LAB, rennet coagulation and ripening characteristic [37, 38]. The rate of changes of milk component values and minerals bound to casein micelles, depending on UF concentration level, are shown in Table 2.2 and 2.3, respectively.

Some basic parameters of the cheese during the production such as coagulation, acidification, rheological properties of the curd and also some other basic parameters during the ripening periods such as the activity of enzymes (mainly rennet), growth and survival rate of spoilage micro-organisms, and water-holding capacity of the cheese were affected by changing in buffering capacity of the milk [40].

Table 2.2. The effects of different concentration factors of UF operation on skim milk component [41]

Volume concentration factor	Total solids content,%	Casein,%	Whey Protein,%	Non protein nitrogenous substances,%	Lactose, %	Ash, %
1 (Skim milk)	8.2	2.8	0.28	0.17	5.1	0.74
2	12.1	5.7	0.65	0.15	5.0	0.99
3	15.5	8.4	0.91	0.20	4.7	1.26
4	18.9	11.5	1.15	0.21	4.7	1.37
5	21.8	13.8	1.41	0.30	4.5	1.70

Table 2.3. The effects of different concentration factors of UF operation on distribution of minerals in the micellar phases of skim milk [41]

Volume concentration factor	Ca,%	Mg,%	Zn,%	Fe,%	Phosphate,%
1 (Skim milk)	71	26	99	16	40
1.5	79	34	100	77	44
2	80	41	99	79	57
3	86	50	99	87	68
4	91	60	100	89	73

2.5 Enzymatic Coagulation Mechanism of Milk

The essential phase of production of cheese is the conversion of milk to cheese curd and this is achieved by:

- The addition of selected coagulated enzymes which are known as chymosin (commercial name of coagulant is rennet)
- Acidification to pH 4.6 (isoelectric point of casein)
- The combination of acidification to about pH 5.2 with heating roughly 90°C.

The majority of cheese varieties (about 75%) is produced by enzymatically coagulation method [19]. These enzymes are obtained from five major sources: mammals stomach,

lysosomes of the cells those contain cathepsin D and E, some tissues that produce chymosin (e.g. sub-maxillary gland and kidney), plants, and the microorganisms [42].

Turkish white cheese is classified as enzymatically coagulated cheeses. Rennet used in this type of the cheese is usually contained about 80% chymosin and 20% pepsin. Casein is the principal milk protein and degradation of this protein by coagulant causes coagulation of milk. The target section of chymosin on casein micelles is κ-casein which exists in a surface layer of micelles and has a stabilizing role of the micelles. It is hydrolysed specifically between the bond of Phe_{105}-Met_{106} by the action of chymosin and this is known as the primary or enzymatic phase of coagulation [19].

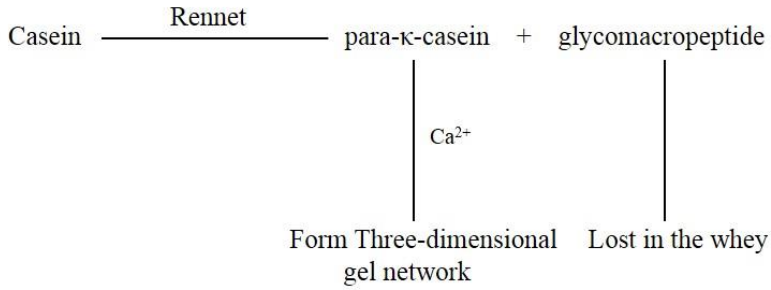

Figure 2.3. Schematic view of rennet coagulation of casein micelle [19]

N-terminal part of the splitted molecule that is referred as para-κ-casein (f1-105) remains attached to rest of the micelles and forms a three-dimensional gel network. This phase is known as secondary phase and occurs in the presence of Ca^{2+} ions and temperature above 20°C. C-terminal of the enzymatically hydrolyzed κ-casein is referred as glycomacropeptide (GMP, f106-169) due to containing the glycosylated residues of κ-casein. Carbohydrate chains content of GMP increases the negative charge and also the hydrophilic properties of C-terminal of κ-casein. Hence, according to soluble properties of GMP, it is lost in the whey after hydrolysis of κ-casein by chymosin [16, 42].

After gel forming, curd subsequently commences to syneresis. The main reason for syneresis is the rearrangement of the gel network (para-casein micelle) [43]. After formation of the gel, cheese curd is subjected to various processes (depending on cheese varieties) such as moulding, shaping, salting, etc. [16].

Cheese curd is a completely dynamic system and after the formation it undergoes significant biochemical and microbiological changes during the process that is referred as ripening period.

2.6 Cheese Ripening

Rennet-coagulated cheese, if consumed freshly, is flavourless and rubbery. Hence, for the development of flavour compounds and improvement in textural features, these kind of cheese must be matured during a period called as “ripening period”. Ripening period of rennet-coagulated cheese, depending on cheese variety may vary from two weeks to more than two years. During this period, various microbiological and biochemical changes lead to enhancement of cheese characteristic features. Several sources are in charge for these changes:

- The cheese milk;
- Coagulant;
- Starter bacteria;
- Non-starter bacteria;
- Adjunct cultures;
- Other exogenous enzymes [16].

During maturation period three principal biochemical events (glycolysis, lipolysis and proteolysis) occur. The products of these biochemical events undergo to several catabolic interactions which are important for the formation of characteristic features of the cheese.

2.6.1 Metabolism of residual lactose and lactate

Lactose is the main carbohydrate exists in the milk. Metabolism of lactose to lactate by starter LAB, and in other words fermentation of lactose, is the main characteristic feature of the cheese production. The amount of residual lactose that remains in the cheese curd is very low (about 0.8–1.0% for Cheddar cheese) and most of it is lost in the whey during cheese manufacture [44]. Fermentation of the lactose and consequently, lactic acid production changes the pH value of the medium and it has several outcomes for the texture and flavour of the product. For instance, the extent and amount of acidification effects demineralization in the cheese curd; so that increase in acidification level leads to increase the susceptibility of casein to proteolysis [45]. The texture of the cheese is directly influenced by the pH level of the medium; so that high pH cheese has a softer texture than products with more acid ones. The pH level also influences cheese texture and flavour indirectly; residual enzymes which are important for cheese ripening is affected by the pH of the medium [46].

Wide-ranging fermentation of lactose and conversion of it to the L-lactate form is very important; this conversion is essential for preventing of undesirable secondary microflora in the cheese [46].

Starter bacteria rapidly metabolize lactose especially to L-lactate in initial stages of ripening but temperature and the salt-in-moisture (S/M) is critical parameters for the activity of starter LAB. Increasing of (S/M) level in dry salting varieties leads to preventing the activity of starter bacteria and remaining unfermented residual lactose which were probably metabolized by NSLAB [46-49].

If there is a significant amount of NSLAB in medium, they can form considerable amount of D-lactate by fermentation of the residual lactose or by racemization of the L-lactate to DL-lactate [49].

Different metabolites that can be produced by lactate metabolism by starter LAB or NSLAB during the ripening period cause characteristic feature of various cheese types. Different pathways of lactate metabolism are shown in Figure 2.4.

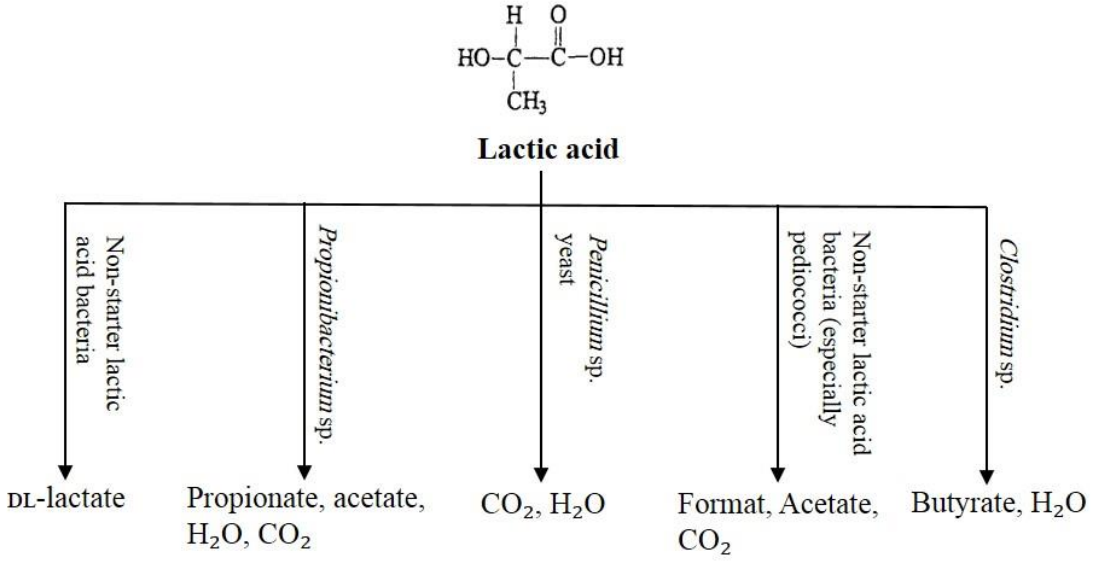

Figure 2.4. Lactate metabolism pathways in cheese during ripening [46]

2.6.2 Metabolism of citrate

Metabolism of citrate is led to production of compounds which are significant for the flavour and quality of some fermented milk products. Same as lactose, most of the citrate is lost in whey during cheese production and especially in whey drainage. There is just slight amount of citrate in cheese curd (approx. 0.1-0.3 g/kg cheese-curd depending on

cheese type). However, this amount is sufficient for production of some important flavour compound such as acetate, acetaldehyde, 2,3-butanediol and probably diacetyl by special LAB and/or NSLAB [46, 50]. Bacteria which contain plasmid for citrate transport are known as citrate positive (Cit^+) bacteria (i.e. *L. lactis* ssp. *lactis* biovar *diacetylactis*, *Leuconostoc mesenteroides* subp. *cremoris* and *Leuconostoc. lactis*) [46]. Citrate participates in the Krebs cycle and LAB can use it as a substrate and ferment it to produce pyruvic acid, CO_2 and acetic acid; so it acts both as a substrate and as a product in Krebs cycle [51].

2.6.3 Lipolysis

Lipids (fats) are considered as one of the main components of milk and hydrolysis of them has some positive and/or negative consequences in milk and milk products. The average amount of fat in cow milk is about 3.5%, but the level of it varies from 3.0 to 5% depending on breed, age, diet, and stage of lactation of animals. Milk fat is composed mainly of triacylglycerols (approx. 98%) and remaining (2%) of the total fat comprises di- and monoglycerides, phospholipids, free fatty acids (FFA), sterols and a trace amount of fat-soluble vitamins [19].

Fats play a significant role in cheese texture so that, reduced-fat cheese has hard and crumbly texture. Also, fats contribute to flavour and aroma of cheese in three ways:

- Fats are known as a source of FA; specifically, short-chain fatty acids (SCFAs) and medium-chain fatty acids (MCFAs) which can be converted to aromatic compounds such as lactone and ketone and so on,
- They can cause undesirable flavour which regarded as a defect in cheese by oxidation which leads to the formation of various unsaturated aldehydes,
- It serves as a carrier for flavour compounds which are produced from fats, protein and lactose [19, 52].

Lipids in food may be degraded by hydrolytic or oxidative pathways. Due to the low redox potential of the cheese medium (about -250 mV), oxidative changes are limited [1, 53].

Lipolysis occurs in almost all type of the cheese varieties. However, degree of lipolysis is different between varieties. For instance, Italian hard cheeses and blue mould cheese have an extensive lipolysis while the amount of lipolysis is limited in Cheddar or Gouda compared with mould ripened cheese. As previously mentioned, lipolysis is important for

the flavour of the cheese but the excessive level of that is not desirable and leads to defects such as rancidity [1, 53].

Lipolytic agents in cheese are:

- Native milk lipase (lipoprotein lipase)
- Lipase/ esterase comes from coagulant such as rennet paste
- Cheese microflora (lipase/esterase of starter, non-starter and adjunct cultures) [46, 52].

Lipoprotein lipase (LPL) is the potent native lipase of the milk. Under optimum condition, it can cause distinctive rancidity in the milk very quickly (within 10 seconds) [54]. But, milk fat globule membrane (MFGM) prevents LPL enzyme to reach the substrate [46]. The LPL has the most important role in cheeses which are made by raw milk. LPL can be completely inactivated by heat treatment (78°C × 10 s). So, LPL has not much effect on lipolysis of the pasteurized milk cheese [55].

Most of the rennet extracts which are produced commercially are free from lipase. However, rennet pastes which are used in some Italian hard cheese and some Greek Feta has lipase activity. Hence, the high level of lipolytic activity of rennet paste and also the existence of pregastric esterase (PGE) is responsible for high level of lipolysis in this type of cheeses [46].

LAB and specifically *Lactococcus* and *Lactobacillus* subsp., in comparison with other species such as *Flavobacterium*, *Acinetobacter*, and *Pseudomonas* are weakly lipolytic. However, the esterolytic/lipolytic activity of LAB plays an important role and determines the specific flavour and aroma of many different types of the cheese. Their enzymes which hydrolyse the ester bonds of triacylglycerides liberate the FFA [52, 53]. Hence, the main lipolysis that occurs in Cheddar and Dutch-type cheese (which are made by pasteurized milk) derives from the esterolytic/lipolytic activity of LAB [19].

The amount of lipolysis in cheeses which contain secondary microflora such as NSLAB or adjunct cultures may vary depending on their esterolytic/lipolytic activities. For instance, *Brevibacterium linens* in surface smear cheese [56], *Penicillium roqueforti* in Blue mould cheese [57] and *Penicillium camemberti* in Camembert cheese [58] increase the lipolysis level in related cheeses.

In a research the effect of adjunct cultures of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* on lipolysis of the low-fat Feta cheese were examined and results indicated that the adjunct treated samples had higher total FFA than control samples [59].

2.6.4 Catabolism of free fatty acid

Lipids can be degraded by hydrolytic or oxidative pathways. However, due to the low redox potential of the cheese, hydrolytic pathways are limited and enzymatic hydrolysis are dominant [53]. The lipolysis of triacylglycerol leads to liberating of FFA which is essential for the development of some cheese flavour. Milk has high contact of SCFAs and MCFAs which directly contributes to cheese flavour. Also, FFAs undergo various catabolic reactions which leading to produce some aroma and flavour compound such as esters, lactones, methyl ketones and so on (Figure 2.5) [53, 60]. For instance, FA esters are produced by the reaction of FA with alcohol (e.g. ethyl esters), thioester is the result of the reaction of FAs with the thiol compound which is formed by the catabolism of methionine. FA lactones (i.e. γ- and δ- lactones) are cyclic compounds formed by intramolecular esterification and have an important role in the flavour of the many cheese varieties [17, 53].

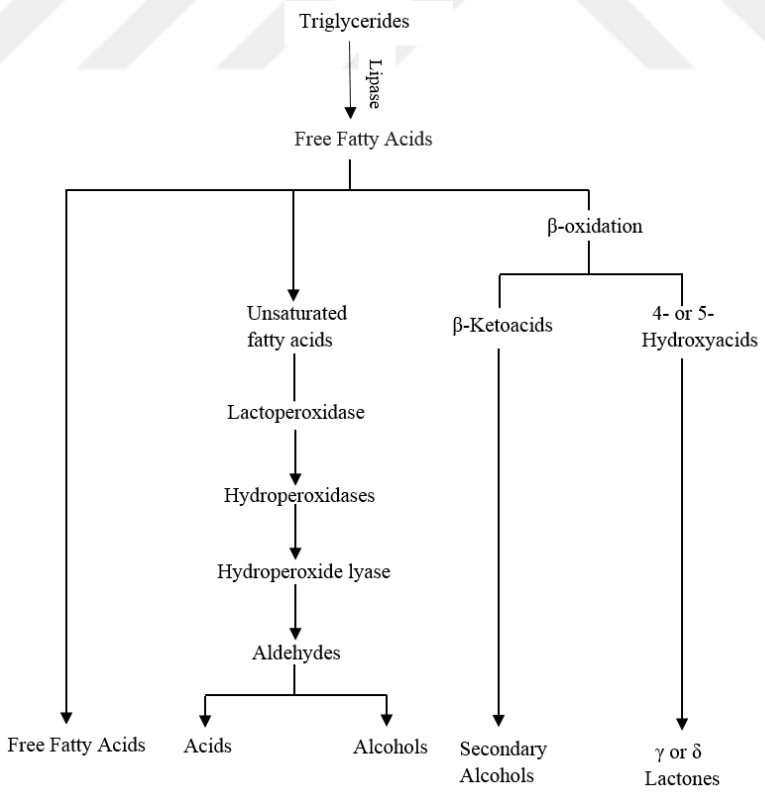

Figure 2.5. Catabolism of FFA on cheese [60]

2.6.5 Proteolysis

Proteolysis, in most type of the cheese varieties, is the most complex and most important degradation event that occurs during ripening. Proteolysis plays a great role in the cheese quality, including:

- Effect on the cheese texture through breaking down the protein network and decreasing water activity (a_w) in the medium;
- Peptides and free amino acids derived from proteolysis have direct impact to the cheese flavour or may cause off-flavour or bitterness in the cheese;
- Amino acids can be substrate for secondary catabolic reactions such as deamination, decarboxylation, and desulphuration. Transamination of the amino acids and the catabolism leads to producing aromatic compounds. Amino acids react with other compounds.
- Degradation of proteins by proteases facilitates the releasing of the pleasant flavour compound during mastication by changing in the cheese matrix [1].

Enzymes acting the proteolysis in cheese originate from different sources such as;

- Coagulant (e.g. chymosin, pepsin or different type of coagulants that originate from microbial or plant sources);
- Residual milk proteinases (e.g. plasmin, different types of cathepsin and somatic cell proteinases);
- Enzymes originate from starter bacteria;
- Enzymes which are secreted from NSLAB or secondary cultures;
- Exogenous proteinases/peptidases used to accelerate of ripening [1, 6].

In the most of the cheese types, primary proteolysis carried out by coagulant and in lesser level by plasmin and enzymes driven from somatic cells such as cathepsin D. The result of the primary proteolysis is the emergence of large and intermediate sized peptides, which are degraded by the enzymes from starter and non-starter bacteria. The result of the bacterial proteinase/peptidase system is the emergence of small peptides and FAAs [1].

The coagulants are usually a combination of selected proteinases, commonly a mixture of chymosin and pepsin or other suitable proteinases which have a considerable proteolytic activity [1, 19]. The principle role of chymosin is the cleavage of a peptide bond between Phe_{105}-Met_{106} of κ-casein. As previously mentioned, κ-casein has a micelle-stabilizing role

and hydrolysis of that is leading to coagulation of milk. Only low amount (0–15%) of residual coagulant activity, depending on different factors (i.e. the type of the coagulant, cooking temperature and pH level) remains in the cheese curd and the rest of that is separated by whey drainage [1, 61].

Plasmin having optimum activity at pH 7.5 and 37°C is the main indigenous protease enzyme in the milk. Plasmin is a trypsin-like serine proteinase and it specifically hydrolyse the peptide bonds of the Lys–X and in lesser extent Arg–X [46]. Plasmin degrades β-casein and α_{s2}-casein more than α_{s1}-casein and also it has no effect on κ-casein. Hydrolysis of the β-casein and α_{s2}-casein leads to the creation of γ-caseins and protease-peptones [1]. Plasmin is a heat-stable enzyme, so its activity is significant in high-cooked cheeses due to inactivation of coagulant proteinase in high temperature [19].

Moreover, the other types of milk indigenous proteinases which are known as cathepsins and elastase can be important in the proteolysis of the cheese curd. Cathepsin D which is an acid proteinase has a similar specificity to that of chymosin with preference degradation for α_{s1}-casein. However, unexpectedly, this enzyme has very low coagulation activity [62]. Nevertheless, the formation of α_{s1}-I-casein in cheeses which are rennet-free is considered as an activity of the acid milk proteinase [63]. Elastase is another milk protease enzyme. It originates from somatic cells and degrades α_{s1}-casein and with a wide-ranging specificity on β-casein [64]. Hence, the activity of that in cheese especially in varieties which are made by raw milk can be significant. Some other protease which originates from the somatic cell can activate plasmin's precursor (plasminogen) and affect the proteolysis level in cheese [1, 6, 65].

LAB (*Lactococcus*, *Lactobacillus*, *Streptococcus)* require a range of amino acids for growth. They have a peptid-transport system and have different types of proteolytic enzymes for degradation of their environmental proteins/peptides [46].

The cell envelope-associated proteinases, lactocepin (CEP) or PrtP, are the principle proteases of the LAB and it is considered as to be a serine proteinase. The principle role of PrtP is the degradation of casein and provides short peptides which are essential for growth of the LAB cells. However, in cheese, the main function of PrtP is the degradation of intermediate-sized peptides which are generated by the action of chymosin and plasmin on casein micelles [46]. PrtP can be divided into two groups according to substrate specificity: PI- and PIII-types [46]. PI-type acts on β-casein more rapidly than α_{s1}- and κ-casein whereas the second group enzymes possess a rapid act on α_{s1}- and κ-casein. LAB possess a

range of intracellular peptidases and proteinases, which former enzymes have a significant effect on the final level of proteolysis and liberating FAA [46]. Some of the significant intracellular enzymes that contribute to liberating FAA after LAB have lysed include; general aminopeptidases (PepN, PepC, PepG), oligoendopeptidases (PepO, PepF, PepE), leucyl aminopeptidase (PepL), glutamyl aminopeptidase (PepA), X-prolyl-dipeptidyl aminopeptidase (PepX), prolinase (PepR), prolidase (PepQ); tripeptidase (PepT), dipeptidase (PepV) [1, 6, 46].

Proteolysis in cheese varieties in which NSLAB are present is greatly influenced by their proteolytic enzymes. The activities of NSLAB are especially important in raw milk cheese varieties. However, they can intentionally be added to cheese-milk due to supplement activity of starter bacteria. For instance, *Propionibacterium* sp. has high peptidolytic activity and presence of it in Swiss-type cheeses plays an essential role in the characteristic flavour of this cheese [46]. Also, the effects of *P. roqueforti, P. camemberti* and *B. linens* on the proteolytic profile of blue cheeses, Camembert/Brie cheeses and surface smear-ripened cheeses have been reviewed by authors, respectively [66, 68].

2.6.5.1 Metabolism of free amino acid

The catabolism of FAA plays an important role in the development of flavour compound in many cheese varieties. The half of potent aroma compound originates from lactate and citrate catabolism and a few from lipolysis and the other half originates from amino acid degradation. The aromatic amino acids (Phe, Tyr, Trp) and branched-chain amino acids (Leu, Ile, Val) and Met, which is a polar amino acid, are the major precursors of cheese aroma compounds. The concentration of various key aroma compounds determines the cheese flavour [67].

Generally, the catabolism of amino acids proceeds by two major pathways. The first one, which results in major sulphur compounds, commence by elimination reaction and it is mainly observed for Met. The second one generally commences by a transamination reaction and resulting α-keto acids, which are then degraded to different aroma compound by one or two additional reactions [67].

Some compounds such as ammonia, amines, aldehydes, phenols, indole and alcohols are produced as a result of the FAA catabolism those contribute to the cheese flavour (Figure 2.6).

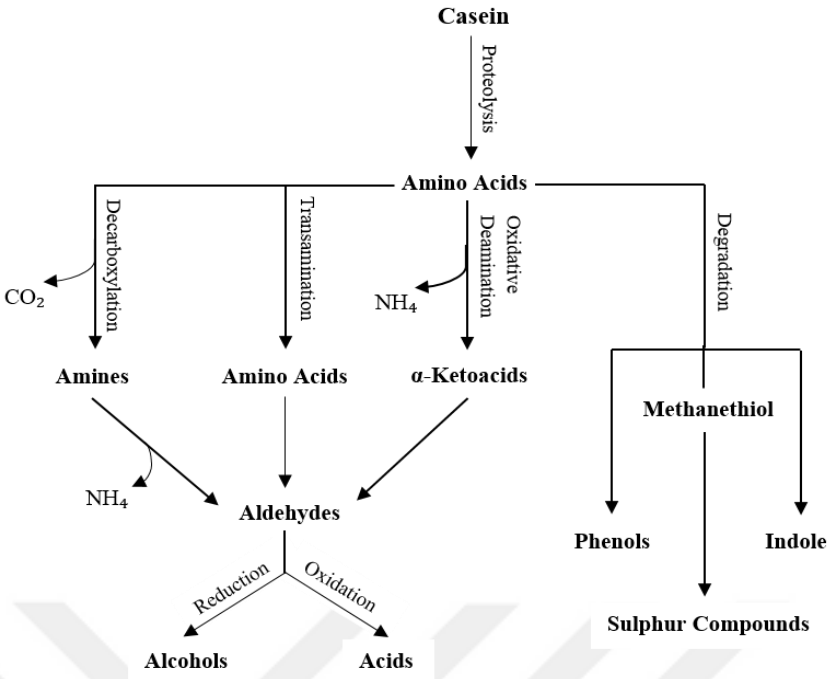

Figure 2.6. Principle pathways of catabolism of FAA [1]

2.7 NSLAB and Adjunct Cultures

Primary and secondary cultures are used in cheese making. Primary cultures which contain starter LAB are involved in the fermentation of lactose and production of lactic acid during cheese manufacture and ripening period [68]. The second group includes yeast, moulds, and bacteria and has a diverse function as compared to primary cultures such as supplementary lipolytic and proteolytic activity, development of aroma compounds and bioactivity effects. The main source of NSLAB is raw milk. It enriches the microflora of the cheese making environment [69]. Natural whey starters also can be the source of the NSLAB [70]. NSLAB also have been isolated from cheese making environment, utensils and equipments [71].

Also, NSLAB and primary cultures are different in terms of kinetic of growth, so that NSLAB shows opposite growth kinetic compared with primary cultures and their number. Unlike primary cultures, they increase during the ripening period. For instance, their number in cheddar cheese just after curd manufacture are <50 CFU/g but they increase very fast to reach approx. 10^7 CFU/g within few weeks to several months of maturation period [72, 73].

Although primary cultures in collaboration with indigenous proteases have a higher role in protein breakdown, NSLAB have a significant role on hydrolysis of peptides to FAAs and

accomplishing of the FAA catabolism (e.i transamination, decarboxylation etc.) which leads to producing significant flavour compounds in cheese [74, 75].

NSLAB have an important role in cheese maturation due to having extensive variety of hydrolytic enzymes [76]. However, in rare cases, defects have also been reported resulting from NSLAB. Those reports were usually related to an unpleasant flavour profile and lactate crystal formation due to racemization of L-lactate [74].

NSLAB are important in raw milk cheeses. Their activity leads to produce peptides with mostly smaller molecular weights and also FAA [75]. Pasteurization destroys most of the NSLAB in milk hence the amount of them are very reduced in cheeses which are produced by pasteurized milk. According to some studies, mature flavour of cheddar cheese, cannot develop without the presence of the NSLAB [77-79]. Hence, in several cheese varieties, some selected NSLAB are added intentionally for development of the cheese flavour. Secondary cultures of *P. roqueforti* and *P. camemberti* contribute the ripening and are used in Blue mould and surface mould cheese (i.e. Roquefort, Camembert, Brie), respectively. Also, coryneform bacteria such as *B. linens* and *Arthrobacter* and several yeast species such as *Geotrichum candidum*, *Debaryomyces hansenii*, are important in surface smear-ripened cheeses and *Propionibacterium freudenreichii* subsp. *shermanii* has a significant role in the ripening of Swiss-type cheese by the production of CO_2, acetate and propionate [6].

Selected bacteria, which are intentionally added to cheese for several purposes such as supplementary lipolytic and proteolytic activity, development of aroma compound and bioactive effects or for other improvements, are defined as adjunct cultures. The main practical difference between adjunct and starter cultures are that the purposes of the addition of the former cultures are not the production of lactic acid [14].

The several impacts of adjunct cultures on cheese have investigated by researchers. The influence of two *Enterococcus faecium* strains as an adjunct culture on quality and redox potential of Turkish white cheese (Beyaz peynir) have been investigated by Bulat [17]. In this study, they demonstrated that usage of two commercial *Enterococcus* species as adjunct culture caused to reduce the redox potential of the cheese medium as compared to control samples. Providing reducing medium enhanced the production of aroma compounds. Hence the rate of proteolysis and lipolysis in cheese produced using *E. faecium* was found to be higher.

In another study, the influence of commercial adjunct culture, containing *L. Lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* on the formation of FFA and volatile compound of low-fat Feta-type cheese has been studied. The results of that study indicated that adjunct culture treated samples had a higher amount of ethanol, acetaldehyde, acetoin and 2-butanone than control low-fat and even full-fat samples [59].

Ristagno [80] had also concluded that using *Hafia alvei* as an adjunct culture on Cheddar cheese effects on lipolysis and it determined that the amount of FFA has increased as a result of this experiment. Furthermore, the level of soluble nitrogen and total FAA were higher in adjunct culture containing samples.

The effect of commercial adjunct culture (CR-213) on the flavour enrichment of low-fat Feta type cheese, was also investigated, and it was concluded that adjunct culture containing samples had a flavour similar to full-fat samples [81]. In addition, improvement of organoleptic properties of reduced-fat Edam cheese [82] and low-fat Caciotta-type cheese [83] by using adjunct cultures was documented.

Flavour enrichment by using adjunct starters is principally based on enhancement of proteolysis and elevation of formation of small peptides and especially FAA in ripened cheeses [10, 75, 84]. Due to poor flavour and rubbery texture of low-fat cheeses, using adjunct cultures are widely considered.

2.8 Using Adjunct Culture in Cheese Manufacture

In the past, using of secondary culture was limited to traditionally manufactured cheeses which mostly were produced by raw milk which is the main source of NSLAB, and also the number of discovered secondary cultures was limited as well. For instance, in blue-veined cheeses, secondary culture (*P. roqueforti*) was added before moulding as a grated, mouldy bread [68]. Since then by the development of hygienic modification in cheese making such as heat treatment and bactofugation of the milk, the amount of indigenous flora dramatically reduced. Nowadays, according to individual features and expected impacts of microorganisms, various type of microorganism (yeasts, moulds and several types of bacteria) can be used as adjunct culture in cheese making.

2.8.1 Yeasts as adjunct cultures

Using yeasts as adjunct culture are common in mouldy and surface-ripened cheeses. Yeasts contribute the ripening of French cheeses (i.e. Brie, Camembert, Pont l'Eveque, Maroilles and Reblochon), Italian cheese (Tallegio) and Belgian cheeses (i.e. Herve and Limburger).

Also, they contribute the maturation of some blue veined cheeses such as Fourme d'Ambert (French blue cheese), Gorgonzola (Italian blue cheese), Danablu (*Danish Blue Cheese)*, Cabrales (Spanish blue cheese) and Stilton (English blue cheese) [68]. The number of yeasts can reach approximately 10^6-10^8 CFU/cm^2 of cheese surface during the first week of ripening. Yeasts could contribute to lactate metabolism. For instance, *Kluyveromyces marxianus* and *Debaryomyces hansenii* are capable of fermentation of the residual lactose and are used commonly as an adjunct culture [68]. Yeasts also have an extensive proteolytic system that includes aminopeptidase, carboxypeptidase and caseinolytic enzymes [68, 85]. For instance, *Saccharomyces cerevisiae*, *Kluyveromyces marxianus* subsp. *marxianus* and *Geotrichum candidum* possess high proteolytic activity [68, 86]. In addition, yeasts contribute to hydrolysis of lipids in cheeses. *Yarrowia lipolytica* possess highest lipolytic activity among the yeasts found in cheese [68]. *Torulaspora delbrueckii, Rhodotorula mucilaginosa* and *Kluyveromyces lactis* are the other yeasts used as adjunct culture due to their proteolytic, lipolytic, glycolytic activity [87].

2.8.2 Moulds as adjunct cultures

Moulds, as an adjunct culture, are used in surface-ripened soft cheese and blue-veined cheeses. Additionally, moulds contribute in the ripening of a few type of semi-hard cheeses such as Tomme and Toma [68].

P. camemberti and *P. roqueforti* which are known as white and the blue-green mould respectively, are the two most used adjunct mould in the cheese production. Moulds play a role in the appearance of the cheeses body (in blue-veined cheeses) and surface (surface mould cheeses). The properties of the mycelium, such as length, colour and density is decisive in choosing the appropriate strain. For instance, *P. roqueforti* strains with light blue or yellowish mycelium colour are used in producing Gorgonzola cheese, whereas for producing of Roquefort cheese, dark green strains are preferred. *P. camemberti* and *P. roqueforti* can utilise lactic acid as an energy source; in another word, they have de-acidification ability which can lead to increase in pH level of the cheese and therefore cause softening in the cheese. *P. camemberti* and *P. roqueforti* can also contribute in proteolysis with their proteolytic enzymes. Both of them possess endopeptidase and exopeptidase enzymes which responsible to proteolysis in cheeses that contain them [68].

Different strains of *P. camembert* and *P. roqueforti* show the diverse level of lipolytic activity. Blue-veined cheeses have more extensive lipolysis level than other cheese varieties. *Penicillium* subsp. lipases are responsible for major lipolysis and characteristic

flavour compounds of mould ripened cheese are methyl ketones and secondary alcohols which are generated from β-oxidation of FFAs [68].

2.8.3 Lactic acid bacteria as adjunct cultures

According to individual feature and expected impacts of microorganisms, a different genus of LAB can use as an adjunct culture. *Streptococcus*, *Pediococcus*, *Lactococcus*, *Leuconostoc*, *Bifidobacterium*, *Carnobacterium* and *Enterococcus* genus have been used in different cheese varieties for different purposes such as flavour and texture enhancement, bioactive aims, and improvements of industrial, biochemical and organoleptic features of the cheeses. It is known that cell autolysis is required to release of proteolytic enzymes into the cheese which leads to the development of flavour compound [88-90]. The positive relation between autolysis of lactococci and proteolysis has been reported by researchers [90, 91]. But, lipolytic activity of LAB is limited and is not greatly contribute to lipolysis of cheese. The effect of using lactococci as a starter culture on lipolysis level of cheese have also been reported [91].

2.9 Lactococcus Genus

Genus *Lactococcus* includes nine species: *L. garvieae*, *L. lactis*, *L. plantarum*, *L. formosensis*, *L. piscium*, *L. fujiensis*, *L. chungangensis*, *L. taiwanensis* and *L. raffinolactis*, but seven species and subspecies of lactococci have been identified that possess physiologic activity [5, 92].

Lactococci are Gram-positive, nonsporulating, homofermentative, microaerophilic bacteria which produce L (+) lactic acid as a result of fermentation of glucose. They appear in individual or pair avoid cell or in the chain and therefore differentiate them among *Streptococcus*, *Enterococcus* and *Leuconostoc* on a morphological basis is difficult [93].

Genus *Lactococcus* have not pathogenicity effect on human. However, it seems that *L. piscium* have an impact on meat spoilage and it is known that *L. garviae* is responsible for mastitis in cow and also regarded as a pathogen in fish [94].

Two species of *Lactococcus* genus, *L. raffinolactis* and *L. lactis*, are registered in 'Inventory of Microbial Food Cultures' with documented usage in food fermentations [95].

L. lactis includes four subspecies: *lactis*, *cremoris*, *hordniae*, *tructae* and a biovar *diacetylactis*. In the production of a fermented dairy product such as cheese and fermented milk, *L. lactis* subsp. *Lactis*, *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* biovar *diacetylactis* are widely used, and they have 'Generally Regarded as Safe (GRAS)' status

[5, 96]. Mainly, *L. lactis* subspecies contribute to organoleptic properties and microbial quality of cheese by fermentation of lactose and proteolytic activity [97, 98].

2.9.1 Protein metabolism by lactococci

The cell envelope-associated proteinases, lactocepin (CEP) or PrtP, is the principle proteases of the *Lactococcus* and it considered as a serine proteinase. The principle role of PrtP is the degradation of casein and provides short peptides which are essential for growth of the lactococci cell, besides PrtP, lactococci proteolysis system possesses significant extracellular peptidase and intracellular enzymes that contribute to liberating FAA after that the cell has lysed. Also as a proteolysis transport system, lactococci cells possess amino acid, dipeptide, tripeptide and oligopeptide transport system [46].

2.9.2 Carbohydrate metabolism by lactococci

In compared with other lactic acid bacteria, *Lactococcus* species shows different action in lactic acid metabolism so that they catabolize glucose and galactose simultaneously. A plasmid-encoded gene in lactococci is responsible for the transfer lactose to the cell and breaking down it. Lactose is phosphorylated by lactococci phosphoenolpyruvate-dependent phosphotransferase system and then hydrolyzed to glucose and galactose subsequently. Galactose and glucose catabolized at the same time by Tagatose and Embden-Mayerhof-Parnas (EMP) pathways respectively. The products of both pathways are converted to pyruvic acid and then to lactate [93].

2.9.3 Lipid metabolisms by lactococci

LAB and specifically *Lactococcus* and *Lactobacillus* subsp., in comparison with other species such as *Flavobacterium*, *Acinetobacter*, and *Pseudomonas* are weakly lipolytic. FFA and especially butyric acid, are detected in cheese matured with *Lactococcus* strains. However, in the case of lactococci cells, producing of FFA from partially hydrolysed lipids is faster than producing them from triacylglycerols [52, 53].

3. MATERIAL AND METHOD

3.1 Material

Raw materials and equipment necessary for cheese production have been provided by Bahçıvan Gıda San. Tic. A.Ş. and production of cheese samples have been performed in the same company by using their UF cheese production steps which were briefly demonstrated below. The only parameter that was changed in production was the addition of adjunct culture to the examined samples. The materials used in this study are summarized below.

In UF process, a semi-permeable polyethersulfone (PES) spiral membrane (Koch Membrane System Inc., Wilmington, MA) with a separation size of 10 kDa were used. The operating pressure was set to approx. 4 bars. The concentration factor of cheese milk in the UF process was adjusted to 3.5. Fermented calf chymosin (CHY-MAX Extra, 600 IMCU) (Chr. Hansen, Denmark) was used as the coagulant enzyme. 130 mL CHY-MAX Extra (600 IMCU) were used for 1300 kg of retentate. CHOOZIT Feta A LYO 100 DCU (Defined multiple-species culture blend of *Lactococcus lactis* subsp. *cremoris, Lactococcus lactis* subsp. *lactis* and *Streptococcus thermophilus, Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus helveticus)* produced by DANISCO (Germany) was used as a starter culture. 100 U of Feta A LYO was added to 1300 kg of retentate.

As an adjunct culture, F-DVS CR-319 (frozen, defined adjunct culture blend of *Lactococcus lactis* subsp. *lactis, Lactococcus lactis* subsp. *cremoris*) produced by Chr. Hansen (Denmark) was used. 150 U of CR-319 as an adjunct culture was added to 1300 kg retentate in addition to starter culture. Experimental design of cheese was as following;

- Cheeses coded as A (control UF cheese) contain Feta A LYO
- Cheeses coded as B (cheese with adjunct culture) contain Feta A LYO + CR-319

The steps of UF cheese production are briefly demonstrated below:

- Quality control of the raw milk
- Pre-treatments (clarification, standardization, etc)
- Pasteurization of the milk (72-74 ° C, 15 s)
- UF treatment at 50-55 ° C (until 31-32 retentate brix)
- Homogenization (100 bar, 50-55 ^{o}C)
- Pasteurization (80 ° C, 20-30 s)

- Cooling to inoculation temperature (33-35 ° C)
- Starter culture and adjunct culture addition
- Rennet addition (the amount of rennet adjusted to obtain the coagulation within approx. 20 min.)
- Filling into the cheese container
- Coagulation in the incubation tunnel (20 min, 33-35ºC)
- Addition of 2% salt to the cheese exited from coagulation tunnel
- Packing hermetically
- Incubation (31-32 ° C, 12-18 hours, until pH 4.7-4.8)
- Storage (6-8ºC)

Cheese production was conducted as to be two replicate.

3.2 Method

3.2.1 Microbiological analyses

Sampling was performed from both trials at day 7, 30, 60, 90 and 120. For performing the analysis, 10 g of the cheese sample was taken in sterile stomacher bags under aseptic conditions and 90 mL of sterile trisodium citrate solution (2 g/100 mL) was added on it. Samples were homogenized for 2 min in Stomacher (Stomacher 400, Seward Laboratory, London, UK). Appropriate dilutions of the obtained suspension were prepared by using physiological saline solution (0.85 g NaCl/100 mL, w/v).

3.2.1.1 Lactic acid bacteria (LAB) count

M17 agar (Merck, GmbH, Darmstadt, Germany) and MRS agar (Merck, GmbH, Darmstadt, Germany) were used for enumeration. LAB count at M17 agar was performed by pour plate method and 2/3 of Petri dish was filled with M17 agar. Cultures were incubated for 3d at 30°C and typical colonies were counted after incubation time [99]. The counting results were multiplied by the dilution factor and given as log CFU/g.

MRS agar was used for counting LAB at the anaerobic condition. Following inoculation, the growth mediums were incubated at 30°C for 3d [100, 101]. At the end of the incubation, typical colonies were counted and the results were multiplied by the dilution factor and given as log CFU/g. All determinations were carried out in duplicate.

3.2.2 Yield calculation

Yield calculations for cheese samples are calculated according to the following equation [102, 103].

$$Yield\ (\%) = \frac{m_{cheese}(g)}{m_{cheese}(g) + m_{whey}(g)} \times 100$$

m_{cheese}: mass of the cheese
m_{cheese}: mass of the whey

3.2.3 Physicochemical and chemical analyses in cheeses

3.2.3.1 Dry matter analysis

At 7, 30, 60, 90 and 120 days of ripening, sampling was made from both trials with two replications. The dry matter was determined by oven drying method at 103°C for about 5 hours [104].

3.2.3.2 pH determination

A digital pH meter with combined glass electrode (Radiometer Analytical, France) was used for measurements. The electrode was immersed directly in the completely mashed and homogenized cheese mass and pH level was read after reaching a fixed value.

3.2.3.3 Fat analysis

The fat determination was made according to the Gerber method by using Van Gulik butyrometer [105].

3.2.3.4 Salt determination

The salt content of samples was determined by the potentiometric method by titration with 0.1 N $AgNO_3$ [106].

3.2.3.5 Titratable acidity analysis

The titration acidity was determined in according to the method given in TS 591 for White Cheese Standard, as a percentage of % lactic acid (% L.a) [107].

3.2.3.6 Protein analysis

The total nitrogen content was determined by the Kjeldahl method according to the IDF method. The percentage of the protein value was calculated by multiplying the obtained total nitrogen content by the factor of 6.38 [108].

3.2.4 Assessment of proteolysis

3.2.4.1 Determination of water-soluble nitrogen (WSN)

Water-soluble nitrogen was analyzed in order to calculate the maturation index in cheese samples [105, 109, 110].

For this analysis, 7 g of completely mashed cheese sample were mixed with 35 mL deionized water and homogenized at 12000 rpm. Samples were held in a 40°C water bath for 60 min. After that, samples were centrifuged at 4°C and 5000 *g* for 30 min. Fat layer from the surface was removed and the supernatant (WSN) was filtered with Whatman 113 and used for nitrogen analysis by the Kjeldahl method. Sediment (water insoluble nitrogen fraction, WISN) was freeze-dried for further analysis. The maturation index value was calculated by the following formula and the results were given as % WSN in total nitrogen (TN).

Maturation index value based on WSN;

$$WSN\ (\%TN) = \frac{WSN}{TN} \times 100$$

3.2.4.2 Soluble nitrogen analysis in 12% Trichloroacetic acid (TCA)

For this analysis, TCA (12% trichloroacetic acid) and the previously prepared WSN solution were mixed in 1:1 ratio and after keeping at room temperature for 2 hours, it was centrifuged. The supernatant was filtered using Whatman No: 42 filter paper. Supernatant was used for nitrogen analysis by the Kjeldahl method [105, 110].

The maturation index value is calculated by the following formula and the results are given as % TCA-soluble nitrogen (SN) in TN.

$$SN - TCA\ (\%TN) = \frac{Soluble\ nitrogen\ in\ TCA}{TN} \times 100$$

3.2.4.3 Determination of total free amino acid

Trinitrobenzenesulphonic acid (TNBS) method was used and analyses were performed according to Ardö and Polychroniadou [105]. The absorbance value of samples was measured at 420 nm by using Thermo Scientific Evolution 201 UV-Visible Spectrophotometer (Shanghai, China). Leucine (Leu) was used to create the calibration curve in a range of 0.05-0.50 mM. The results were expressed as mg Leu/g cheese.

3.2.4.4 Examination of proteolysis in cheese samples by urea-PAGE (Polyacrylamide Gel Electrophoresis) method

Electrophoresis was used to detect proteolysis levels in cheeses according to method of Andrews [111], Shalabi and Fox [112] and Bulat [17]. The separation gel concentration adjusted to be (T = 12.5%, C = 4%, pH 8.9). For the preparation of samples, 10 mg of the freeze-dried WISN fraction were resolved in 1 mL of electrophoresis sample buffer. 8 μL of the prepared samples were loaded into the gels. Protean II XL vertical gel unit system (Bio-Rad Laboratories Ltd., Watford, UK) was used for gel running. Before loading samples, the gels subjected to the run at 280 V for 40 min and then after loading samples, then a constant voltage of 300 V was applied. Obtained gels were held in the dye solution for overnight and protein bands were stained [113] after that, were left in pure water for 1 night for destaining. The gels were scanned using an Agfa Arcus 1200 scanner controlled with Agfa Fotolook v3.5. Changes in the bands of casein fractions were densitometrically determined by using TotalLab Quant v12.2 (TotalLab Limites, Newcastle upon Tyne, UK).

3.2.4.5 Determination of proteolysis in cheese samples by RP-HPLC

The previously prepared WSN fraction was mixed with deionized water containing 0.2% TFA (trifluoroacetic acid, v/v) in a 1:1 ratio. The mixture was taken up in HPLC vials after filtration by 0.45μm filter and was analysed. For this analysis, ThermoFinnigan SpectraSystem HPLC system (ThermoFinnigan Inc., CA, USA) was used. The system components were given at Table 3.1.

Table 3.1. The RP-HPLC system used for determining proteolysis in cheese samples

Sampler:	Autosampler (AS3000) with 100μL sample injection volume and 30°C column temperature
Degasser:	SCM 1000
Pump:	P4000 gradient pump
Detector:	UV 6000LP DAD

Chromatographic separation was performed by using Phenomenex Jupiter C18 (250×4.6 mm, 5 μm, 300 A^o) reverse phase column (Phenomenex, Torrance, CA, USA). The elution was performed with a gradient [110]. Measurements were performed at 214 and 280 nm. The data were evaluated using the ChromQuest 5.0 software.

3.2.5 Free fatty acid extraction and analysis

In cheese samples, FFAs were extracted according to De Jong and Badings method [114] with some modifications [17, 143]. Briefly, 4 g of homogenized cheese samples and 12 g anhydrous sodium sulphate (Merck) were mixed until a homogenous mixture was obtained. 0.3 mL 2.5 M H_2SO_4, and 1 mL internal standard (a mixture of pentanoic acid ($C_{5:0}$), nonanoic acid ($C_{9:0}$) and heptadecanoic acid ($C_{17:0}$)), and 15 mL diethyl ether/heptane was added. The prepared mixture was vortexed for 1 min and then centrifuged in 500 g at 4°C. The supernatant was transferred to the Schott bottles. This step was repeated 2 more times and the supernatant was collected in the same bottle. Prepared supernatants (with a volume of approximately 45 mL) were passed through solid phase extraction aminopropyl column (500 mg/3 mL Strata NH_2 (55µm, 70 A^o), Phenomenex, California, USA) that conditionalized by using 10 mL heptane. In the next step, 20 ml of hexane/2-propanol (3:2, v/v) were passed through the column to remove neutral triacylglycerols. At the end, the fatty acids bounded to aminopropyl column were eluted into the vials by passing 4 mL ether solution containing 3% formic acid. The extracted FFAs were analyzed by ThermoScientific TRACE 1310 GC and data were evaluated by ChromQuest 5.0. Analysis conditions and GC system properties are given in Table 3.2.

Table 3.2. The GC system properties and conditions in used for determining FFA in cheese samples

Sampler:	Autosampler (ThermoScientific TriPlus RSH, Switzerland)
Column type:	TR-FFAP column (30 m length × 0.25 mm inner diameter × 0.25 µm film thickness, Thermo Fisher Scientific, Bellefonte PA, USA)
Mobile phase:	Helium
Flow rate:	2 mL/min
Split ratio:	20:1
Injection temperature:	250°C
Detector:	Flame ionisation detector (FID), 260°C
Separation temperature:	The column temperature was initiated at 90°C, and after holding at this temperature for 1 min, it was increased to 240°C (10°C/min) and kept at this temperature for 10 min.

3.2.6 Organic acid and sugar analysis

Cheese sample (5 g) were homogenized in 25 ml 0.013 N H_2SO_4, then centrifuged at 5000 g at 4 ^oC for 15 min supernatant filtered through a 0.45µm filter and analyzed by HPLC. For this analysis, ThermoFinnigan SpectraSystem HPLC (ThermoFinnigan Inc., CA, USA) was used. Citric, pyruvic, lactic, formic and acetic acid were measured at 210 nm wavelength and orotic, uric acid and acetoin were measured at 280 nm wavelength. Analyses are performed isocratically [115, 116]. Calculation of the associated organic acid peaks was made according to Zeppa et al [117]. For identification of organic acid and sugar amount in each sample, standards with high purity were used. Citric acid, lactic acid, pyruvic acid, formic acid, uric acid, acetoin, glucose, lactose and galactose which were used as a standard, were supplied from Fluka, Sigma and Merck. The HPLC system properties and conditions used for organic acid and sugar analysis are given in the Table 3.3.

Table 3.3. The HPLC system properties and conditions in used for determining organic acids in cheese samples

Sampler:	AS3000 Autosampler
Pump:	P4000 gradient pump
Column type:	Rezex ROA H^+ column (300×7.8 mm. ID), Phenomenex, USA
Column temperature:	65°C
Mobil phase:	0.013 N H_2SO_4
Flow rate:	0.50 mL/min
Detector (for organic acid):	Photo-diode Array (PDA) 210 nm and 280 nm,
Detector (for Sugar)	Refractive index (RI), Shodex RI-101 (Showa Denko, NY, USA)

3.2.7 Volatile component analysis

The SPME (Solid Phase Microextraction) method was used to extract the volatile compounds of the cheese samples. For this analysis, 3 g of cheese samples were taken in vials (20 mL) and 80 µL internal standard (2-methyl-3-pentanone with a concentration of 20 ppm) was added to cheese samples. DVB/CAR/PDMS (50/30 µm, 1 cm StableFlex fiber, divinylbenzene/Carboxen/polydimethylsiloxane, Supelco, Bellefonte, PA, USA) was used as the SPME fibre. For this analysis, ThermoScientific ISQ-QD GC-MS system (ThermoFisher Scientific, USA) was used. The cheese sample in the vial was equilibrated for 30 min at 45 °C, and then SPME was held in vial headspace for 30 min (45 °C) for the

adsorption of the volatile component. The properties of the system and the condition of the analysis are given in Table 3.4 [118, 119]. The WILEY, NIST GC-MS library and external standard were used for the identification process. The retention index of peaks was calculated by using C_8-C_{20} alkane standard (Supelco, Bellefonte, PA, USA). The results are given as peak area of the volatile component/peak area of the internal standard. GC-MS system properties and conditions for determining volatile components are given at Table 3.4.

Table 3.4. GC-MS system properties and conditions for determining the volatile components

Sampler:	Autosampler (ThermoScientific TriPlus RSH, Switzerland)
Desorption:	The SPME fibre was held at 260°C for 3 min.
Injector	Splitless mode
Detector:	ISQ-QD MS
Column:	TR-WaxMS column (60 m length × 0.25 mm inner diameter × 0.25 µm film thickness, Thermo Fisher Scientific, Bellefonte PA, USA)
Carrier gas*:*	Helium (1 mL/min)
Mass range:	35-350 m/z (mass/charge)
Temperature program:	After waiting at 40 °C for 10 min, it was increased to 250°C at a rate of 5°C/min and was kept at this temperature for 10 min.
Ion source temperature:	260°C
Transfer line temperature:	260°C

3.2.8 Texture profile analysis

For instrumental textural profile analysis (TPA), TA Plus Texture Analyzer device (Ametek Lloyd Ins. Ltd., UK) was used. Hardness, Springiness, Gumminess, Chewiness, surface adhesiveness and Cohesiveness properties of the cheese samples were examined during ripening. Cheese samples were cut into 25 mm diameter cylinders and analyzed at ambient temperature. The diameter of the cylindrical probe in used was 10 mm and the velocity of that was adjusted to 0.5 mm/s. Two consecutive compression operations were applied in the texture analysis and the compression ratio was set at 33% [110]. Texture analysis parameters of the cheese mass were calculated from the obtained graphic. These parameters are explained below.

- Hardness: The maximum force (N) applied in the first compression.
- Springiness: The rate of returning the sample to its original size after the first compression (mm).
- Chewiness: The chewing force required to make a solid food ready to swallow (Nmm)
- Gumminess: The breaking force (N) required to prepare a semi-solid food for swallowing
- Adhesiveness: The force (Nmm) required to overcome the adhesive force between food and probe.
- Cohesiveness: The force between internal bonds of the food samples [120, 121].

3.2.9 Sensory evaluations

Sensory evaluation of the cheese samples was carried out by 5 panelists. Three panelists were from Hacettepe University Food Engineering Department and other two panelists were from Bahcivan Dairy Company. Cheeses have been assessed for their appearance, structure, smell, taste, bitterness level and total acceptability (overall impression). Sensory evaluation scores for cheese trials are given in Appendix 1. The scoring test technique was applied for total acceptability levels (overall impression level) from "don't like (1)" to "very like (5)". The intensity of bitterness was also evaluated and scored on a scale from "0 (not bitter)" to "4 (very strongly bitter)" [17, 110].

3.2.10 Statistical evaluation of research results

SPSS 16.0 program was used in the evaluation of the data obtained in this study. The significance control between variances differences was determined by using ANOVA.

Principle component analysis (PCA) was applied to the data obtained from RP-HPLC chromatograms of WSN fractions of the cheeses. The applied numerical modification for this purpose is described by Piraino et al [122]. Briefly, HPLC chromatograms (The height of the peaks at 214 nm/peak area in 280 nm and retention time) were divided into 91 sections with a class width of 1 min and chromatograms between 5 and 90 min were taken for analysis. The shape parameter was set to 248.6. PCA analysis was calculated as the covariance matrix.

4. RESULTS AND DISCUSSION

In this section, experimental results of milk and cheese are presented. Results are also discussed in detail.

4.1 Compositional Properties of UF Cheese Milk

Compositional properties of the UF milk used to manufacture of UF white cheese were given at Table 4.1. There is an increase of 3.5x fold in protein and fat content compared with standard cheese milk which is extremely important for cheese yield.

Table 4.1. Component properties of the UF milk used in the production of white cheese

Properties	Values
Fat (%)	14.5±0.7
Protein (%)	11.8±0.6
Lactose (%)	4.28±0.1
Dry matter (%)	32.71±0.2
Brix	31.7±0.4
pH	6.52±0.1

4.2 Microbiological Results of UF White Cheese

LAB counts in cheese samples were carried out by using M17 agar and MRS agar growth medium and obtained results are given in Table 4.2. Generally, M17 agar is used for enumeration of starter lactococci by authors. In our study, viable numbers of lactococci on M17 agar at day 7 of ripening, in A and B cheeses, were 7.66 and 8.47 log CFU/g, respectively (Figure 4.1). It has been observed as a decreasing trend during the ripening. At 120 d of ripening the numbers of lactococci was 5.11 and 6.90 log CFU/g for the cheese A and B, respectively. Almost 2.5 log cycle decrease was observed in cheese A while there was only 1.5 log cycle decrease in cheese B.

The addition of the adjunct culture was found a significant effect on the LAB count between two trials at 30, 60, 90 and 120 days of ripening ($P < 0.05$). Changes of Lactococci number in cheese samples were also statistically significant ($P < 0.05$).

Table 4.2. Mean values of the LAB counts of white UF cheese at M17 agar and MRS agar, during the ripening (log CFU / g cheese)

Samples	Ripening (Days)	M17 agar	MRS agar
Cheese A	7	7.66±0.48	6.18±0.04
	30	6.37±0.42	5.53±0.20
	60	6.36±0.12	4.98±0.11
	90	6.56±0.10	3.38±0.35
	120	5.11±0.00	3.21±0.19
Cheese B	7	8.47±0.09	7.68±0.45
	30	8.17±0.15	8.19±0.08
	60	7.58±0.10	7.70±0.27
	90	7.27±0.04	7.16±0.02
	120	6.90±1.27	7.27±0.13

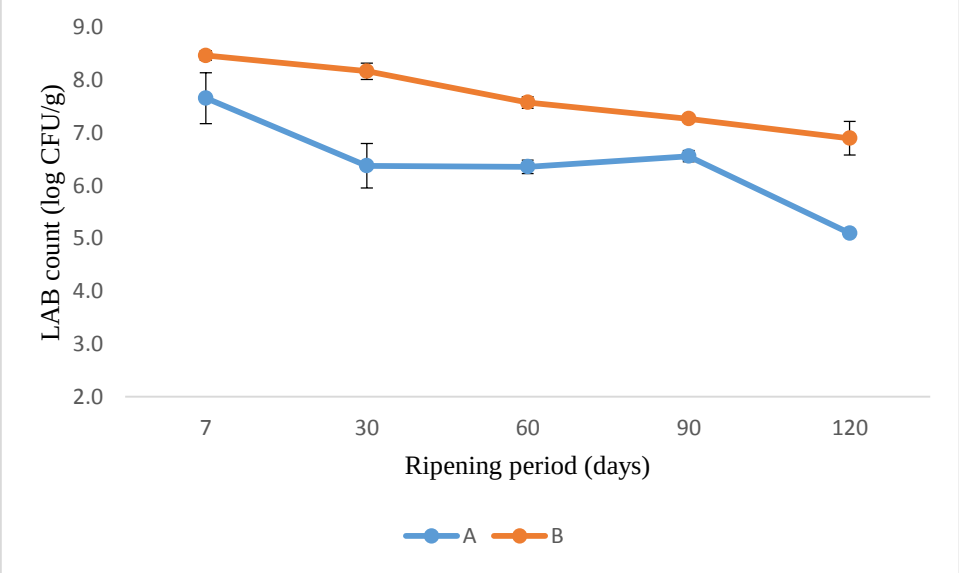

Figure 4.1. LAB (log CFU/g) on M17 agar medium during ripening period

As it can be seen from Figure 4.1, the amount of bacterial count of cheese B was higher than those of cheese A at all stages of ripening. This is entirely logical because cheese B contains adjunct culture in addition to starter culture so the amount of lactococci in cheese B shows the higher quantity.

Lactococcus, *Lactobacillus* and *Leuconostoc* subsp. can be grown at MRS agar medium [124]. LAB count of control cheese on MRS agar medium was found 6.18 (log CFU/g) at the day 7 for cheese A. This value decreased almost to half at 120 d of ripening. The effect of aging was found significant in LAB count of control cheeses at MRS agar medium ($P < 0.05$). LAB count for cheese B was found more stable; it was found 7.68 (log CFU/g) at day 7 of ripening and this value decreased to 7.27 (log CFU/g) at day 120 (Table 4.2, Figure 4.2). The high aminopeptidasic activity of CR-319 adjunct culture may produce amino acids and peptides that may stimulate the growth or survival of starter cocci, and lactobacilli during ripening.

By statistical evaluation of the obtained data, it was found that the addition of the adjunct culture had a significant effect on the LAB count between two trials on MRS agar medium ($P < 0.05$). The changes in LAB count of cheese A during ripening was also statistically important ($P < 0.05$).

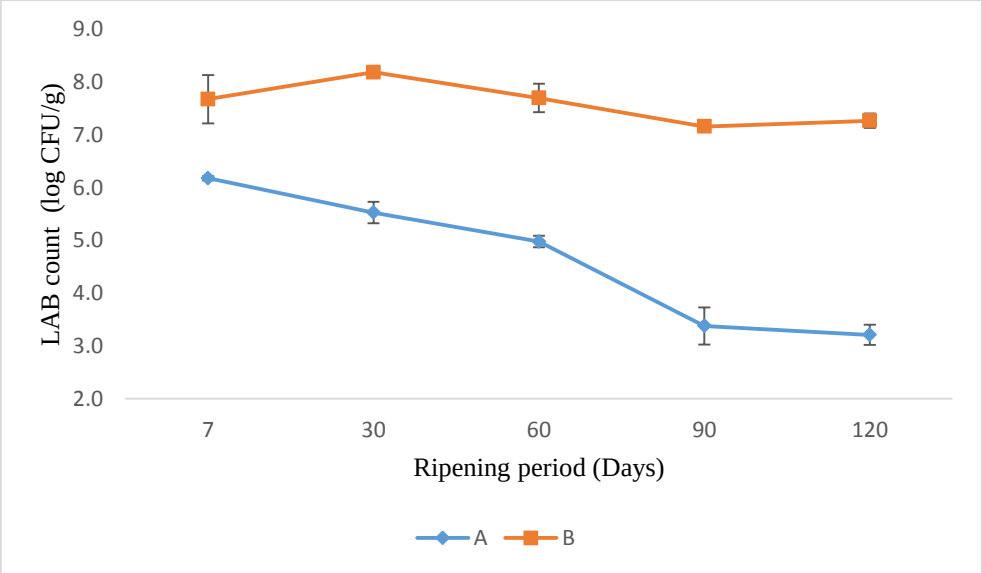

Figure 4.2. LAB (log CFU/g) on MRS agar medium during ripening period

4.3 Physicochemical and Chemical Analysis Results

During the ripening period, mean values of compositional results of UF cheese samples are given separately, as follows

4.3.1.1 pH values of the cheese samples

The mean pH values for control cheese and cheese with adjunct culture on day 7 of ripening were 4.54±0.01 and 4.64±0.01 respectively. These values were changed to 4.56±0.00 and 4.69±0.01 for A and B cheese after 120 d of ripening. Cheeses with adjunct culture were shown higher pH level than control cheese at all stages of ripening ($P < 0.05$). Slight increases and decreases in pH values of the both trials during the ripening period were identified probably depending on buffering capacity. During the UF concentration, mineral salts attach to the casein micelle (Ca, Mg, P) and buffering capacity of retentate increases. Hence, it may prevent significant changes in pH value during ripening [37]. Salt diffusion into the cheese may also cause those elevations. The pH results of our study are higher than Karami et al [125] which they found between 4.20-4.30, but in agreement with Soltani et al [126]. It can be related to specific buffering properties of UF cheese. Changing in pH values of cheese A and B during ripening days is shown in Figure 4.3.

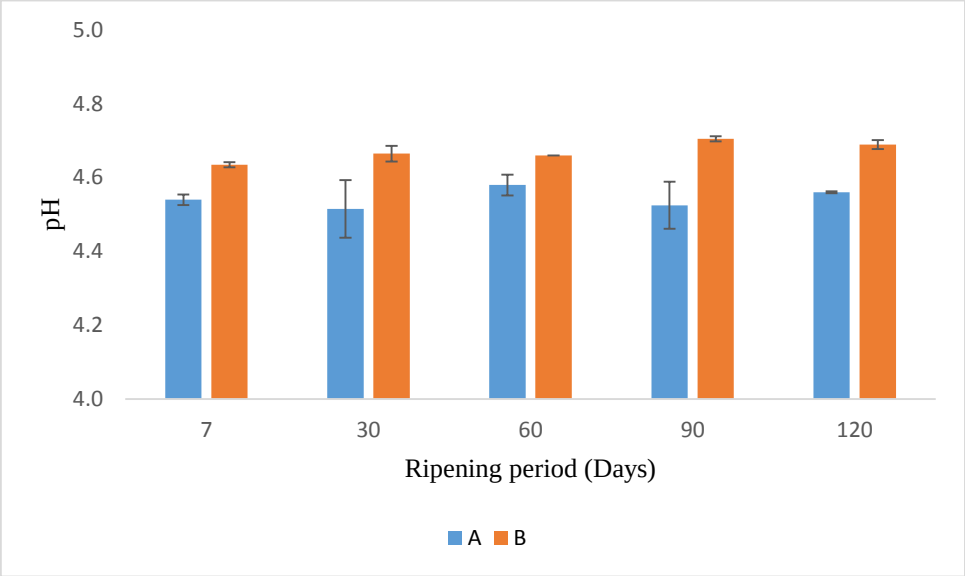

Figure 4.3. Changing in pH values of cheese A and B during ripening

As it can be concluded from Figure 4.3, cheese with adjunct culture shows higher pH value in all days of ripening in compared with control cheese. It might be attributed to the difference in proteolysis level between cheese A and B. Hydrolysis of proteins to peptides and then amino acids and consequently liberation of alkaline compounds such as NH_3 during ripening has an important impact on increasing in pH value of the cheese. [126,127]. Adjunct culture may also suppress the metabolic activities of starter LAB.

4.3.1.2 Results of the titratable acidity of the cheese samples

The changes in titratable acidity values of the UF white cheese samples during ripening days are shown in Figure 4.4. Titratable acidity values in cheeses A were between 0.88±0.02 and 1.17±0.03 as % of lactic acid (% L.a). In both trials, fluctuations in titratable acidity were observed during ripening days but there was a slightly increasing trend of % L.a for both cheese trials. However, in cheese A, a higher value of % L.a was observed than cheese B. The differences between both trials were found significant at 30 and 120 days of ripening ($P < 0.05$).

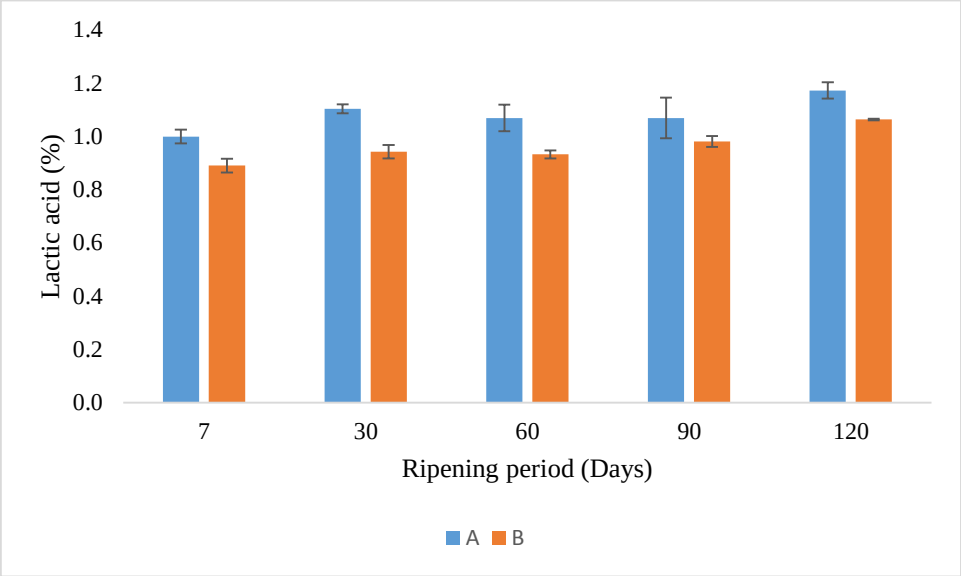

Figure 4.4. Titratable acidity (% L.a) values of the UF white cheese samples during ripening

Fermentation of lactose in soft cheese and conversion of that to lactic acid is fast in first days of ripening and then it becomes slower towards the end of the ripening period because the amount of lactose diminish during ripening and this conversion can even completely stop at last days [128]. However, we did not observe any complete diminish in lactose content for all samples. The high osmotic pressure of UF cheese may limit the acid production.

4.3.1.3 Dry matter (DM) values of the cheese samples

DM (%) values of the UF cheese samples during ripening vary between 34.20±0.35 and 36.59±0.93. The minimum value belongs the day 7 of the B sample and maximum value belongs to day 120 of the control cheese. As it can be concluded from Figure 4.5, the DM values of the both cheese was approximately same at the 7 d of the ripening but after 120

days DM of sample A, showed the higher amount in compared with cheese B. It means that cheese with adjunct culture contains more moisture content in compared with control cheese at the end of the ripening period. The differences between groups and days were not significant ($P > 0.05$). The high moisture content causes that the excess amount of coagulant enzyme retain in the cheese structure and it leads to more hydrolysis of the proteins and peptides. As previously mentioned, the release of amino acids can raise the pH value [129] and it can lead to increase of water absorption of the cheese structure [19]. Hence according to high pH value and high liberation of amino acids in cheese B (the results will be shown in next section) differences in DM (%) values between cheese A and B are logical and it is in good agreement with results reported by Karami et al [125]. Also, Poveda et al had been evaluated the effect of adjunct culture on Manchego cheese; they have reported that the trials which contained *Lactobacillus paracasei* subsp. *paracasei* as an adjunct culture, showed high proteolysis level and less DM (%) as compared to control cheese [130]. Increasing in moisture content of the Feta cheese with adjunct culture were also reported by Kumar et al [131].

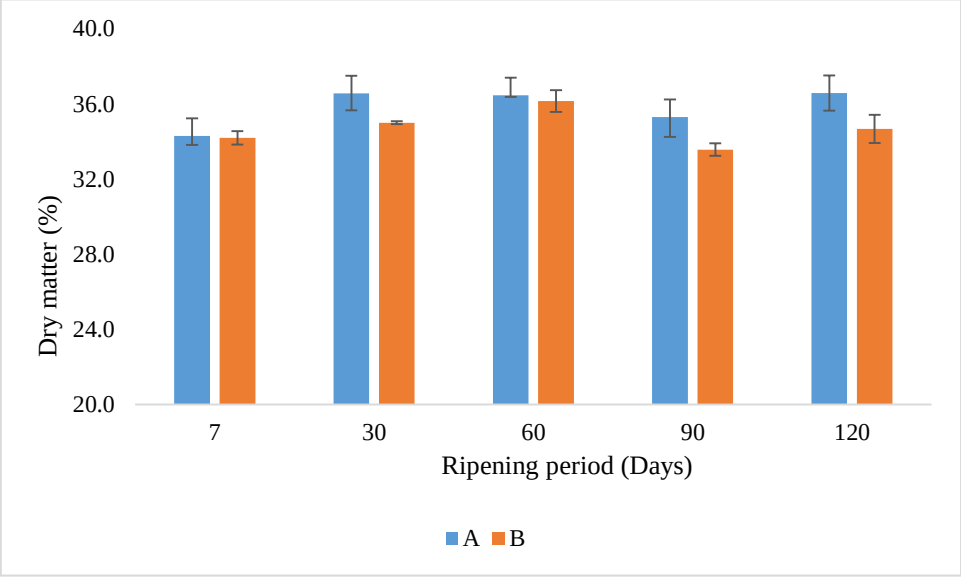

Figure 4.5. Dry matter (% DM) values of cheese A and Cheese B during ripening

4.3.1.4 Fat in dry matter (%) values of the cheese samples

The mean Fat/DM (%) values of the cheese samples are shown in Figure 4.6. Fat/DM (%) of the cheese samples varies between 43.07±2.07 and 45.64±0.72 during ripening days.

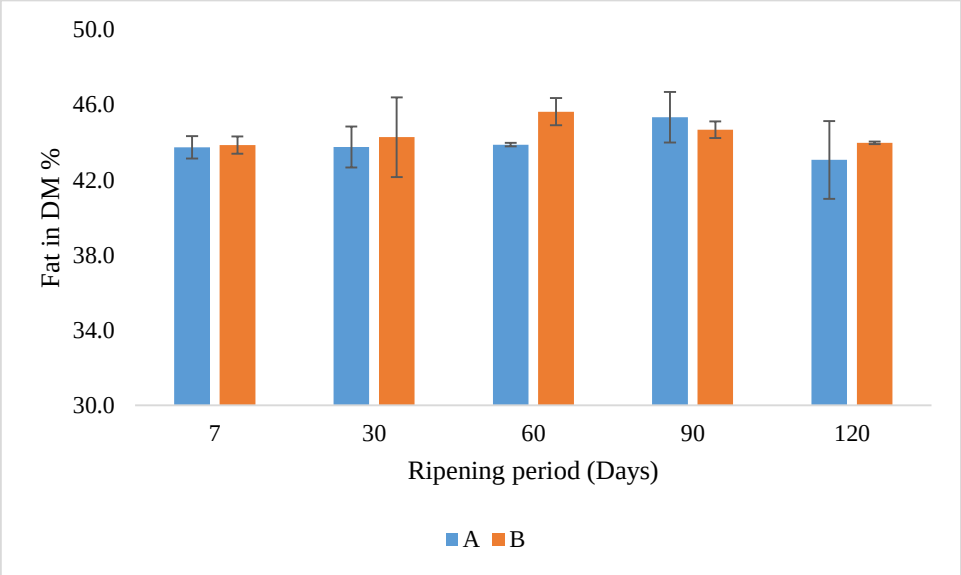

Figure 4.6. Fat in dry matter (%) values of the cheese samples during ripening

According to Figure 4.6, there is a fluctuation during ripening in fat in DM values and after 120 days of ripening the amount of fat in DM in cheese B is higher than control cheese. The difference between trials was not found significant ($P > 0.05$). Change in the fat content of two cheeses that are produced with different cultures is normal. The change in fat in DM values can be related to differences between moisture content of the samples.

4.3.1.5 Salt in DM (%) values of the cheese samples

According to Turkish white cheese codex, maximum salt in DM of the white cheese should not be more than 6.5 %. As it can be seen from Figure 4.7, the salt in DM percentages of the cheese samples is between 5.66±0.16 and 6.11±0.02 during the ripening period. The salt in DM (%) values after 120 days are approximately same in both trials. According to Figure 4.7, the salt in DM (%) value increased and decreased during ripening days. It can be related with the changes in DM (%) values of the samples during ripening or salt diffusion. The difference between cheeses was not found significant ($P > 0.05$) except at day 90 that difference was found significant ($P < 0.05$). Also, ripening days had no significant effect on salt in DM (%) values ($P > 0.05$).

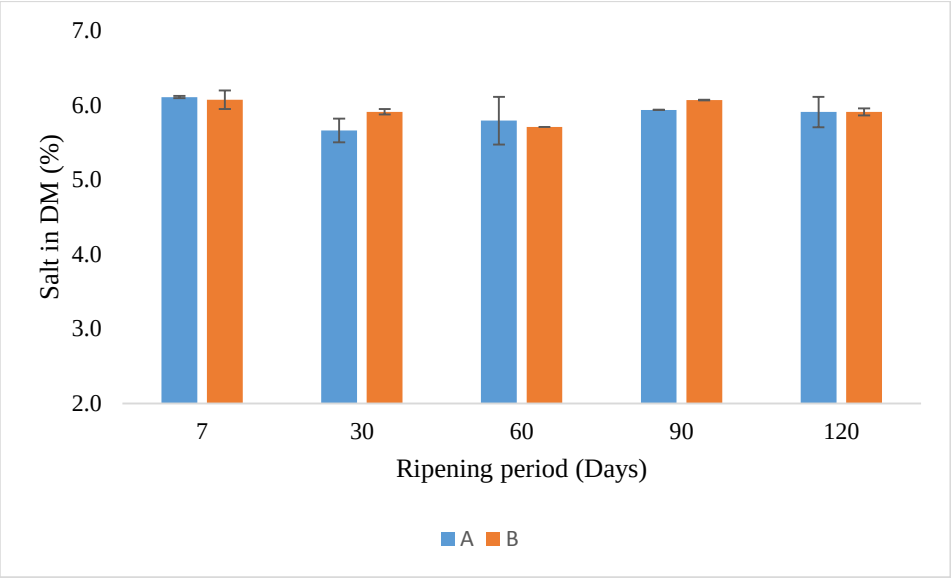

Figure 4.7. Salt in dry matter (%) values of cheese samples during ripening

4.3.1.6 Total protein (%) values of the cheese samples

Detection of % protein level in cheese samples is an important criterion in determining the proteolysis level in cheese during the ripening period. Figure 4.8 is demonstrates protein level changes during the storage. According to the Figure 4.8, despite the slightly high level of the total protein level of cheese with adjunct culture in compared with control cheese, the level of both trials have not shown significant differences ($P > 0.05$). According to proteolytic properties of CR-319 commercial culture, which noted that it has not significant effect on the first stage of proteolysis, these results are normal and logical. To understand the effect of adjunct culture on the second stage of proteolysis, WSN and TCA soluble nitrogenous substance of the cheeses were analyzed during ripening.

In cheese samples, protein contents varied from 11.89±0.06 to 12.33±0.06. As it can be concluded from Figure 4.8, protein values show some fluctuations during ripening. These may be related with the diffusion of some water soluble peptides into the whey.

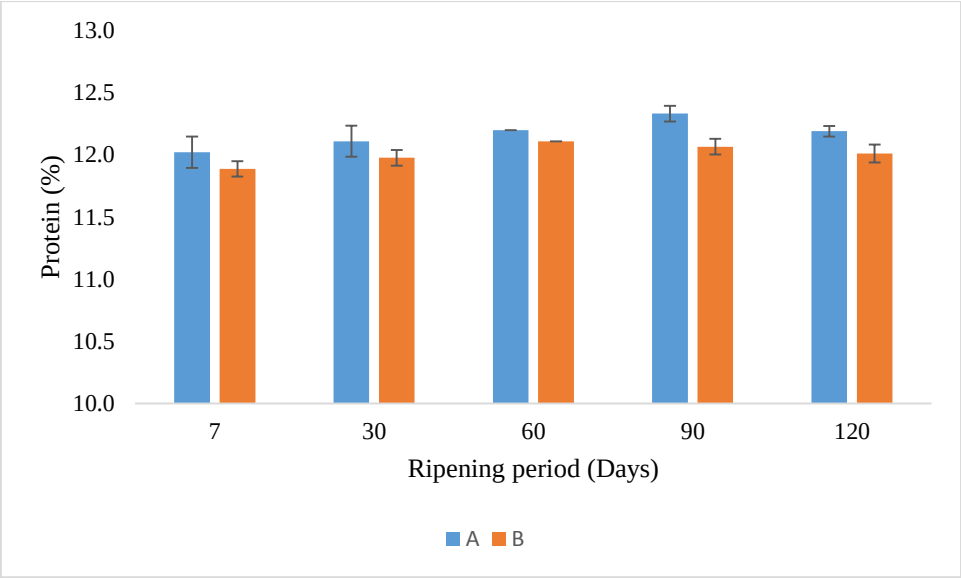

Figure 4.8. Protein (%) values of cheese samples during ripening

4.4 Yield (%) of the Cheese Samples

Figure 4.9 demonstrating yield (%) values of the cheese trials during various ripening days.

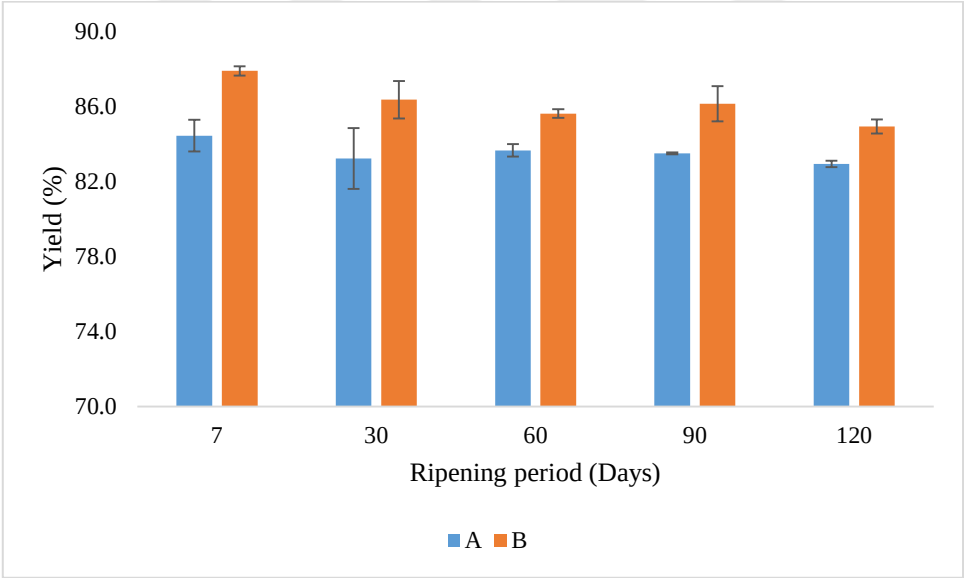

Figure 4.9. Yield (%) values of the cheese samples during ripening

As it can be concluded from Figure 4.9, the yield (%) of the cheese with adjunct culture, at the end of the ripening, is higher than control cheese. The reason of this can be high pH value of the cheese with adjunct culture which can keep more water in cheese mass. In addition, high level proteolysis can increase small peptides which have water binding ability. The differences between yield results of cheese A and B were found significant during 7, 30, 60, 90 and 120 days of ripening ($P < 0.05$).

4.5 Assessment of Proteolysis

Various analyses were performed for determining proteolysis level of cheese samples. For this purpose, WSN fraction analysis, 12% TCA-SN analysis, total free FAA analysis, urea-PAGE Electrophoresis of water insoluble fraction and RP-HPLC analysis of the WSN were performed. The result of WSN fraction, 12% TCA-SN and total FAA are shown in Table 4.3. All proteolysis results are evaluated in following pages separately.

Table 4.3. The mean values of soluble nitrogen fractions in cheese A (control) and cheese B (adjunct culture contained) during ripening

Analysis	Ripening (Days)	A	B
WSN (% of TN)	7	13.15±0.92	13.48±0.07
	30	14.89±0.93	15.43±0.18
	60	15.69±0.00	15.81±0.52
	90	16.79±0.85	18.82±0.62
	120	17.87±0.67	20.30±0.11
12% TCA-SN (% of TN)	7	6.41±0.43	6.52±0.39
	30	8.13±0.24	8.10±0.17
	60	8.80±0.05	9.04±0.10
	90	9.86±0.31	10.37±0.21
	120	11.01±0.11	11.62±0.43
Total FAA (mg Leu/g cheese)	7	2.30±0.42	2.75±0.26
	30	3.06±0.03	3.01±0.05
	60	3.51±0.0	3.53±0.08
	90	3.93±0.24	4.08±0.09
	120	4.59±0.20	5.06±0.40

WSN: Water Soluble Nitrogen, **TCA-SN:** Trichloroaceticacid Soluble Nitrogen, **TN:** Total Nitrogen, **FAA:** Free Amino Acid

4.5.1 Analysis of water soluble nitrogen fractions (WSN)

WSN fraction is used to evaluate the peptides with high molecular weight mostly obtained by primary proteolysis and degradation of casein micelle [132]. The peptides in this fraction are mostly obtained by the action of chymosin and in less amount of plasmin on casein submicelles [66]. The pH, temperature, moisture content of the cheese and differences in drainage pH values has an effect on WSN values during the manufacturing

and ripening. WSN is a parameter that gives important clues about ripening tendency in terms of cheese texture and flavour, which are developed by proteolysis during ripening.

WSN results of UF cheese samples (Table 4.3) varies between 13.48±0.07 and 20.30±0.11 which is in agreement with results reported by Soltani et al [126]. In the production of the UF white cheese, all starter culture and coagulant enzymes remain in cheese structure due to the absence of curd cutting and whey drainage steps. As it can be also seen from Figure 4.10, the ripening index in both trials increased during the storage. At day 60, cheese B has shown more proteolysis level compared with control cheese A. Differences between trials are not significant at 7, 30, 60 and 90 days ($P > 0.05$). The differences at day 120 were found significant ($P < 0.05$). These results are in agreement with data reported by Michaelidou et al [133]. They studied the proteolytic effect of adjunct culture containing *L. lactis* subsp. *cremoris* and subsp. *lactis* on low-fat Feta cheese and concluded that WSN increased during ripening until 120 days and then got stable until 180 days of ripening. They found no significant differences in WSN analysis between trials.

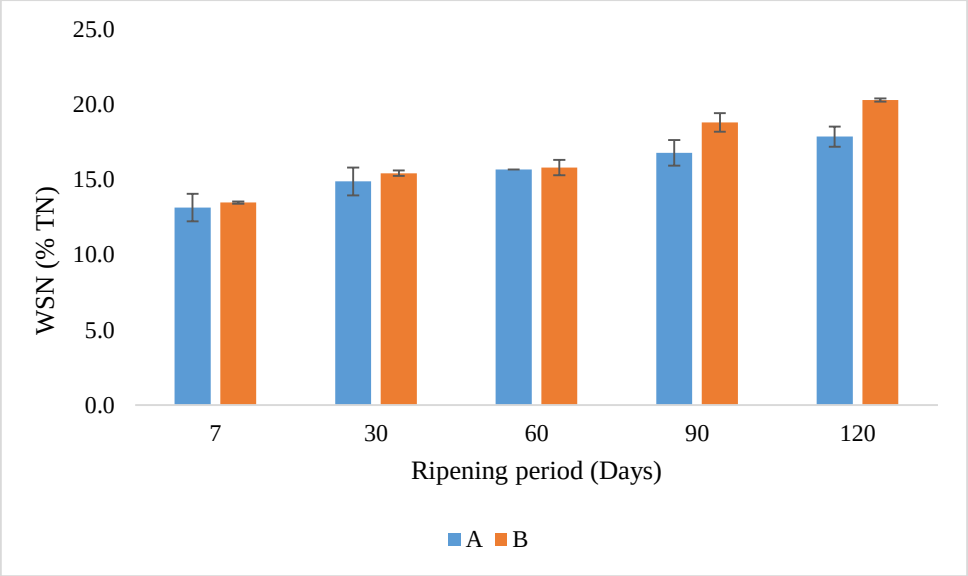

Figure 4.10. Changes of WSN fraction of cheese samples during ripening

4.5.2 Analysis of 12%trichloroacetic acid soluble nitrogen fractions (TCA-SN)

The evaluation of 12% TCA-SN fraction mainly determines the amount of the amino acids and/or low molecular weight peptides (usually between 2-22 amino acid) accumulated during ripening period by the action of protease and peptidase of the LAB, NSLAB, adjunct cultures, or residual milk indigenous enzymes [19, 134, 135]. Nitrogenous substances in term of 12% TCA fraction mainly resulted from the secondary proteolysis.

TCA soluble fractions mostly are results of the degradation of α_{s1}-casein. It has been reported that starter proteinases hydrolyze peptide bonds between Gln_9-Gly_{10} and Gln_{13}-Glu_{14} in the polypeptides and peptides generated from the action of chymosin and plasmin to form a FAA and low molecular weight peptides [66]. Changes of the TCA-SN fraction during ripening period are given in Figure 4.11. As it can be seen, there is an increasing trend of TCA-SN during the ripening for both cheese samples. The effect of ripening on TCA-SN level for both trials were found significant ($P<0.05$). The TCA-SN level in experimental cheeses was affected by adjunct addition at the end of ripening probably depending on aminopeptidase activity. However, no significant differences existed between treatments (except at 120 d). The results are in agreement with other studies [126, 133, 143].

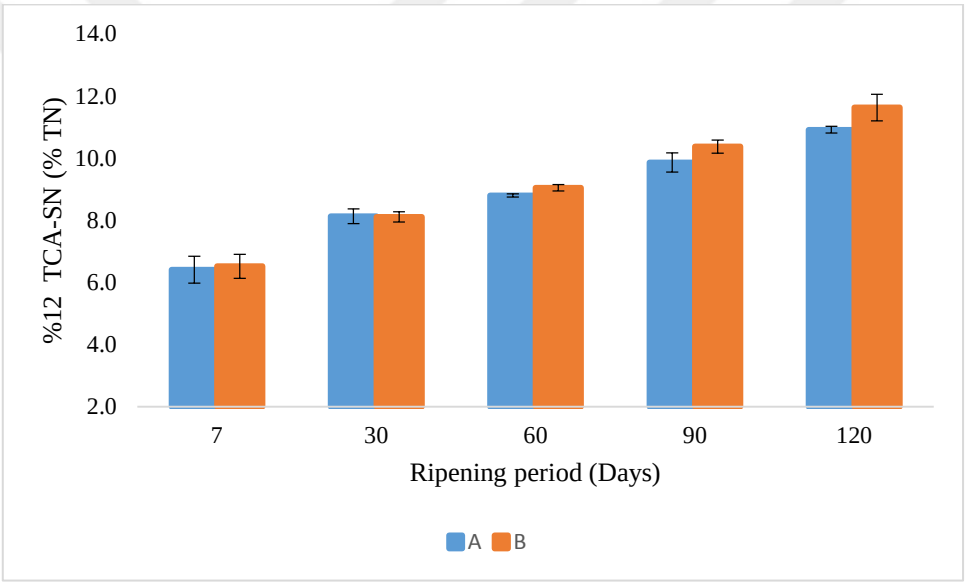

Figure 4.11. Changes of %12 TCA-SN fraction of cheese samples during ripening

4.5.3 Analysis of total free amino acids

The total FAA values of the cheese samples are given in Figure 4.12. By examining the figure, it can be seen that the total free amino acid content of both trials increases during ripening. In generally, the trend of FAA values was in agreement to that observed for TCA-SN. These values for both cheeses were close until 90 days. The total FAA of cheese B shows higher amount than cheese A at 90 and 120 days. It can be related to aminopeptidase activity of adjunct culture. Lynch et al [136], reported an increase in total FAA after 90 days and can be related to peptidase activity of adjunct culture or reduction in utilization of FAA by bacteria after 90 days, due to a reduction in viable bacteria count.

Differences between trials were not significant (P > 0.05) except at 120 d of ripening. In many studies, using of adjunct culture has been reported to cause an increase free amino acid formation in cheeses [133, 137, 138].

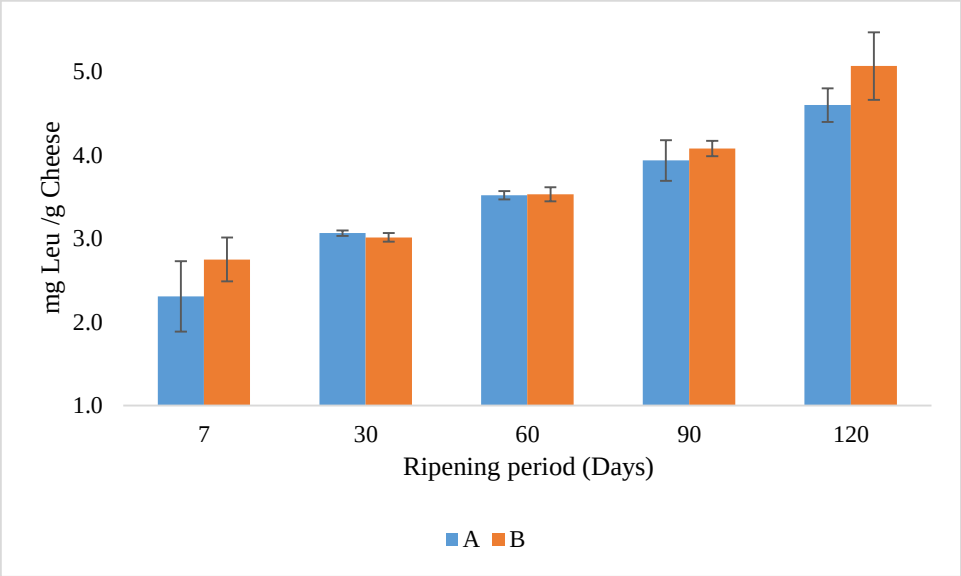

Figure 4.12. Change of total free amino acid concentrations (mg Leu/g cheese) of the cheese samples during ripening

4.5.4 Results of Urea-PAGE electrophoresis analysis of the cheese samples

The electrophoretograms of the cheese trials that performed to determine the proteolysis at 7, 30, 60, 90 and 120 days of ripening are given at Figure 4.13 and 4.14.

Proteolysis, which occurs in cheese during ripening can be carried out by the action of coagulant (e.g. chymosin), indigenous proteinase of the milk (e.g. plasmin), proteinase and peptidase of LAB, proteolytic enzymes of the NSLAB and proteolytic enzymes and exogenous proteinases and peptidases of the secondary and adjunct cultures. Casein micelles, with dependent on the type of the proteolytic enzyme, were degraded to long and medium peptides and then these peptides were hydrolyzed to short peptide and amino acids by exogenous proteinases and peptidases of the NSLAB, secondary, and adjunct cultures [61]. Proteolysis in this study was thought to be the result of residual coagulant, indigenous proteinase in the milk and proteinase and peptidase of the starter and adjunct culture.

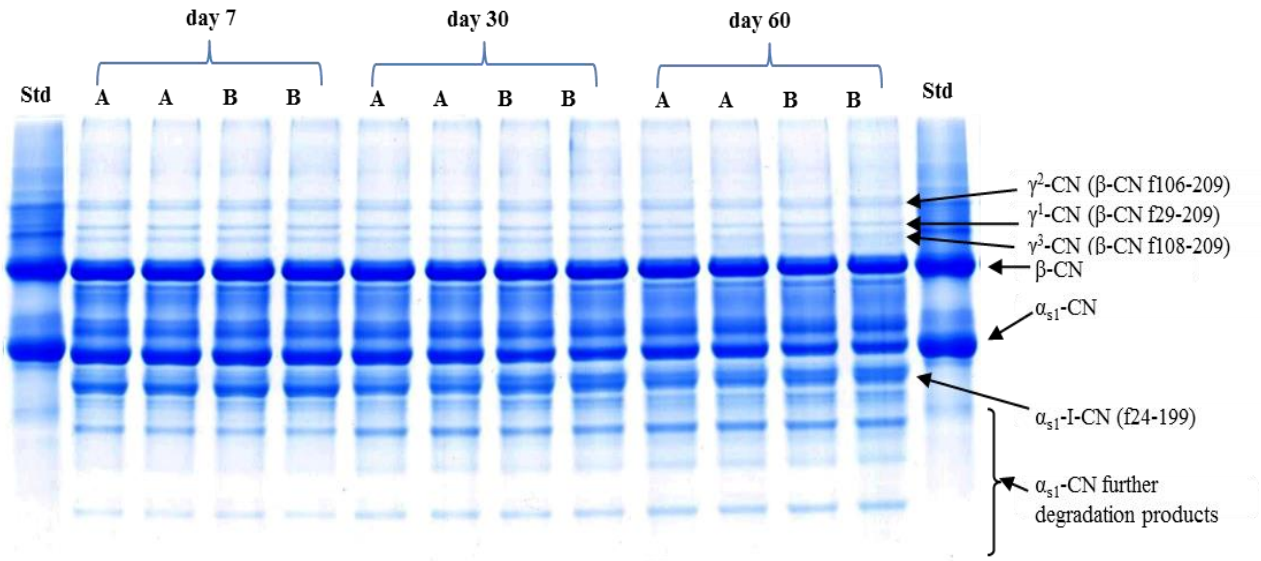

Figure 4.13. Urea-PAGE electrophoretograms of the 7, 30, 60 days of ripening (Std: Standard (sodium caseinate), A: control UF cheese, B: adjunct culture contained UF cheese) (identification of bands have done according to [105])

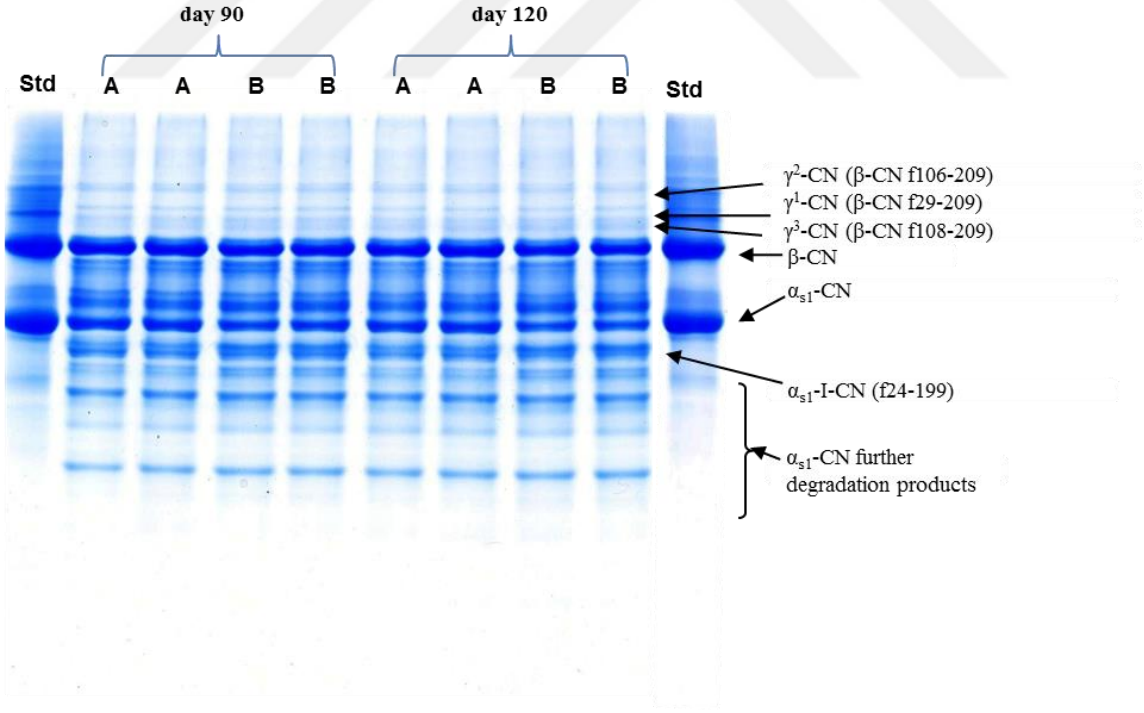

Figure 4.14. Urea-PAGE electrophoretograms of the 90 and 120 days of ripening (Std: Standard (sodium caseinate), A: control UF cheese, B: adjunct culture contained UF cheese) (identification of bands have done according to [105])

The electrophoretograms were also evaluated densitometrically and the changes of the residual β-casein and α_{s1}-casein (%) at 7, 30, 60, 90 and 120 days of ripening were given in Table 4.4.

Table 4.4. The mean values of residual β-casein and α_{s1}-casein during ripening

	Ripening (Days)	**Cheese A**	**Cheese B**
Residual β-casein (%)	7	100±0.00	100±0.00
	30	94.21±1.15	88.81±1.78
	60	87.07±1.16	78.59±1.30
	90	86.75±4.96	79.04±1.32
	120	81.44±3.20	69.65±3.52
Residual α_{s1}-casein (%)	7	100±0.00	100±0.00
	30	85.58±3.32	73.01±4.61
	60	73.82±0.11	59.44±0.16
	90	74.68±4.70	59.57±0.95
	120	65.23±3.06	46.51±2.40

The changes in the residual β-casein (%) at 7, 30, 60, 90 and 120 days of ripening are shown at Figure 4.15. Degradation level at cheese B was higher than cheese A at 7, 30, 90 and 120 days of ripening and changes were found statistically significant ($P < 0.05$) for both trial during the ripening period.

Maximum degradation levels of the β-casein fraction for cheese A and cheese B were found at 120 days of ripening as expected. The residual β-casein fraction level was 81.5% and 69.6% for cheese A and cheese B, respectively.

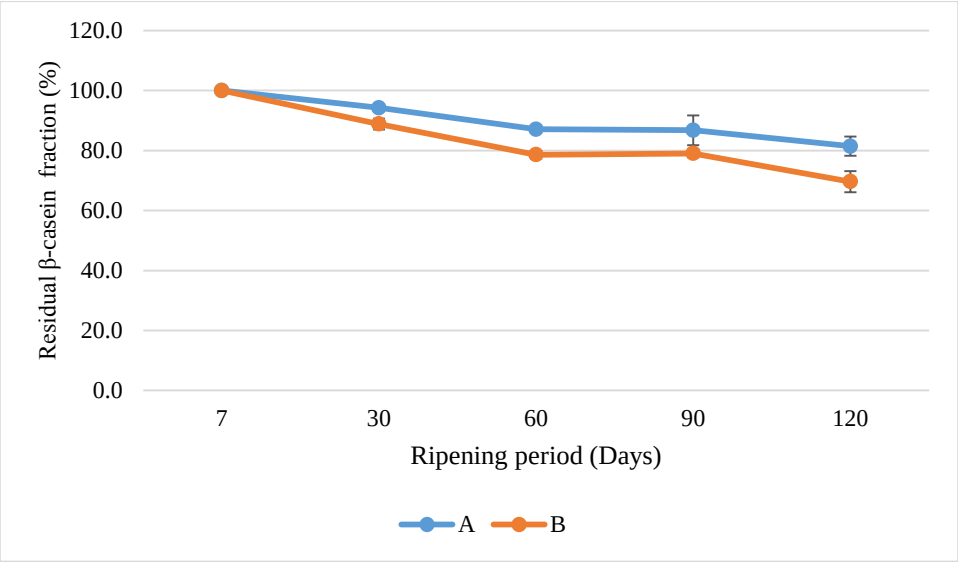

Figure 4.15. The mean values of the change in the residues β-casein fraction (%) in the cheese samples during ripening

As it can be seen from Figure 4.13 and 4.14, the formation of α_{s1}-I-casein (f24-199) at day 7 in both trials is revealed. Chymosin retained in cheese structure, hydrolysis the 40% of αs_1-casein from the peptide bond between Phe23 and Phe24 at the first 24 hours of ripening and consequently forms αs_1-casein (f1-23) and α_{s1}-I-casein (f24-199) fractions [139, 140]. The degradation of α_{s1}-casein during ripening is increased in both trials. Figure 4.16 demonstrates the electrophoretograms of cheese A during ripening.

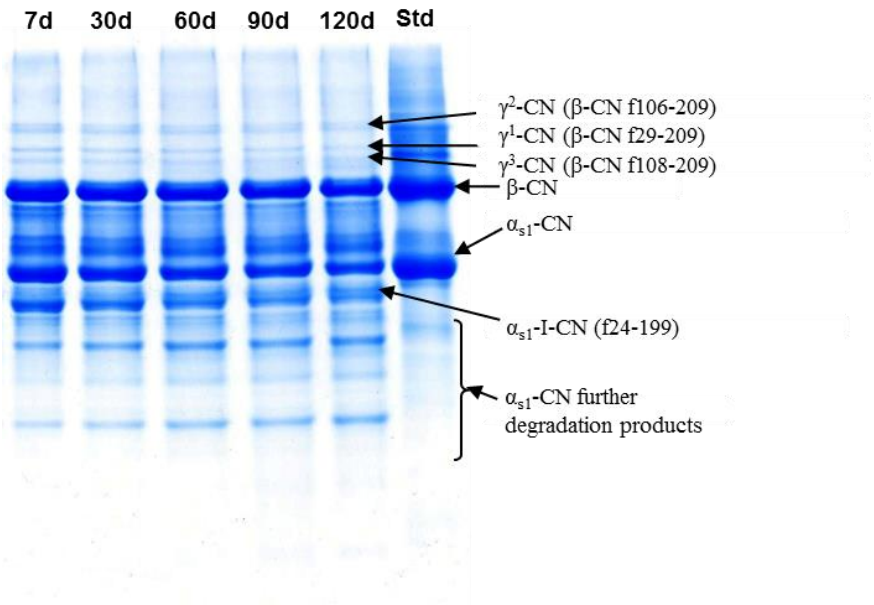

Figure 4.16. Urea-PAGE electrophoretograms of cheese A (control UF cheese) at 7, 30, 60, 90 and 120 days of ripening. (Identification of bands have done according to [105])

As previously mentioned, the degradation of αs_1-casein in cheese starts at first days of the cheese production and then increase during ripening. This is an expected change in the cheese ripening [110, 141]. This degradation in cheese B was found higher than cheese A. For a better description of the α_{s1}-casein degradation, the percentage of the residual αs_1-casein in 7, 30, 60, 90 and 120 days of ripening were evaluated and results are given in Table 4.4 and Figure 4.17.

According to Figure 4.17, the reduction of αs_1-casein in cheese B was found higher than control cheese and this may be related with the proteolytic activity of adjunct culture. The differences between residual α_{s1}-casein of the cheese A and B were found significant at 60, 90 and 120 days of ripening ($P < 0.05$). These results are in agreement with the results reported by Wishah [22] and Bulat [17].

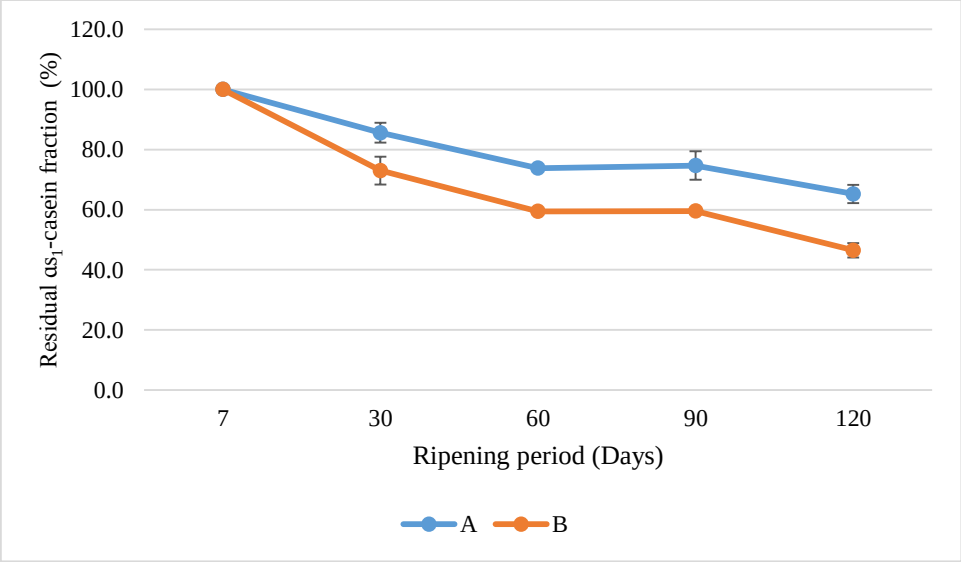

Figure 4.17. The mean values of the change in the residues αs_1-casein fraction (%) in the cheese samples during the ripening

The γ-casein fractions are seen just above the β-casein band in urea-PAGE electrophoretograms. With the progress of ripening, no noticeable change was detected in the γ-casein fractions. It is known that the presence of plasmin or some heat-resistant proteinases of the milk such as natural alkaline proteinases or proteinases belonging to psychrotrophic bacteria can lead to hydrolysis of β-casein and consequently leads to increase in γ-casein fractions. But, UF cheese with pH range between 4.42±0.08 and 4.70±0.00 and with approximately 6% S/DM is not suitable medium for the activity of

plasmin. Hence it can be the reason of detection of no significance increasing in γ-casein fractions during ripening days. These results are also in good agreement with data reported by Wishah [22] and Bulat [17].

4.5.5 Results of RP-HPLC analysis of the WSN fractions of the cheese samples

RP-HPLC is used for water soluble peptide analysis. It is the method for characterization and comparison of the proteolysis level of cheeses with different maturity grades and qualities, and to investigate the effect of various cheese production parameters on proteolysis level [142]. The α_{s1}-casein (f1-23) is hydrolysed at Gln_9-Gly_{10}, Gln_{13}-Glu_{14}, Glu_{14}-Val_{15} and Leu_{16}-Asn_{17} bonds. The resulting α_{s1}-casein (f1-9), α_{s1}-casein (f1-13) and α_{s1}-casein (f1-14) peptides and other water-soluble peptides and amino acids can be analysed by RP-HPLC [61, 66, 143].

RP-HPLC chromatograms of the water-soluble extracts (WSE) of UF cheeses at 214 and 280 nm during the ripening days are shown in Figure 4.18 and 4.19, respectively. As it can be concluded from RP-HPLC chromatograms, on the first day of storage the peak numbers and the peak heights are low and these values are increased during ripening days. As the concentration of peptide and amino acids increased the peaks of RP-HPLC chromatograms becomes bigger and higher. It can observe that, at the both trials, the number of peaks and the areas or heights of some peaks increased with the progression of proteolysis until 120 days. The peaks that recorded at approximately 80, 84 and 86 min are α-lactalbumin, β-lactoglobulin B and β-lactoglobulin A respectively. There is no significant change in height or area of those three peaks during ripening days. The peak at 36.1 min represents tryptophan and the peaks after tryptophan represent hydrophobic peptides. As it can be concluded from RP-HPLC chromatograms, there is an evident decrease in the hydrophobic area of the cheese B in compared with cheese A at 120 days of ripening. This difference is more manifest at 280nm peaks. Hydrophobic peptides can lead to bitterness in cheese and the reduction in the concentration of the hydrophobic peptides at cheese B can be related to the debittering properties of CR-319 commercial adjunct culture.

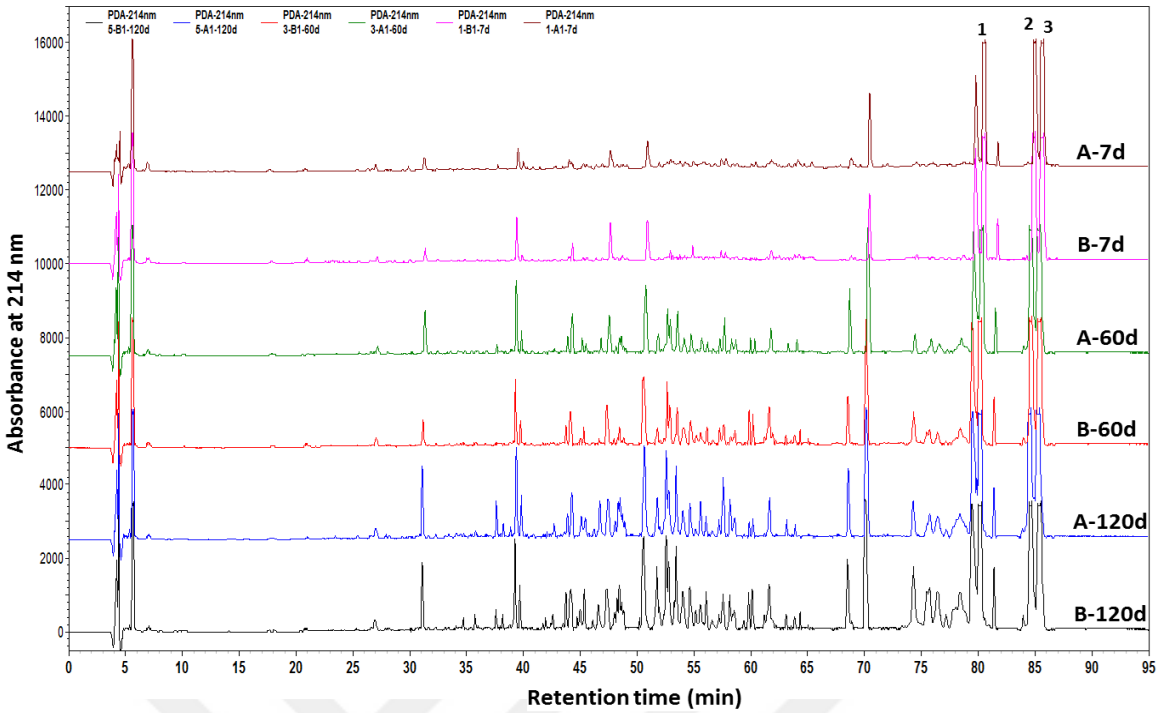

Figure 4.18. Reversed-phase HPLC chromatogram of the water-soluble extracts of UF cheeses at 214 nm during ripening (peak 1: α-lactalbumin, peak 2: β-lactoglobulin B, peak 3: β-lactoglobulin A) (A: control cheese; B, cheese with adjunct culture)

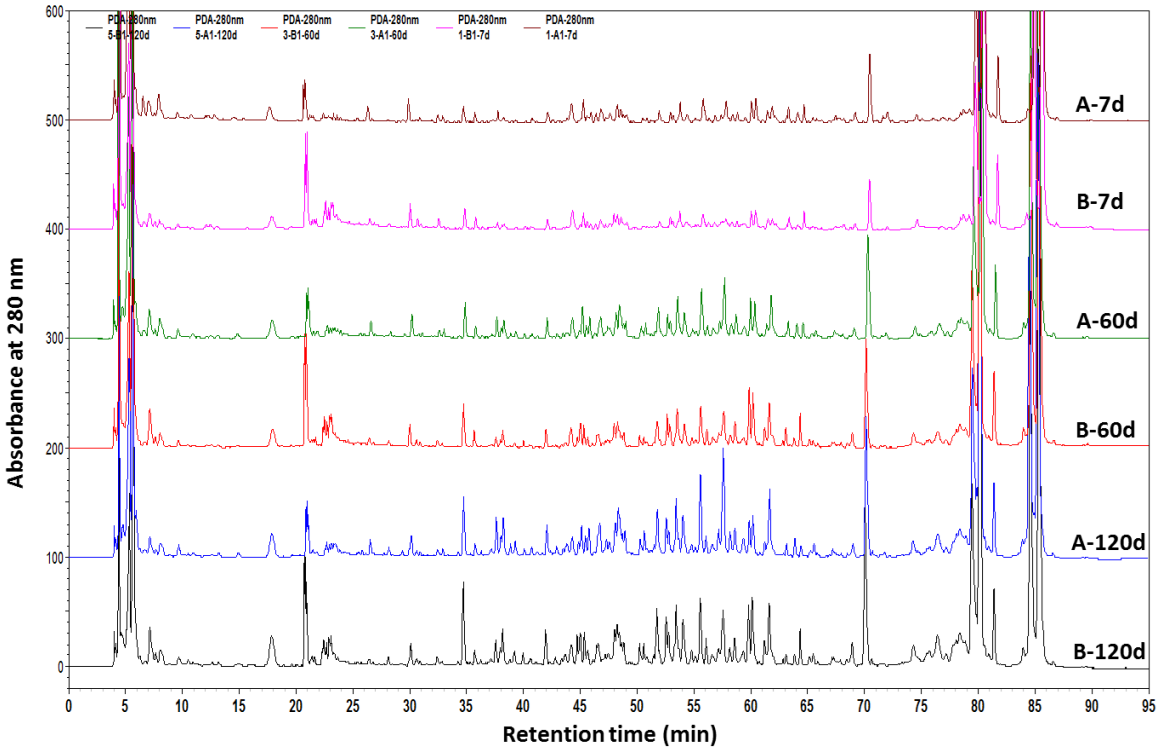

Figure 4.19. Reversed-phase HPLC chromatogram of the water-soluble extracts of UF cheeses at 280 nm during ripening (peak 1: α-lactalbumin, peak 2: β-lactoglobulin B, peak 3: β-lactoglobulin A) (A: control cheese; B, cheese with adjunct culture)

In the RP-HPLC analysis, at both 214 nm and 280 nm, many data, related to proteolysis level in cheese, were obtained. Therefore, the differences in the RP-HPLC chromatograms of the WSN fraction during ripening were determined by using principle component analysis (PCA). The obtained results are shown in Figure 4.20.

The first principal component (PCA1) divided and described cheese samples according to ripening period and the second principle component (PCA2) grouped the cheeses according to the culture type (with or without adjunct culture). According to PCA Figure 4.20b at 280 nm, it can be understood that cheese B is separated by two blue circles. Cheese B at 7 and 30 days are classified in one group and day 60, 90 and 120 takes place in the other blue circle. Also, cheese A at 7 and 30 days are separated with Day 60, 90 and 120 by two red circles. If the figure is divided into upper and lower parts by imaginary line in the middle of the figure, it will be seen that cheese B (blue circles) will be at the lower part and A (red circles) will be at the upper side of the figure. That result indicates that RP-HPLC results of WSE (proteolysis and peptidolysis) were greatly affected by the adjunct addition. These differences could be mainly due to strain variations in aminopeptidase activities.

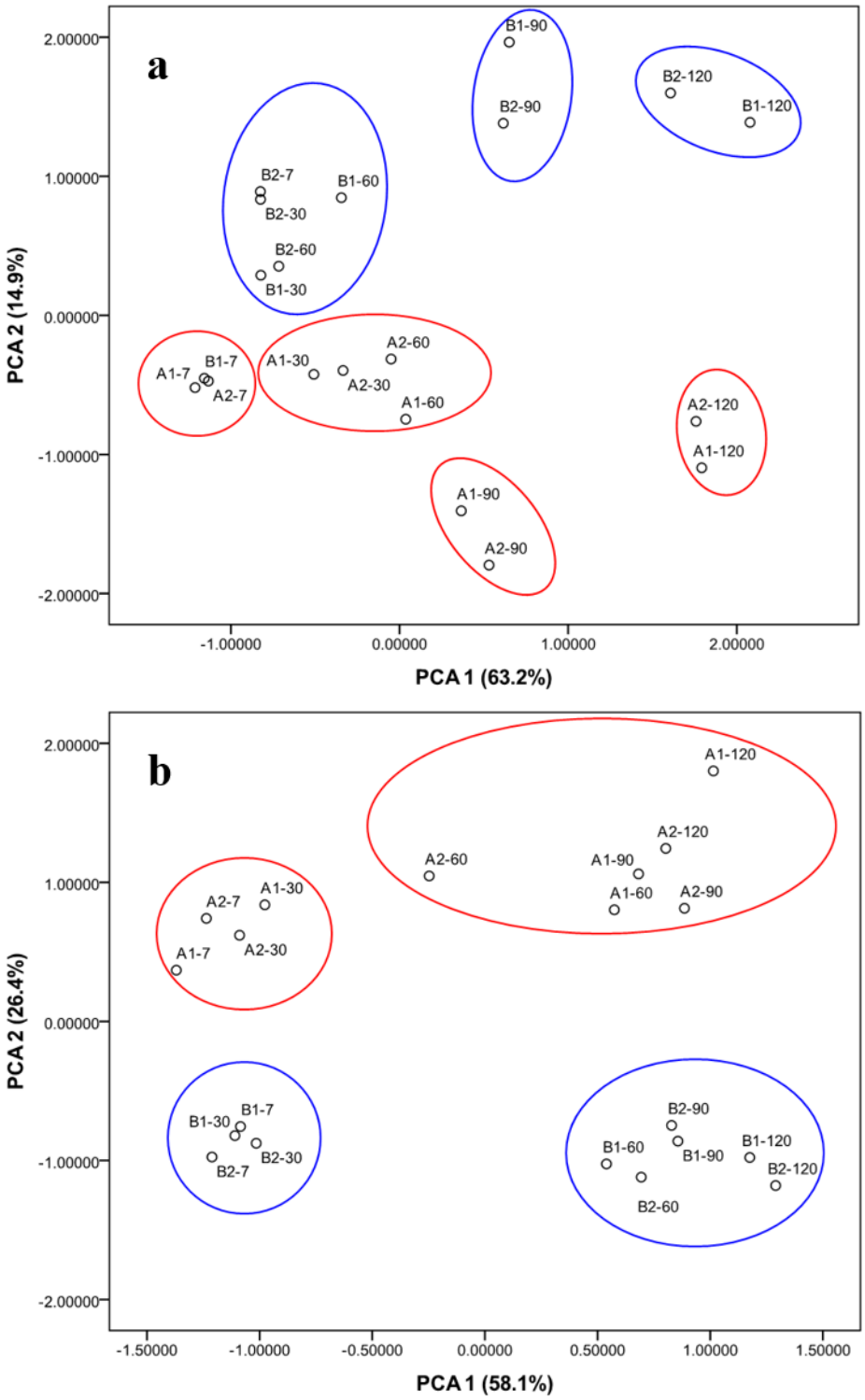

Figure 4.20. a) Principal Component Analysis of peak heights obtained at 214 nm from RP-HPLC analysis of water-soluble fractions of UF cheeses b) Principal Component Analysis of peak areas obtained at 280 nm from RP-HPLC analysis of water-soluble fractions of UF cheese

4.6 Results of Free Fatty Acid Analysis in Cheese Samples

Lipolysis in cheese is the outcome of the activity of lipase/esterase enzymes which leads to hydrolysis the ester bond between a fatty acid and the glycerol [46, 144]. *Lactococcus* subsp. and *Lactobacillus* subsp. have low lipolytic activity in compared with other bacteria such as *Pseudomonas* or moulds. However, in Cheddar and Dutch type cheeses produced with pasteurised milk, LAB are the dominant microflora of the cheese. Hence, in the absence of strong lipolytic agents and in a long period of time, such as cheese ripening time, lipase/esterase enzymes of lactococci and lactobacilli are the major lipolytic agents in cheese [46]. It was determined that FFA concentration in aseptic cheese that acidifies with glucono delta-lactone instead of starter bacteria was low and that concentration did not increase through ripening period. The lipase/esterase activities of lactic acid bacteria are considered to be completely intracellular [53]. It has been determined that strains are different from each other in the term of lipase/esterase activity and that some strains have two esterases. LAB can hydrolyse the mono- and diglycerides produced by milk lipoprotein to FFA [143].

In this study, analysis of FFA was carried out with GC system and the chromatograms of control cheese at 7 and 120 days are given at Figure 4.21 and 4.22, respectively. The chromatograms of the cheese B at 7 and 120 days are shown at Figure 4.23 and 4.24, respectively. Figure 4.25 shows all four chromatograms together.

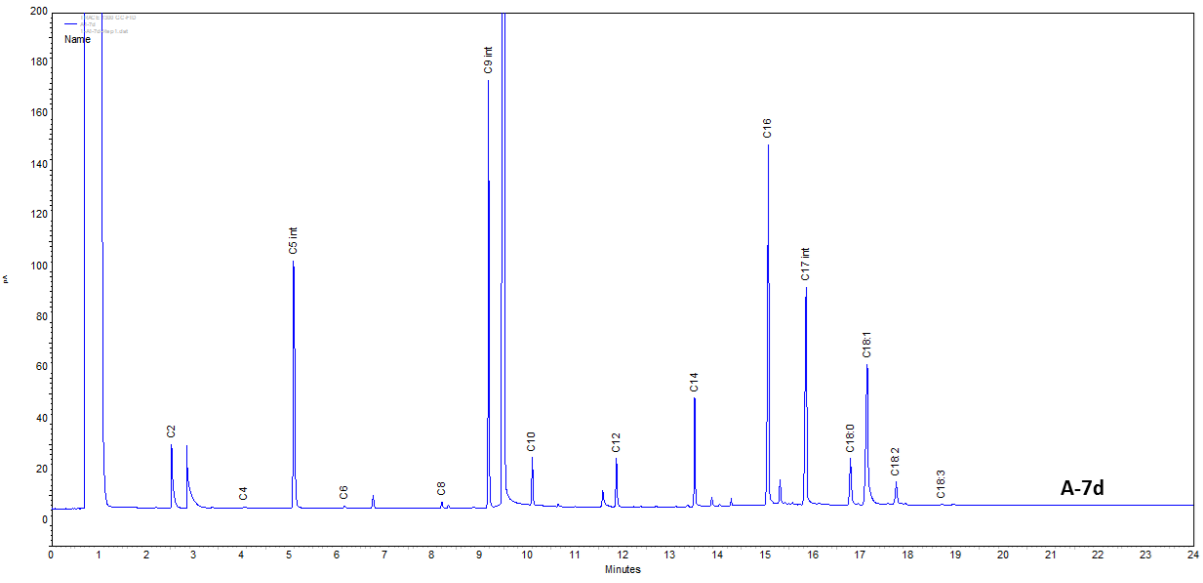

Figure 4.21. GC chromatogram of FFAs analysis of the cheese A at 7 days of ripening

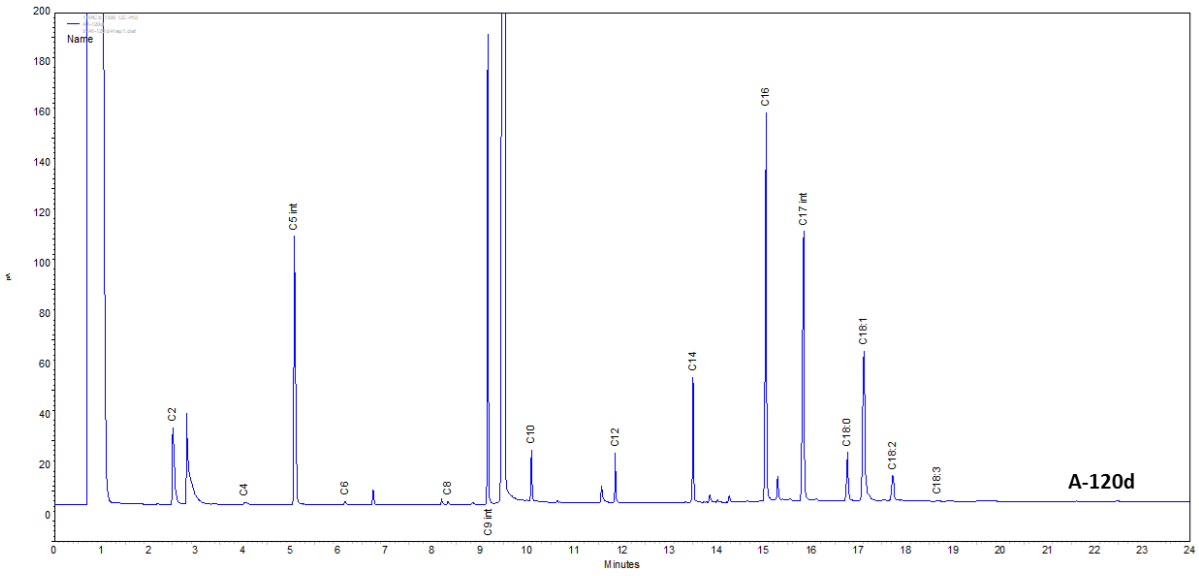

Figure 4.22. GC chromatogram of FFAs analysis of the cheese A at 120 days of ripening

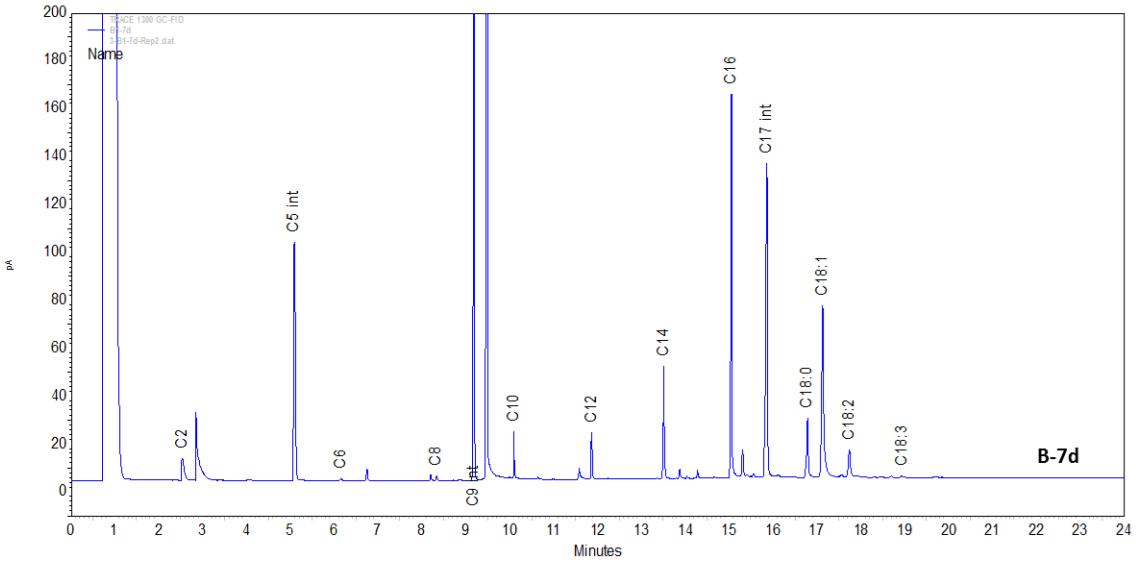

Figure 4.23. GC chromatogram of FFAs analysis of the cheese B at 7 days of ripening

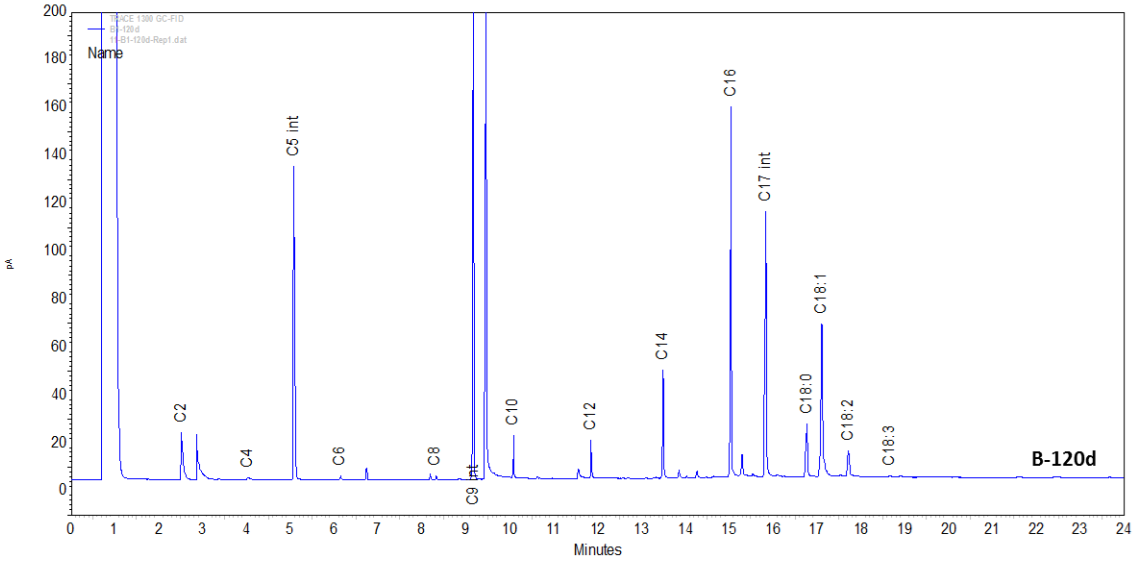

Figure 4.24. GC chromatogram of FFAs analysis of the cheese B at 120 days of ripening

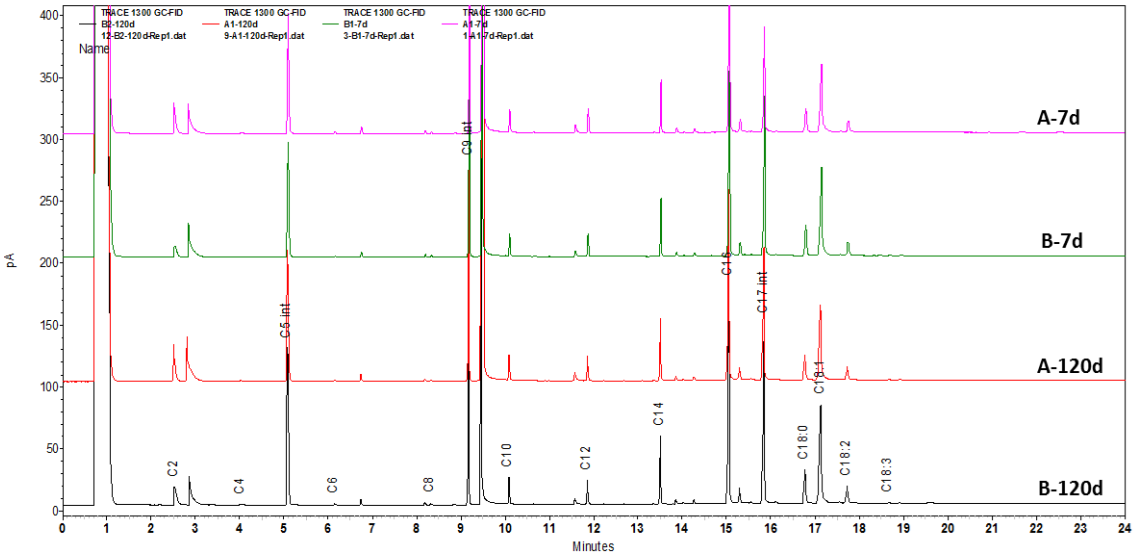

Figure 4.25. GC chromatograms of FFAs of UF cheeses at 7 and 120 days of ripening (A: control cheese; B: cheese with adjunct culture)

FFA values of both samples at 7, 60 and 120 days of ripening are listed in Table 4.5. For more clearly understanding the changes in FFA amounts between samples during ageing of cheese trials, the values are shown as a diagram at Figure 4.26.

Table 4.5. The amount of different FFAs (mg/kg cheese), found in cheese A (control) and cheese B (contained adjunct culture) at 7, 60 and 120 days of ripening

	Ripening period (Days)					
	7		60		120	
FFA	**A**	**B**	**A**	**B**	**A**	**B**
C4:0	3.55±0.44	4.21±0.92	3.92±0.17	5.17±0.52	5.20±0.43	5.45±0.63
C6:0	3.46±0.34	4.09±0.31	4.02±0.27	5.10±0.97	4.84±0.48	5.88±0.36
C8:0	6.17±0.44	7.59±0.53	6.99±0.19	7.83±0.30	7.33±0.68	8.28±0.39
C10:0	36.27±3.63	41.51±3.31	42.46±6.50	47.91±1.40	42.44±1.23	55.61±1.27
C12:0	40.65±1.14	46.34±1.35	42.95±3.20	55.50±7.12	44.11±1.63	61.87±6.02
C14:0	120.67±0.94	114.82±15.05	143.00±3.73	149.52±1.99	150.51±5.83	181.86±8.32
C16:0	571.30±5.73	592.51±5.45	637.84±20.05	703.66±7.84	764.60±15.31	803.03±23.14
C18:0	95.20±1.58	107.29±2.26	105.78±7.30	115.39±12.65	125.10±4.88	127.28±13.81
C18:1	364.55±21.88	371.15±18.05	385.90±21.59	466.81±59.57	457.96±25.11	482.01±24.86
C18:2	57.83±7.08	55.53±5.25	61.83±6.56	60.54±5.81	74.46±3.11	75.92±6.11
Total	1299.63±13.5	1345.04±36.8	1434.73±21.9	1617.43±79.9	1676.37±26.7	1807.18±82.9

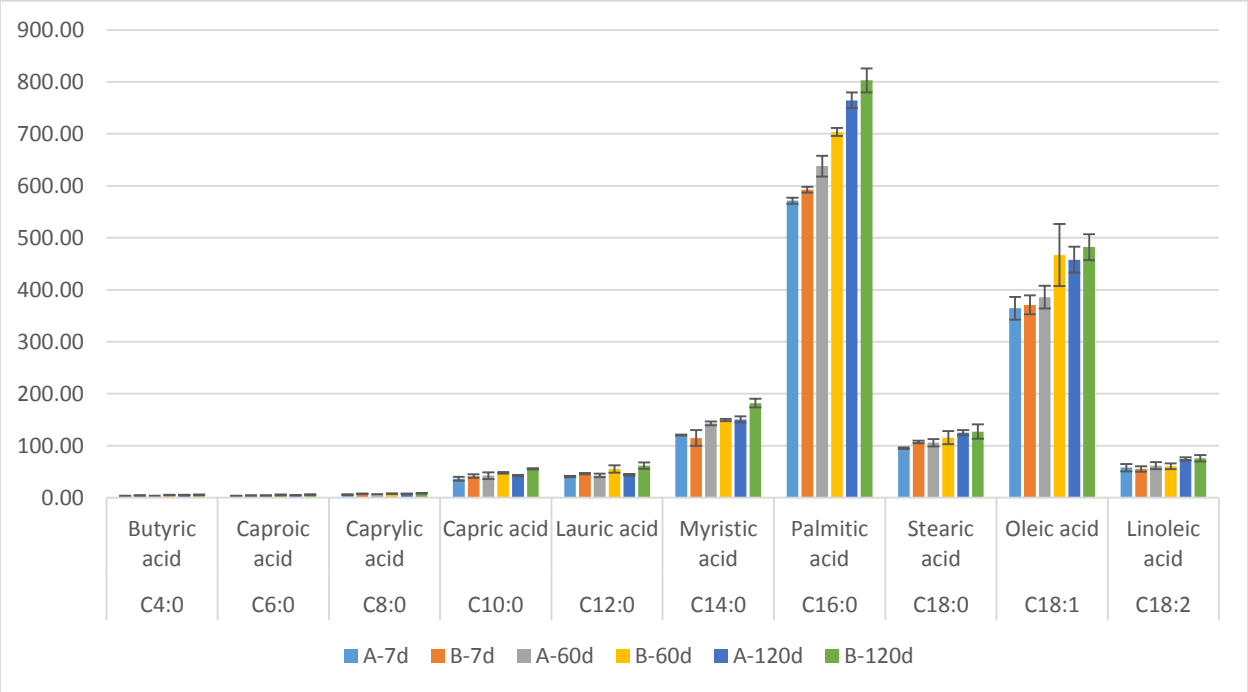

Figure 4.26. The mean values of different FFA, found in cheese A (control) and cheese B (contained adjunct culture) at 7, 60 and 120 days of ripening

As it can be concluded from Figure 4.26, palmitic acid ($C_{16:0}$) is the major FFA content of the both trials and after that oleic acid ($C_{18:01}$) and myristic acid ($C_{14:0}$) are the foremost FFA of the trials respectively. These data are in agreement with the results reported by Kirmaci [145]. In another study, palmitic acid and oleic acid were found the most abundant FFA in Cheddar cheese which produced with *L. lactic* subsp. *cremoris* AM2 [91]. In the same study, authors found that fast lysing culture of *L. lactic* subsp. *cremoris* AM2 has a higher FFA level then the slow lysing culture of *L. lactic* subsp. *cremoris* HP in Cheddar cheese [91]. The evidence was provided for starter cell autolysis and level of lipolysis in that study. In our study, the amount of FFAs increased during ripening days in both trials. Compared with cheese A, CR-319 adjunct culture added samples had higher level of lipolysis. The rise in the amount of FFAs in cheese samples by progressing ripening days was reported formerly in ripened cheeses [146, 147]. In another study, the effect of the adjunct culture of CR-213 (*L. lactic* subsp. *lactis*, *L. lactic* subsp. *cremoris*) on lipolysis at Feta-type cheese was investigated, and it was concluded that total FFA level was higher in adjunct added cheese samples than control cheese [59].

4.7 Results of Organic Acid and Sugar Analysis in Cheese Samples

Organic acids in dairy products were originated from normal animal metabolism, or produced by the destruction of citrate, proteins, fat and lactose during ripening. Under ideal conditions, sugars in cheese should be converted to lactic acid. However, during the maturation of the cheese, the development of bacteria may grow under inappropriate conditions such as low pH, low water activity and high salt concentration and this forces the bacteria to use alternative biochemical pathways where other organic acids are produced [148]. Organic acids play a major role in the development of flavour compounds of milk products. For instance, acetic acid is the major flavour compound of the Feta cheese or specific flavour of Emmental cheese is related to the propionic acid [149]. The amount of organic acids can also be used to determine starter activity and bacterial growth during ripening.

Some of the main organic acids of the UF cheese trials are given in Table 4.6. In addition, the organic acid contents of the UF milk retentate are 1819.34±72.18, 161.84±7.11, 205.96±53.91, 34.37±12.69, 4.28±0.12 and 0.02±0.01 for citric acid, lactic acid, acetic acid, pyruvic acid, lactose, and galactose respectively.

In a study, the effect of the commercial culture of *L. lactis* strains, on the organic acid content of low-fat Feta type cheese was investigated. In that study, the addition of adjunct

culture had an increasing effect on the amounts of butyric acid, propionic acid and acetoin. Lactic acid, citric acid, and acetic acid were found the major organic acid in all trials during ripening [115]. In our study, the effect of ripening period on acetoin, acetic acid and pyruvic acid was found statistically significant ($p<0.05$). However, the changes of lactose and galactose were not significant. In addition, treatment effect on galactose and acetoin was important ($P<0.05$).

Table 4.6. Sugars and organic acids of the UF cheeses made with or without adjunct culture during ripening

Organic Acid	Ripening period (Days)	Cheese A	Cheese B
Lactose (%)	7	1.28±0.09	1.54±0.04
	30	1.24±0.18	1.54±0.01
	60	1.29±0.00	1.49±0.02
	90	1.26±0.12	1.53±0.01
	120	1.25±0.02	1.52±0.00
Galactose (%)	7	0.32±0.01	0.16±0.00
	30	0.28±0.10	0.15±0.00
	60	0.26±0.01	0.17±0.01
	90	0.28±0.09	0.15±0.00
	120	0.30±0.01	0.15±0.01
Acetoin (mg/kg)	7	188.26±18.04	109.12±0.37
	30	179.91±18.59	200.51±21.16
	60	180.98±6.77	233.75±13.76
	90	178.10±2.75	303.29±9.53
	120	197.34±4.33	417.01±29.26
Lactic acid (mg/kg)	7	12314.88±268.58	11587.29±120.12
	30	12721.52±554.36	11739.23±111.03
	60	12471.27±80.59	11798.56±65.93
	90	12479.60±516.74	11812.08±67.23
	120	12506.52±115.87	1179.24±3.68
Citric acid (mg/kg)	7	1350.54±50.36	1402.68±35.00
	30	1387.48±23.27	1432.05±4.61
	60	1377.79±14.25	1380.03±36.74
	90	1338.80±19.95	1399.22±1.33
	120	1350.05±6.88	1383.53±33.58

Table 4.6 (continue)

Acetic acid (mg/kg)	7	140.24±0.98	132.75±3.67
	30	152.89±1.52	153.34±1.11
	60	155.91±5.30	159.90±3.38
	90	158.01±1.12	170.08±3.12
	120	161.68±5.30	179.07±4.20
Pyruvic acid (mg/kg)	7	66.24±1.85	51.44±2.04
	30	150.20±25.28	93.76±39.02
	60	196.75±11.23	151.88±19.58
	90	209.78±50.18	185.83±12.44
	120	232.47±8.61	189.43±7.34

4.8 Results of Volatile Compounds Analysis in Cheese Samples

Volatile flavour compounds of the cheese are the results of three main pathways; glycolysis which causes conversion of lactose to lactate or lactic acid, lipolysis which cause hydrolysis of lipids and proteolysis which cause degradation of protein to peptides and amino acids [150]. The changes in volatile compound obtained during ripening days in cheese were detected by SPME-GC-MS. The results of the volatile compounds analysis of the cheese samples are given in Table 4.7, 4.8, 4.9, 4.10, 4.11 and 4.12 as the ratio of the peak area of the volatile component in the peak area of the internal standard.

The acids found in cheese can contribute to cheese flavour directly and/or indirectly (as precursors of alcohols, ketones, lactones and esters) [151]. The results of the changing in volatile compound (acids) of the cheese samples are given in Table 4.7. The main volatile carboxylic acids detected in cheese trials during ripening are acetic acid, 2-methylpropanoic acid, butanoic acid, 3-methylbutanoic acid, hexanoic acid, 2-ethylhexanoic acid, heptanoic acid, octanoic acid, and decanoic acid. The amount of all detected volatile carboxylic acids at Day 120 at cheese B was higher than cheese A. FFAs may derive from different sources such as lipid catabolism, metabolism of lactose or lactic acid (e.g., acetic acid, butanoic acid and propionic acid) and metabolism of amino acid, generally from leucine and valine [152]. The differences in detected volatile acids between two trial, especially in the amount of acetic acid, may be related to adjunct culture possession of cheese B that were caused more participate in acid formation at corresponding cheese.

Table 4.7. Some of the main volatile compounds (acids) of the UF cheeses made with or without adjunct culture during ripening

Acids	RI	Ripening period (Days)	Cheese A	Cheese B
Acetic acid	1444	7	33.16±2.76	41.89±5.41
		30	32.57±8.01	36.64±7.90
		60	34.33±1.39	45.15±1.78
		90	34.78±3.80	44.93±2.37
		120	56.64±8.52	81.97±3.51
Propanoic acid, 2-methyl-	1567	7	1.34±0.24	ND
(isobutyric acid)		30	0.63±0.29	0.70±0.27
		60	0.70±0.08	0.90±0.04
		90	0.55±0.03	0.78±0.23
		120	0.61±0.14	0.60±0.10
Butanoic acid	1622	7	24.58±3.85	27.83±4.73
		30	29.65±3.59	29.63±3.73
		60	27.40±0.92	34.96±1.10
		90	27.83±2.83	38.13±0.53
		120	33.58±2.18	41.58±3.64
Butanoic acid, 3-methyl	1668	7	1.78±0.03	2.02±0.20
(isovaleric acid)		30	0.88±0.40	1.17±0.52
		60	1.11±0.03	1.40±0.05
		90	0.84±0.11	1.36±0.53
		120	0.95±0.32	1.20±0.20
Hexanoic acid	1839	7	29.29±3.82	30.62±6.50
(caproic acid)		30	29.96±4.66	32.81±4.31
		60	34.15±1.72	37.04±0.63
		90	34.40±2.33	43.71±7.47
		120	41.61±4.75	40.53±5.00
Hexanoic acid, 2-ethyl	1943	7	2.09±0.25	2.29±0.52
(ethylhexanoic acid)		30	0.29±0.42	0.71±0.23
		60	0.74±0.00	0.91±0.20
		90	0.66±0.09	0.42±0.59
		120	0.55±0.02	0.50±0.20
Heptanoic acid	1949	7	3.89±0.30	3.34±0.64
		30	1.61±0.78	1.72±0.75
		60	1.50±0.07	1.81±0.03
		90	1.22±0.16	1.59±0.37
		120	1.27±0.33	1.20±0.20

Table 4.7 (continue)

Octanoic acid	2053	7	11.27±3.42	11.02±0.60
(caprylic acid)		30	11.53±2.45	12.74±1.44
		60	11.87±0.42	13.34±0.15
		90	9.84±1.39	14.72±2.18
		120	14.41±3.11	16.94±1.30
Decanoic acid	2280	7	3.40±1.07	3.13±0.12
(capric)		30	3.56±0.75	3.94±0.66
		60	3.49±0.12	4.19±0.18
		90	2.89±0.24	4.31±0.85
		120	5.20±0.53	5.94±0.41

RI: retention index. The results are given as an arbitrary unit after normalization of each volatile compounds with IS peak area. Some of the volatiles (having low concentration) are not given in the Table.

Esters which are responsible for the fruity taste in cheese are formed by esterification of alcohol and carboxylic acids or by alcoholysis [153]. Some of the main esters those were detected in the cheese trials during the ripening period are given at Table 4.8. Acetic acid ethyl ester and hexanoic acid ethyl ester were detected at both trials. Acetic acid ethyl ester has a fruity and pineapple-like aroma, whereas butanoic acid ethyl ester has fruity and apple, banana-like aroma [143]. As previously observed the amount of acetic acid and hexanoic acid were found higher in compared with other carboxylic acids; also it is known that esters can be formed by esterification of carboxylic acid. Hence the amount of these three esters can be related to the higher amount of their corresponding carboxylic acids in cheeses.

Methyl ketones such as 2-propanone, 2-butanone, 2-pentanone, 2-heptanone, 2, 3-butanedione (diacetyl) and its reduction form 3-hydroxy-2-butanone (acetoin) are formed by enzymatic oxidation of FFA and then decarboxylation of obtained β-ketoacids [1, 154, 155]. Diacetyl and acetoin are also formed from citrate by the activity of LAB [151]. Ketones (except diacetyl, heptan-2-one and oct-1-en-3-one that gives buttery, musty and mushroom notes, respectively) give fruity or off floral notes [151]. Ketones, which are intermediate compounds, can be reduced to alcohols and they contribute to the formation of the characteristic aroma of the Roquefort, Camembert and etc. cheeses [155, 156]. Some of the main ketones that are detected in the cheese trials during the ripening period are given in Table 4.9. The amount of diacetyl, acetoin, 3-hydroxy-2-pentanone and 2-hydroxy-3-pentanone of the cheese B were found obviously higher than cheese A. it can be related to flavour enhancement characteristic of the adjunct cultures in the cheese B.

Table 4.8. Some of the main volatile compounds (esters) of the UF cheeses made with or without adjunct culture during ripening

Esters	RI	Ripening period (Days)	Cheese A	Cheese B
Acetic acid ethyl ester	880	7	1.53±0.10	1.30±0.08
		30	0.96±0.52	0.98±0.61
		60	1.29±0.01	1.14±0.09
		90	1.03±0.37	0.78±0.09
		120	0.88±0.03	0.90±0.40
Hexanoic acid ethyl ester	1228	7	ND	ND
		30	ND	ND
		60	0.25±0.03	ND
		90	0.24±0.11	0.23±0.33
		120	0.62±0.33	0.40±0.00

RI: retention index. The results are given as an arbitrary unit after normalization of each volatile compounds with IS peak area. Some of the volatiles (having low concentration) are not given in the Table.

Table 4.9. Some of the main volatile compounds (ketones) of the UF cheeses made with or without adjunct culture during ripening

Ketones	RI	Ripening period (Days)	Cheese A	Cheese B
2-Propanone	809	7	21.28±1.49	22.58±2.77
		30	12.72±5.84	13.26±6.97
		60	16.40±0.73	16.00±1.38
		90	15.27±4.45	11.60±0.79
		120	13.46±0.73	14.20±5.70
2-Butanone	895	7	12.76±0.95	11.52±0.01
		30	7.95±3.67	8.96±3.14
		60	10.14±0.29	9.53±0.89
		90	9.43±2.66	6.79±0.53
		120	8.08±0.40	8.10±3.40
2-Pentanone	970	7	2.07±0.17	1.33±0.43
		30	1.57±0.85	0.97±0.82
		60	1.97±0.04	0.98±0.14
		90	1.94±0.66	0.70±0.03
		120	1.74±0.02	0.90±0.50

Table 4.9 (continue)

2,3-Butanedione (Diacetyl)	973	7	7.96±0.76	10.04±3.88
		30	5.24±0.34	10.89±2.92
		60	6.69±1.58	11.76±0.82
		90	6.62±0.98	10.81±2.35
		120	6.88±0.53	15.80±2.80
2,3-Pentanedione	1057	7	1.67±0.10	3.78±0.20
		30	0.76±0.30	1.84±0.67
		60	0.81±0.08	2.23±0.41
		90	0.61±0.13	2.12±1.24
		120	0.70±0.37	1.60±0.00
2-Heptanone	1176	7	5.37±2.51	5.32±0.59
		30	6.15±0.86	5.86±2.49
		60	10.72±6.07	9.11±0.06
		90	5.62±1.48	7.01±2.55
		120	7.00±0.12	5.20±1.00
2-Butanone, 3-hydroxy-(acetoin)	1280	7	92.37±6.47	66.25±5.11
		30	48.48±19.54	56.39±18.62
		60	61.14±9.29	76.85±12.76
		90	61.84±19.21	71.21±2.96
		120	51.60±4.35	94.40±38.60
3-Hydroxy-2-pentanone	1337	7	13.27±1.45	35.00±0.12
		30	7.21±2.64	19.87±9.50
		60	8.56±0.80	25.17±0.17
		90	6.06±0.42	24.35±11.27
		120	7.11±2.86	20.10±1.30
2-Hydroxy-3-pentanone	1353	7	7.18±0.24	18.42±0.43
		30	3.44±1.40	10.62±5.32
		60	4.20±0.39	12.88±0.12
		90	2.84±0.26	12.94±6.74
		120	3.26±1.39	10.60±0.90

RI: retention index. The results are given as an arbitrary unit after normalization of each volatile compounds with IS peak area. Some of the volatiles (having low concentration) are not given in the Table.

Aldehydes are generally produced by catabolism of FFA (straight-chain aldehydes) and/or transamination of amino acids (branched-chain aldehydes). Aldehydes of the cheese are transitory compounds and rapidly are converted to alcohols and acids so they do not accumulate in cheese structure. Aldehydes have different perception thresholds and give divers aromatic notes. For instance, hexanal gives the green note of unripe fruit; octanal, nonanal and decanal give orange-like note and benzaldehyde gives bitter almond note

[151]. The main aldehydes which were found at cheese trials during ripening period were hexanal, octanal, nonanal and benzaldehyde. The amount of detected aldehyde at cheese trials during ripening are given at Table 4.10. As previously mentioned aldehydes are transitory compounds and do not accumulate in cheese. Hence, as it can be understood form Table 4.10 the amount of them is low at Day 120.

Table 4.10. Some of the main volatile compounds (aldehydes) of the UF cheeses made with or without adjunct culture during ripening

Aldehydes	RI	Ripening period (Days)	Cheese A	Cheese B
Hexanal	1076	7	8.33±0.41	14.33±0.62
		30	4.87±1.42	5.64±1.84
		60	4.62±0.23	6.17±1.26
		90	3.99±0.02	6.35±1.98
		120	4.11±0.31	7.80±2.20
Octanal	1283	7	2.58±0.44	2.39±0.45
		30	2.13±0.77	2.30±0.76
		60	2.01±0.72	ND
		90	0.87±1.23	1.02±1.21
		120	1.43±2.02	1.70±0.60
Nonanal	1388	7	3.76±4.20	2.59±0.83
		30	0.26±0.06	0.31±0.13
		60	0.19±0.01	0.56±0.08
		90	0.22±0.13	0.30±0.07
		120	0.29±0.08	0.30±0.20
Benzaldehyde	1521	7	4.50±3.03	4.49±1.06
		30	1.00±0.39	1.82±0.69
		60	0.78±0.04	1.68±0.80
		90	0.83±0.02	1.39±0.80
		120	1.58±0.12	2.40±0.50

RI: retention index. The results are given as an arbitrary unit after normalization of each volatile compounds with IS peak area. Some of the volatiles (having low concentration) are not given in the Table.

Alcohols are synthesised by different pathways. For instance, ethanol, which has an important role in the formation of esters, is produced by the catabolism of the alanine and/or lactose fermentation. Butane-2, 3-diol can be formed from diacetyl and acetoin by dehydrogenase enzymes. The presence of branched-chained primary alcohols such as 2-methyl-1-butanol, 2-methyl-1-propanol and 3-methyl-1-butanol indicates the conversion of the aldehydes produced by the catabolism of the isoleucine, valine and leucine respectively

[154]. Fermentation of lactose and the catabolism of Ala may have led to the production of ethanol [157]. Alcohol compounds that were detected in cheese trials are given in Table 4.11. The amount of ethanol were found higher in cheese B. Kondyli et al [59] were investigated the effect of commercial adjunct culture on reduced-fat Feta type cheese and they reported that the amount of ethanol in cheese with adjunct culture were found higher than control reduced-fat and also even more than control full-fat cheese. According to the research reported by Soltani et al [157], which they were investigated the effect of different protease on Iranian UF cheese volatile compound, ethanol was also found as the most abundant alcohol compound in the Iranian white cheese.

Table 4.11. Some of the main volatile compounds (alcohols) of the UF cheeses made with or without adjunct culture during ripening

Alcohols	**RI**	**Ripening period (Days)**	**Cheese A**	**Cheese B**
Ethanol	925	7	20.99±1.13	22.75±2.59
		30	11.38±4.30	12.10±6.39
		60	14.08±0.31	13.14±1.15
		90	12.83±3.58	9.90±1.17
		120	10.91±1.47	12.20±3.90
1-Butanol	1143	7	2.49±0.66	2.74±0.22
		30	1.35±0.61	1.55±0.79
		60	1.56±0.13	1.59±0.08
		90	1.18±0.04	1.63±0.53
		120	1.32±0.40	1.30±0.10
1-Pentanol	1243	7	2.96±4.18	7.01±0.58
		30	3.30±1.19	4.13±2.53
		60	3.63±0.02	4.33±0.46
		90	3.11±0.45	4.07±1.70
		120	3.50±0.86	3.40±0.20
1-Hexanol	1343	7	6.00±0.37	4.71±0.56
		30	7.14±2.96	4.33±0.76
		60	8.29±0.47	6.70±0.32
		90	8.55±0.81	5.67±1.37
		120	10.29±3.38	5.00±0.60
1-Octen-3-ol	1440	7	0.68±0.12	0.54±0.09
		30	0.75±0.23	0.58±0.06
		60	0.87±0.04	0.81±0.07
		90	0.89±0.01	0.80±0.21
		120	0.92±0.26	0.70±0.10

Table 4.11 (continue)

1-Hexanol, 2-ethyl-	1478	7	16.85±14.64	9.86±3.19
(2-ethylhexanol)		30	2.15±0.97	2.82±1.21
		60	1.80±0.03	2.20±0.07
		90	0.70±0.99	1.91±0.70
		120	0.83±1.17	1.30±0.30
Benzenemethanol	1861	7	2.08±1.56	ND
		30	0.38±0.21	0.43±0.19
		60	0.30±0.01	0.37±0.04
		90	0.25±0.03	0.11±0.16
		120	0.29±0.08	ND
1,4-Butanediol	1895	7	0.94±0.03	1.12±0.28
		30	0.35±0.18	0.41±0.16
		60	0.34±0.03	0.41±0.04
		90	0.24±0.01	0.31±0.11
		120	0.25±0.04	0.20±0.00

RI: retention index. The results are given as an arbitrary unit after normalization of each volatile compounds with IS peak area. Some of the volatiles (having low concentration) are not given in the Table.

The compounds that are classified as miscellaneous compounds are shown in Table 4.12. These compounds are generally in trace quantity and include lactones, sulfur compounds and etc. Generally, cheese B has higher values then that of cheese A. However, the effect of adding adjunct culture on the formation of miscellaneous compounds was found not significant between two trails ($P > 0.05$).

Table 4.12. Some of the main volatile compounds (miscellaneous compounds) of the UF cheeses made with or without adjunct culture during ripening

Miscellaneous compounds	**RI**	**Ripening period (Days)**	**Cheese A**	**Cheese B**
δ-Hexalactone	1792	7	0.52±0.05	0.47±0.03
(Delta-hexalacton)		30	0.20±0.09	0.23±0.09
		60	0.23±0.01	0.26±0.01
		90	0.15±0.01	0.23±0.09
		120	0.16±0.06	0.20±0.00
Methane, sulfonylbis-	1889	7	1.49±0.26	1.65±0.05
(Dimethyl sulfone)		30	0.53±0.24	0.72±0.37
		60	0.70±0.08	0.78±0.08
		90	0.36±0.00	0.63±0.26
		120	0.40±0.17	0.50±0.10

Table 4.12 (continue)

δ-octalactone	1968	7	0.74±0.02	0.84±0.07
(Delta-octalactone)		30	0.38±0.17	0.44±0.17
		60	0.41±0.05	0.45±0.01
		90	0.28±0.03	0.39±0.15
		120	0.30±0.10	0.30±0.00
δ-decalactone	2202	7	1.37±0.05	1.48±0.05
(Delta-decalacton)		30	0.88±0.39	0.93±0.32
		60	1.03±0.08	1.17±0.03
		90	0.80±0.14	1.06±0.42
		120	0.94±0.30	0.90±0.00

RI: retention index. The results are given as an arbitrary unit after normalization of each volatile compounds with IS peak area. Some of the volatiles (having low concentration) are not given in the Table.

4.9 Results of Texture Analysis in Cheese Samples

Cheese texture is affected by different factors such as fat, protein, moisture, concentration and redistribution of salt and pH [46, 158]. Usually high acidity, high protein content and dry matter making cheese texture harder and more resistance to deformation [159]. Texture development of the cheeses is the result of hydrolysis of α_{s1}-casein during ripening. As it is known, water binding in casein network of the cheese makes the product more elastic. On the other hand, increasing the amount of citric acid and sodium chloride in the cheese structure reduces the flexibility and increases the hardness of the cheese texture [22, 160]. Changing in some textural properties of the cheese samples during ripening period are given in Table 4.13.

Changing in hardness (N) of the cheese samples during ripening days are also demonstrated in Figure 4.27. As it can be concluded from Figure 4.27, the hardness of the both trials decreased during the ripening days ($P<0.05$). Those results are in agreement with Katsiari et al. [81].

The maximum hardness value was found in 7 days for both trials and minimum hardness was found in cheese A in 120 days. The manifest decrease in hardness value was seen in 30 days. The early falling in hardness value (until 30 days) may result from the degradation of α_{s1}-casein to α_{s1}-1-casein [131]. The hardness values of cheese B were lower than cheese A.

The changes in cohesiveness, springiness, gumminess and adhesiveness of cheese samples during ripening days are demonstrated at Figure 4.28, 4.29, 4.30 and 4.31 respectively.

Table 4.13. Textural properties of the cheese samples during ripening

Variables	Days	Cheese A	Cheese B
Hardness (N)	7	4.10±0.26	4.08±0.06
	30	3.22±0.21	3.15±0.44
	60	2.90±0.14	2.65±0.15
	90	2.80±0.06	2.81±0.07
	120	2.77±0.05	2.52±0.25
Cohesiveness	7	0.14±0.03	0.12±0.04
	30	0.14±0.02	0.18±0.04
	60	0.17±0.02	0.15±0.03
	90	0.16±0.01	0.15±0.05
	120	0.12±0.03	0.11±0.05
Springiness (mm)	7	4.52±0.34	3.95±1.25
	30	4.29±0.97	4.94±0.56
	60	4.89±0.55	5.47±0.42
	90	4.83±0.52	4.41±0.31
	120	3.68±1.15	4.79±0.66
Gumminess (N)	7	0.59±0.13	0.49±0.16
	30	0.46±0.13	0.60±0.16
	60	0.45±0.05	0.43±0.08
	90	0.45±0.04	0.42±0.13
	120	0.31±0.08	0.30±0.14
Adhesiveness (Nmm)	7	0.09±0.02	0.14±0.01
	30	0.07±0.04	0.07±0.02
	60	0.17±0.06	0.11±0.03
	90	0.08±0.06	0.07±0.03
	120	0.12±0.03	0.12±0.04

The cohesiveness values raised until 30 days and then fallen until 120 days and at the end of 120 days, the cohesiveness value of cheese B was found lower than control cheese.

Springiness values increased and decreased during ripening days. Degradation of calcium para-κ-caseinate and also the liberation of calcium ions may affect the springiness of the

cheese [131]. Maximum springiness was found in cheese B at 60 days (5.47±0.42) and the minimum value was 3.68±1.15 for cheese ‘A’ at 120 days of ripening.

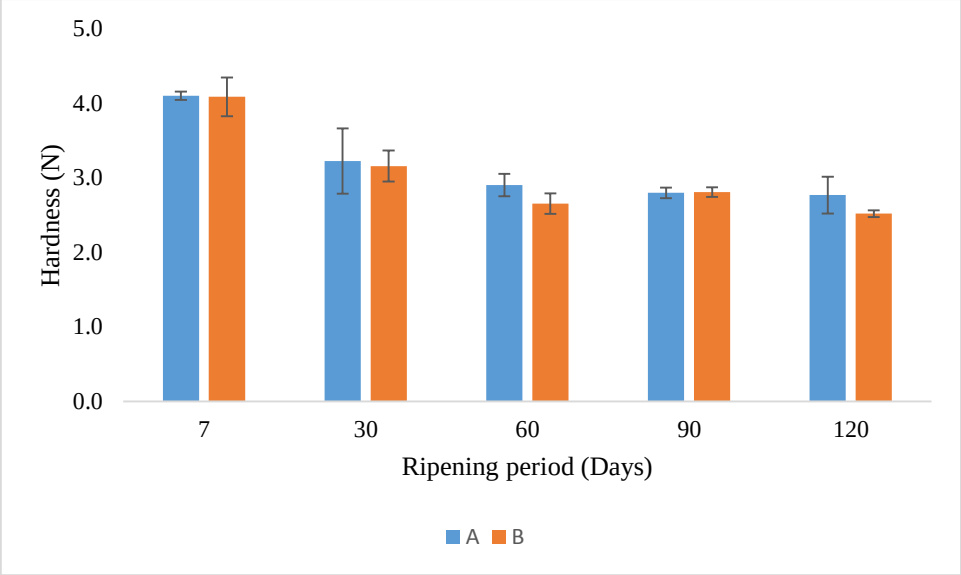

Figure 4.27. Changing in hardness (N) of the cheese samples during ripening

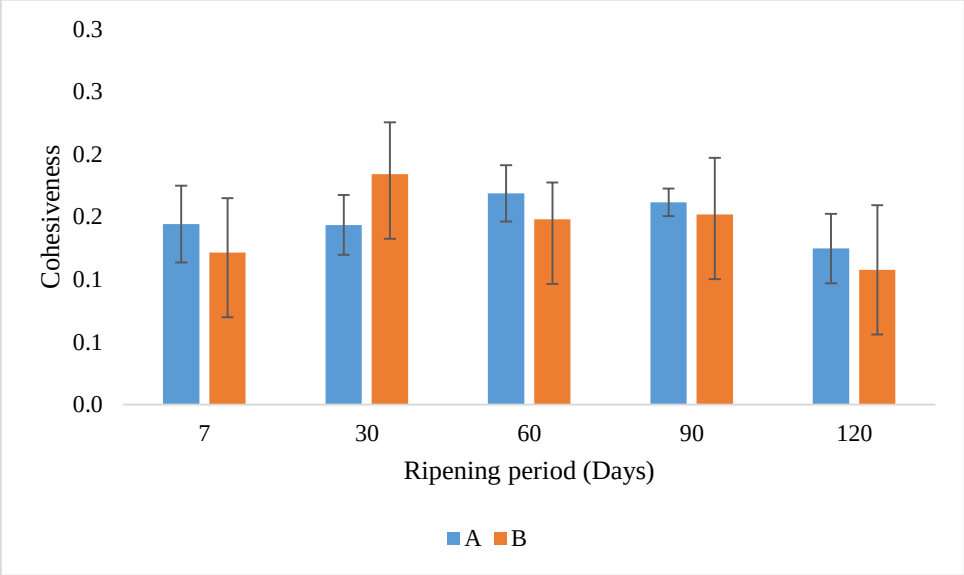

Figure 4.28. Changing in cohesiveness of the cheese samples during ripening

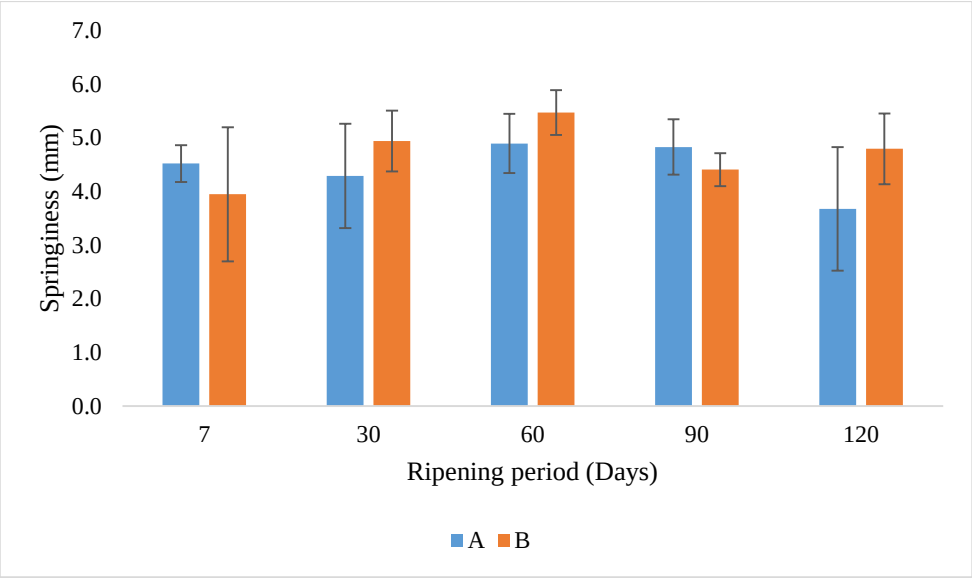

Figure 4.29. Changing in springiness (mm) of the cheese samples during ripening

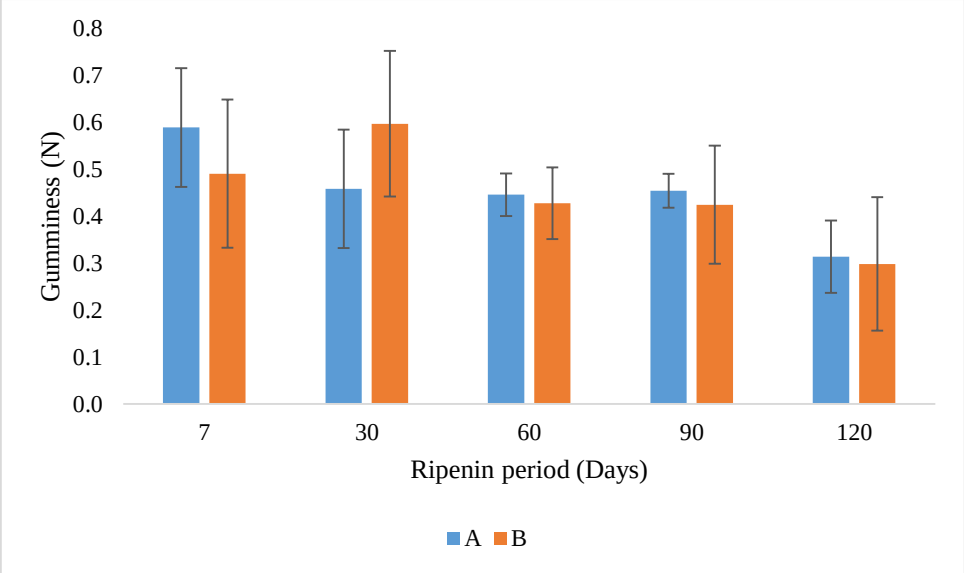

Figure 4.30. Changing in gumminess (N) of the cheese samples during ripening

Gumminess is defined as the energy needed to split a food product to parts that make it ready for swallowing [161]. Gumminess values were decreased in both trials during ripening days. They were found between 0.30±0.14 and 0.60±0.16 during the ripening. Reduction of gumminess is the expected result because increasing of gumminess is a reflection of increasing in proteolysis level in cheese structure. The results are in line with data reported by Sahingil et al [162].

Adhesiveness during ripening days was shown fluctuation. It was varied between 0.07±0.02 and 0.14±0.11. Adhesiveness is severely related to the fat content and DM in cheeses [163].

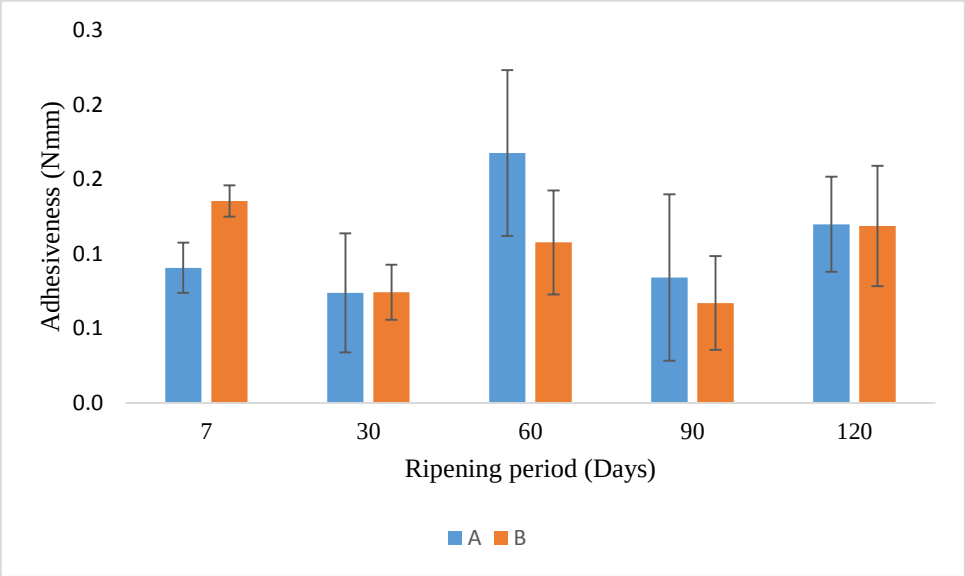

Figure 4.31. Changing in adhesiveness (Nmm) of the cheese samples during ripening

4.10 Results of Sensory Evaluation of the Cheese Samples

5 trained panelists have evaluated cheese samples in the term of appearance, body and texture, odour, taste, bitterness level and total acceptability (overall impression). Mean values of sensory evaluation results are given in Table 4.14, 4.15, 4.16, 4.17, 4.18 and 4.19. The template used in the sensory evaluation of cheese samples is given in appendix 1.

Table 4.14. The mean values of the appearance scores of the UF cheese samples

Ripening period (Days)	Cheese A	Cheese B
7	10.00±0.00	10.00±0.00
30	10.00±0.00	10.00±0.00
60	10.00±0.00	10.00±0.00
90	10.00±0.00	10.00±0.00
120	10.00±0.00	10.00±0.00

According to Table 4.14, no changes were found in the appearance of both trials during ripening days. It is in good agreement with results reported by Bulat [143].

Table 4.15. The mean values of the body and texture scores of the UF cheese samples

Ripening period (Days)	Cheese A	Cheese B
7	9.80±0.20	10.00±0.00
30	9.25±0.95	9.80±0.05
60	10.00±0.00	10.00±0.00
90	9.40±0.40	9.80±0.20
120	9.30±0.57	9.67±0.50

As it can be seen in Table 4.15, cheese B has higher scores that showing higher acceptability compared with cheese A. Panelists have reported that cheese B were more creamy texture than cheese A.

Table 4.16. The mean values of the odour scores of the UF cheese samples

Ripening period (Days)	Cheese A	Cheese B
7	9.00±1.41	8.50±0.70
30	9.50±0.50	10.00±0.00
60	9.60±0.50	10.00±0.00
90	9.50±0.50	10.00±0.00
120	9.00±0.00	9.30±0.50

Minimum odour score was in cheese B at 7 days (8.50±0.70), then that cheese trials get highest scores during the ripening. However, the difference between them was not significant, though ($P > 0.05$).

Table 4.17. The mean values of the taste scores of the UF cheese samples

Ripening period (Days)	Cheese A	Cheese B
7	9.00±0.00	8.50±0.70
30	8.50±0.58	10±0.00
60	9.50±0.50	10±0.00
90	8.00±0.57	8.75±0.57
120	7.00±0.00	8.00±0.00

The minimum value of taste score was found in cheese A at 120 days and the highest score was belong to cheese B at 30 and 60 days. As it can be concluded from Table 4.17, taste scores in both trials were increased until 60 days and then decreased and at the end of the 120 days, cheese B had higher score in compared with cheese A. Improvement in the taste of the cheese samples until 60 days can be related with catabolism of FAA and FFA which

leads to creation of flavour compounds and consequently improvement in taste of the cheese. The decrease in taste scores after 90 days can be the result of the creation of bitter amino acids and short hydrophobic peptides which adversely influence the taste of the cheese.

Table 4.18. The mean values of the total acceptability scores of the UF cheese samples

Ripening period (Days)	Cheese A	Cheese B
7	4.50±0.70	4.00±0.00
30	5.00±0.00	5.00±0.00
60	4.80±0.28	5.00±0.00
90	4.00±0.00	4.50±0.57
120	3.00±0.00	4.00±0.00

Total acceptability values of the cheese samples increased until 60 days for cheese A and 90 days for cheese B and then fallen with progressive of the days (Table 4.18). Adjunct culture contained cheese samples were found higher than control cheese at 120 days of ripening. This may be related with the enzymatic system of adjunct culture that let them contribute to the development of flavour compound and consequently their positive effects on overall cheese properties. Bitterness values are shown in Table 4.19. The bitterness was detected at 90 and 120 days in both trials. Bitterness in control cheese at 90 and 120 days was found higher than cheese B. The difference between them was significant ($P<0.05$). Formation of bitterness can be related with accumulation of peptides that are responsible for bitter off-flavour. The balance between the creation of peptides and their consequent degradation to amino acids is very significant. The ability of cultures to degrade peptides that are responsible for bitterness is important to prevent this defect in cheese. Low bitterness scores of cheese B may be attributed to the high aminopeptidase activity of CR319 adjunct culture that leads to debittering of the cheese [2].

Table 4.19. Mean values of the bitterness scores of the UF cheese samples

Ripening period (Days)	Cheese A	Cheese B
7	0.00±0.00	0.00±0.00
30	0.00±0.00	0.00±0.00
60	0.00±0.00	0.00±0.00
90	2.12±1.03	1.00±0.00
120	2.34±0.57	1.00±0.00

5. CONCLUSION

The aim of this study was to investigate the effect of commercial adjunct culture having high aminopeptidase activity on the properties of the UF white cheese which is one of the most popular cheese varieties in the Mediterranean region. CR-319 which contains *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* is used as a commercial adjunct culture. The effects of adjunct culture on the microbiological, chemical, biochemical, textural and sensorial properties were investigated. For this purpose, UF white cheese production has been performed and trials are coded as A and B. Cheese A (control) was produced by using standard LAB culture and cheese B was manufactured like as cheese A except for the addition of adjunct culture of CR-319 to the standard LAB culture. After the production, cheeses were stored at 6-8^{o}C, and were analyzed at 7, 30, 60, 90 and 120 days of ripening.

The microbiological analysis was performed at M17 and MRS agar growth mediums. The results of LAB count on M17 agar were found between 5.11±0.00 and 7.66±0.48 for cheese A. Those values were between 6.90±1.27 and 8.47±0.09 for cheese B. The addition of the adjunct culture was found a significant effect on the LAB count between two trials at 30, 60, 90 and 120 days of ripening ($P < 0.05$). The number of LAB on MRS agar was found between 3.21±0.19 and 6.18±0.04 for cheese A and between 7.16±0.02 and 8.19±0.08 for cheese B. The addition of the adjunct culture had a significant effect on the LAB count between two trials on MRS agar growth medium ($P < 0.05$). The LAB count at both medium was shown decreased during ripening days but this decrease was found more intense at the control cheese and the amount of viable LAB at cheese B at the day 120 was found higher at the both medium. The existence of the high amount of LAB on M17 and MRS agar in cheese B (due to the adjunct culture) may be resulted from the synergist effect of adjunct culture by producing free amino acids that catalysis the LAB growth.

Cheese B at all analysis stages were shown higher pH values in compared with cheese A and the difference between cheese pH values statically was found significant ($P < 0.05$). This result can be related to high proteolysis level of cheese B or antagonistic effect of CR-319 on starter LAB. The results of titratable acidity values are in line with pH values so that the amount of titratable acidity of the cheese A was found more than cheese B at all analysis days. The differences between the titratable acidity of the trials were found significant at 30 and 120 days of ripening ($P < 0.05$).

Dry matter contents (%) of the UF cheese samples during ripening vary between 34.20±0.35 and 36.59±0.93. At all analysis stages, DM (%) values of cheese A were found higher than cheese B. However, the differences between groups and days were not significant ($P > 0.05$). This result is may be related to the difference in pH values between two trials (cheese with high pH level can bind more water at its structure and also, high proteolytic activity enhance water holding capacity). The addition of adjunct culture had not significant effect on salt/DM (%) and fat/DM (%) values between two trials, though ($P > 0.05$).

The yield (%) of cheese with adjunct culture were found higher than control cheese at all analysis days and the differences between yield values of two trials found significant ($P < 0.05$).

The amount of WSN fraction of both trials were shown increases during ripening and at the day 120, the WSN values of cheese A and B was found 17.87±0.67 and 20.30±0.11 respectively. Differences between trials were not significant except at the day 120 ($P > 0.05$). %12 TCA soluble nitrogenous substances of both trials were also increased by progressing the ripening days. The differences between days were found significant for both trials ($P < 0.05$). Briefly, the presence of the adjunct culture of CR-319 enhanced the level of primary and secondary proteolysis as measured by the concentration of WSN, 12% TCA-SN and FAA.

According to urea-PAGE electrophoretograms, the amount of the residual β-casein (%) were decreased to 81.44±3.20 for cheese A at day 120 and this value for cheese B was found 69.65±3.52 at 120 days of ripening. The Same trend was observed for α_{s1}-casein degradation. The residual α_{s1}-casein (%) values of cheese A and B was found 65.23±3.06 and 46.51±2.40 at 120 day, respectively. According to urea-PAGE results, the effect of CR-319 treatment on proteolysis was statistically important ($P < 0.05$). Likewise, according to the RP-HPLC analysis that was performed for determination of proteolysis and peptidolysis levels of the cheese. It was shown that adjunct addition greatly influenced RP-HPLC profile as expected. Furthermore, RP-HPLC chromatograms indicated that hydrophobic peptides of the cheese B were lower than cheese A.

FFA analysis was performed for determining the lipolysis level of the cheese. The lipolysis was limited for both trials but the level of lipolysis was higher in cheese B. The dominant individual FFA of both trials was palmitic acid and oleic acid respectively.

Volatile compound analysis of cheese samples was performed by GC-MS system and the number of various volatile compounds such as acids, esters, ketones, aldehydes, alcohols and miscellaneous compounds was determined. Generally, the volatile component of cheese B was found higher than cheese A. Especially, ketones such as diacetyl, 2,3 pentanedione, acetoin etc. were at high concentration in cheese B. Those volatiles contribute buttery notes to cheese samples.

The hardness of both trials was decreased during ripening days. The amount of cohesiveness, springiness, gumminess and adhesiveness values of both trials were shown fluctuation during ripening. The sensory analysis also indicated that the utilization of adjunct culture of CR-319 had a positive effect on overall acceptability in UF cheese without causing bitterness and improved aroma properties of cheese samples.

In conclusion, present results showed that CR-319 adjunct culture contributes to the acceleration of flavour development of UF white cheese and reduce bitterness during the 120 days of ripening. The effect of adjunct was clear after 60^{th} or 90^{th} day of ripening. It also improves the chemical and sensorial properties of the cheese. It leads to the more creamy-like texture of the cheese. At the industrial view, utilization of adjunct culture of CR-319 may help to improve cheese yield and may help to decrease cheese defects. So, it can be beneficial for the improvement of financial features of the production.

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APPENDIX

SENSORY EXAMINATION EVALUATION SCORES FOR CHEESE SAMPLES

EVALUATION CRITERIA	Score
APPEARANCE AND COLOUR	
Uniform, intact bright white, homogeneous and uniform prismatic cheese	10
Mat. Pale white color and a few holes and pores on the cross-section surface	9
Non-homogenous appearance	8
Non-uniform color distribution	7*
Uneven prismatic appearance	6*
Fractured cracks, excess perforations and pores, browning and abnormal color, mouldy appearance	≤ 5
TASTE	
Unique and usual taste	10
Yeasty or cooked taste	9
Salty taste	8
Sour taste	7*
Sweety and crude taste	6*
Metallic, mouldy, bitter, rancid or ammonia taste	≤ 5
ODOUR	
Unique and usual odour	10
Yeasty odour	9
Sour odour	8
Mouldy odour	7*
Strange, animal or grassy odour	≤ 6
BODY AND TEXTURE	
Smooth, spotless, homogeneous cross section view, not too hard or too soft	10
Dry and hard texture	9
Crumbly texture	8
Spotted cross section view	7*
Slippery texture	6*
Sandy, elastic, soft and wet, slit and cracked, molten structure	≤ 5

TOTAL ACCEPTABILITY	Score
Liked it very much	5
Can be consumed	4
Should be corrected	3
I do not like it at all	1-2

BITTERNESS LEVEL	Score
No bitterness	0
Very little bitterness	1
Apparent bitterness	2
High bitterness	3
Very high bitterness	4

CURRICULUM VITAE

Credentials

Name, Surname : Sattar EGHBALIAN

Place of Birth : TABRIZ / IRAN

Marital Status : Single

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Address : Hacettepe University Food Engineering Department 06800, Beytepe, Ankara, Turkey

Education

High School : Shahed high school

BSc. : Azad University of Sofian

MSc. : University of Hacettepe

Foreign Languages

English (Fluent)

Turkish (Fluent)

Persian (Fluent)

Work Experiences

-

Areas of Experiences

Dairy Science and Technology

Project and Budget

Hacettepe University Scientific Research Project Coordination Unit, Project Number: FHD-2016-11071, Utilization of Commercial Adjunct Culture In The Manufacture of Ultrafiltrated White Cheese And Its Effects On The Cheese Properties. Researcher 2016-2017

Publications

-

Oral and Poster Presentation

-

HACETTEPE UNIVERSITY
GRADUATE SCHOOL OF SCIENCE AND ENGINEERING
THESIS/DISSERTATION ORIGINALITY REPORT

HACETTEPE UNIVERSITY
GRADUATE SCHOOL OF SCIENCE AND ENGINEERING
TO THE DEPARTMENT OF FOOD ENGINEERING

Date: 02/06/2017

Thesis Title / Topic: **Utilization of commercial adjunct culture in the manufacture of ultrafiltrated white cheese and its effects on the cheese properties**

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I respectfully submit this for approval.

02/06/2017
Date and Signature

Name Surname:	Sattar EGHBALIAN
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ADVISOR APPROVAL

APPROVED.

Assoc. Prof. Ali TOPCU
(Title, Name Surname, Signature)