UNIVERSITE DE LILLE- SCIENCES ET TECHNOLOGIES

Ecole doctorale Science de la Matière, du Rayonnement et de l'environnement

THESE DE DOCTORAT

Spécialité : Biotechnologies agroalimentaires, sciences de l'aliment, physiologie

Présentée par

Mustapha NAJIB

Pour l'obtention du grade de

DOCTEUR DE LUNIVERSITE DE LILLE ET DE L'UNIVERSITE LIBANAISE

Étude du procédé de fabrication et caractérisation des interactions moléculaires lors de la fabrication de la Qishta et évaluation de la stabilité du produit alimentaire

Manufacturing process study and characterization of molecular interactions during the production of Qishta and the stability assessment of the food product

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Préparée au laboratoire :

Unité Matériaux et Transformations CNRS UMR

Equipe : Processus aux Interfaces et Hygiène des Matériaux

Président du jury de la thèse : Romdhane KAROUI

Soutenue le 17 Juillet 2020 devant le jury composé de :

Nour-Eddine CHIHIB, Professeur, Université de Lille	**Directeur**
Monzer HAMZE, Professeur, Université Libanaise	**Directeur**
Adem GHARSALLAOUI, Maitre de conférences, Université de Lyon 1	**Rapporteur**
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Loubna FIRDAOUS, Maitre de conférences, Université de Lille	**Invitée**
Samer HALLAB, CEO, Hallab 1881 s.a.l.	**Invité**

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Scientific valorizations

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Oral presentations

1) M. Najib, M. Hallab, K. Hallab, Z. Hallab, G. Delaplace, M. Hamze, N-E. Chihib. (2019). The mechanisms of the heat-induced interactions of milk components during the process of Qishta: A Lebanese cream. Journeé des jeunes chercheurs, Université de Mons, Belgique, 7 November, 2019.

2) M. Najib, M. Hallab, K. Hallab, Z. Hallab, G. Delaplace, M. Hamze, N-E. Chihib. (2019). Effect of heat treatment on cross-linking of milk proteins during the heat treatment of milk: Qishta's case. Second European food chemistry and nutrition congress. Amsterdam, 27-28 July, 2019.

3) M. Najib, M. Hallab, K. Hallab, Z. Hallab, G. Delaplace, M. Hamze, N-E. Chihib. (2018). Traitements thermiques et produits laitiers cas de la Qishta, le Kajmak et la Khoa. Sécurité et Qualité alimentaire 2. Tripoli, Lebanon, 12-14 April, 2018.

4) M. Najib, M. Hallab, K. Hallab, Z. Hallab, G. Delaplace, M. Hamze, N-E. Chihib. (2017). Characterization and understanding of the mechanisms involved in the production of "Qishta". Journeé des doctorants. Lille, 14 November, 2017.

ABSTRACT

Manufacturing process study and characterization of molecular interactions during the production of Qishta and the stability assessment of the food product

Abstract

Qishta is a Lebanese dairy product obtained by heating whole milk for 2 to 3 hours in an open shallow vessel. During the process, milk is added to readjust its level and compensate the amount of evaporated water. The process consists of harvesting the coagulum formed at the milk surface. Our findings showed that these aggregates result from the denaturation of milk proteins and their interactions with the fat globules. This work has demonstrated that Qishta has almost the same amount of proteins and fat estimated at 12% and therefore the product has a composition similar to that of Ricotta cheese made from whole milk. The characterisation of Qishta using mass spectrometry has allowed to determine and quantify the proteins present in Qishta. These analyses have demonstrated also the presence of "Cross links" between proteins which are involved in the Qishta coagulum formation. Lanthionine and lysinoalanine were identified as neoformed amino acids during the heat treatment. These amino acids are involved in the cross links formed during the heat treatment of milk. These amino acids will establish covalent bonds between the proteins of Qishta coagulum. The confocal laser scanning microscopy analyses of Qishta samples previously treated with Fast Green and Nile red dyes have allowed to highlight the protein-fat interactions considered as the source of coagulum formation.

Our findings showed the significant effect of the fat amount present in the initial milk on the process and the yield of Qishta. Increasing the amount of milk fat leads to an increase in the Qishta yield. A milk with 3.2% of fat has given the maximum yield. However, the use of skim milk in the production did not nor lead to any coagulum formation.

The effect of ageing during Qishta storage for 20 days at 4 °C on the fat oxidation and therefore on the Qishta shelf life has been realized. The results of the primary and secondary indicators of oxidation analyses have showed the fat present in Qishta was not oxidized during the study period. In addition, the emission spectra of tryptophan, riboflavin and the excitation spectra of vitamin A were directly recorded using frontal fluorescence on Qishta stored at 4 ° C for 20 days.

The analysis of these spectra by multidimensional statistical tools such as principal component analysis and discriminant factor analysis has allowed to significantly differentiate Qishta samples. Indeed, these analyses have allowed to significantly distinguish between the samples and subsequently predict their shelf life of the product.

Key words: Qishta, Milk, Heat Treatment, Casein, Whey protein, Cross-links, Lysinoalanine, Lanthionine, Fat, Oxidation, LC-MS/MS, Fluorescence spectroscopy, Confocal Scanning Laser Microscopy.

RESUME

Résumé

La Qishta est un produit laitier Libanais obtenu par traitement thermique de 2 à 3 heures du lait entier dans un plateau peu profond. Durant le procédé, le lait est ajouté ponctuellement afin de réajuster le niveau du lait et de compenser la quantité d'eau perdue suite à l'évaporation. Le procédé consiste à récupérer le coagulum qui se forme à la surface du lait. Les agrégats formés proviennent de la dénaturation des protéines et de leurs interactions avec les globules gras. Ce travail a permis d'établir que la Qishta est composée de 12% en protéine et 12% en lipide, composition similaire à celle de la Ricotta. La caractérisation de la Qishta par spectrométrie de masse a permis d'identifier et de quantifier les protéines présentes dans le produit. Ces analyses ont permis aussi de démontrer la présence de « Cross links » entre les protéines. Les résultats montrent la formation de lysinoalanine et de de la lanthionnine lors du traitement thermique. Ces acides aminés vont établir des liaisons covalentes entre les protéines du coagulum de la Qishta. L'analyse par microscopie confocale de la Qishta préalablement traitée par le Nile Red et le Fast Green a permis de mettre en évidence les interactions lipoprotéiques à la base de la formation du coagulum.

Les études menées ont permis aussi de démontrer l'effet significatif de la quantité de la matière grasse sur la production et le rendement de la Qishta. L'augmentation de la concentration de la matière grasse dans le lait conduit à une augmentation du rendement. Le rendement maximal est obtenu pour un lait entier à 3,6% en matière grasse. Cependant, l'utilisation du lait écrémé pendant le procès n'aboutît pas à la formation de la Qishta.

L'effet du vieillissement lors du stockage de la Qishta pendant 20 jours à 4 °C sur l'oxydation de la matière grasse et par la suite sur sa durée de vie a été réalisé. Les résultats des analyses des indicateurs primaires et secondaires d'oxydation montrent que la matière lipidique n'a pas été oxydée pendant la durée étudiée. Par ailleurs, les spectres d'émission du tryptophane, de la riboflavine et les spectres d'excitation de la vitamine A ont été enregistrés directement au moyen de la fluorescence frontale sur de la Qishta stockée à 4°C pendant 20 jours. L'analyse de ces spectres par des outils statistiques multidimensionnelles telles que l'analyse en composante principale et l'analyse factorielle discriminante ont permis de différencier d'une manière significative les échantillons de la Qishta. En effet, ces analyses ont permis de distinguer d'une manière significative entre les échantillons et par la suite de prédire leur durée de vie.

Mots clés : Qishta, Lait, Traitement thermique, Caséine, protéine sérique, Cross-links, Lysinoalanine, Lanthionnine, Globule Gras, Oxydation, LC-MS/MS, Fluorescence spectroscopie, Microscopie Confocale.

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GENERAL INTRODUCTION

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General introduction

Milk and dairies are considered as ones of the most demanded products worldwide. The increase in demand on dairy product could be attributed to the population growth and to the life style in the developed countries which resulted in higher per capita milk consumption (Muehlhoff, Bennett & MacMahon, 2013). Milk heat treatment is considered as a critical step in reducing the bacterial load and therefore increasing the shelf life of the product (Muir, 1996). Regardless the final product, the process of dairying which relies on the heat treatment shares some similarities such as protein denaturation, fat coalescence and Maillard reaction (Al-Saadi, Easa, & Deeth, 2013). Interestingly, milk has been a subject for huge investigations and studies, which facilitated the understanding of many mechanisms and molecular interactions undergone during its heat treatment. However, there is still a lot to investigate in this complex matrix (Heiner, Wilson, & Lahey, 1964). India, USA and China represent the main milk producers with more than 35% of the total cow milk produced in 2016 (FAO-Infographic-milk-facts-en). Despite the development of programs that aim to improve the milk sector and motivate the population to the importance of national milk supply, cow milk production in Lebanon covers a small part of the need (Muehlhoff, Bennett & MacMahon, 2013). Lebanon imports milk powders from France, Denmark, Netherlands, and Eastern Europe such as Czech Republic to cover the deficit (Arja, Haddad, Mouawad, & Serhan, 2001). Laban, Labneh, Qishta, are such an example of Lebanese traditional dairy products highly consumed and demanded. Qishta is a Lebanese dairy product obtained by heating whole milk, for couple of hours, in a specific artisanal way practiced only in Lebanon. Despite being present in almost every day's Lebanese menu, Qishta was not well studied neither for its fabrication process and its composition nor for its shelf life, except one article which

describes briefly the chemical composition of the product (Kassaify, Najjar, Toufeili, & Malek, 2010). The process of Qishta consists of heating milk in an open shallow vessel for more than 3 hours from one side. This vessel has a diameter of 1 meter, a capacity of almost 10 liters and a thickness that varies between 0,2 cm and 0,7 cm (Figure 1). During the process, milk is added in order to compensate the amount of evaporated water due to the intense heat treatment applied and to readjust the level of milk in the plate. At the end of the process, Qishta which consists of the coagulum formed at the milk surface will be gathered, drained, packed and stored at 4°C. According to Hallab 1881 company; which is considered as one of the biggest producers of oriental sweets and Qishta in Lebanon and in the Middle East, a production day is divided into three cycles of production, where the residual milk at the end of the first cycle will be used in the second cycle of production. A production cycle begins from milk addition's step to the Qishta gathering. This will create, in addition to the different variable during the process of Qishta, such as milk addition, temperature, milk hydration and heat intensity, a difference in the composition of the Qishta obtained from each cycle. Milk powder is used for most of the Qishta production in Lebanon. However, the hydration process of milk differs from a producer to another, yet the final product is visually the same. In this context, we have established a research project with the participation of Hallab 1881 company, This study represents to our knowledge the first study that focus on the processing, the mechanism of formation and the chemical stability of the Qishta. This study aims to characterize the mechanisms involved in the Qishta formation during milk heat treatment, to master the process and therefore control the consistency issue of the final product. We aim to understand the factors impacting the shelf life of Qishta estimated nowadays for 4-5 days

according to the producers in Lebanon. Lastly, we attempted to improve the procedure of Qishta resulting in a difficult work environment that can affect negatively the shelf life of the Qishta.

In that context the first goal was to find out some key points such as similar products in the literature on which we can built our strategy, the focus was done on a research carried out on thermal processing of milk as a main tool in the production, this part corresponds to the first chapter of this PhD.

The second objective was to analyze, to study and to understand the Qishta black box processing procedure mainly base on a on heat treatment and to characterize the composition of the product, this part corresponds to the second chapter of this PhD.

The third objective was to understand the molecular interactions which take place during the heat treatment and which are involved in the coagulum formation, this part corresponds to the third chapter of this PhD.

The fourth objective was focus on the chemical stability of the product using classical and alternatives strategy based on the front-face fluorescence spectroscopy for monitoring lipid oxidation during Qishta ageing, this part corresponds to the fourth chapter of this PhD.

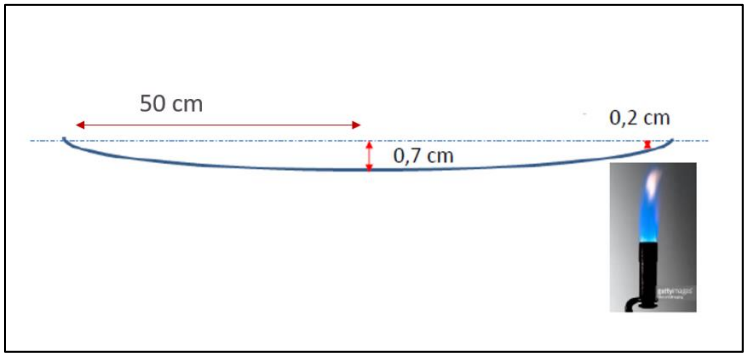

Figure 1: Dimension of the plate used at Hallab 1881 factory during the Qishta production and position of the burner. The plate has a diameter of 1 meter and a thickness of 0,2-0,7 cm.

CHAPTER : I

ARTICLE I- LITERATURE REVIEW

Thermal processing of milk as a main tool in the production of Qishta, Khoa and Kajmak

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Abstract

Since their beginning, dairy industries have experienced different level of expansion. From adapting heating-milk process to applying spray drying process, a long journey of innovation has been gone through. Despite all the innovations made and all the new technologies implemented in this sector, heat treatment of milk, either for extending its shelf life or for creating new products, has been a key factor in that manner. Qishta, Khoa and Kajmak are typical examples of traditional dairy products respectively in Lebanon, India and Serbia and which only depend on the temperature as a main tool of their process. These three products share at the same time some similarities and some dissemblance. We believe that these products descend maybe from one ancestor, yet little changes have been implemented to the process of each, in order to suit the traditions and the practices applied in each country. While Kajmak is rich in fat (47-60%), Khoa and Qishta contains only 27 and 12% respectively. The effect of heat treatment of milk on the interactions between its components, in addition to the description of the process and the composition of these traditional products are discussed in this paper.

Keywords: Heat Treatment, Milk, Qishta, Khoa, Kajmak.

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Introduction

Milk and dairy products are considered nowadays essential elements in each one's life and an important need for all humans in general. Before they are even capable of digestion, infant mammals use milk as a primary and unique source of nutrition [1]. The nutritional value of milk is determined according to the balance of nutrients that it contains. Regardless of their origin, fat, proteins and minerals are present in milk from any species, but within different values [2]. Table 1 shows the milk composition variations according to species [3,4].

Milk can be directly consumed, or valorised, through different treatments, into milk by-products. Historically, human civilization has always been searching for ways to store food for more scarce times. Henceforth, technologies to preserve food have been adapted for many years. These technologies did not only induce changes to the product's shelf life, but also left it with a new set of physiochemical and sensorial attributes. There are several principal categories of milk products such as liquid/beverage milk, cheese, milk powders, concentrated milks, fermented milk products, butter, ice-cream, infant formula, creams, protein-rich products…

Table 1: Chemical composition of milk in various species [3,4] .

Composition	Goat	Sheep	Cow	Human	Camel
Fat (%)	3.8	7.9	3.6	4.0	2.9
Solids-non-fat (%)	8.9	12.0	9.0	8.9	8.7
Lactose (%)	4.1	4.9	4.7	6.9	4.91
Protein (%)	3.4	6.2	3.2	1.2	2.5
Ash (%)	0.8	0.9	0.7	0.3	1.3
Moisture (%)	79	68.1	78.8	78.6	79.8

ARTICLE 1 CHAPTER 1 LITERATURE REVIEW

Milk by-products are defined according to the process they have been subjected to. Milk can be altered by acids, enzymes, temperature or microorganisms. When heated, the chemical constituents can undergo major changes depending on the intensity and the time of treatment applied. Several alterations in heat treatment in terms of time, temperature, pre- and post-treatments of milk, type of heat-exchanger used and others, will determine a unique final product. Through a specific treatment, a unique product is made with specific sensorial, chemical and physical properties [5]. Products obtained from heated milk remained widely accessible because heat treatment was mastered by all cultures around the world. Importing a food product to a new geographical area required sometimes adaptations depending on culture, climate and raw material availability. Lebanese Qishta ,Turkish Kaymar, Indian Khoa, Serbian Kajmak and Iraqi Geymer are examples of almost similar products present in different countries, probably descending form one ancestral [5,6] and obtained mainly by heating milk. Indian Khoa and Lebanese Qishta are subjected to almost the same heat treatment technique. Traditionally, these products are slightly different because of their making process techniques. During Khoa preparation, milk is stirred vigorously, while in Qishta production milk is not stirred leading to a much more soft and creamy texture; not forgetting the characteristic skin development (solids clotting on the liquid surface) rather than a complete transformation of the entire product [7-9]. Therefore, both humidity and solid dry matter of these two products are different. The concept of Kajmak preparation is different from those of Qishta and Khoa, however its texture can be similar to theirs. In certain areas, traditional products still have a major indigenous production, while in other places, industrial production is on the rise to take over the market [11]. In this paper, the characteristics of production and final composition of some of these traditional products will be investigated and compared.

This review aims to describe the process and the chemical composition of three partially dehydrated dairy products, all of which relying on heat treatment as the main and only tool during their processing. First of all, the making process of each of these popular traditional dairy products will be described. Then, an investigation on the effect of heat treatment on milk components will be done. Finally, those products will be compared in terms of parameters, composition and sensory aspects.

Industrial versus indigenous technology

During the last century, science invaded the dairy industry and succeeded, to a certain level, in clarifying and explaining the craft traditions of the past. The combination of old practices with technical innovations made the development of traditional products (such as cheeses and yogurts) possible. Innovative technology has created new products, such as spray dried milk products, milk protein concentrates and whey protein concentrates, that meet the needs of today's society and has increased the shelf life of certain dairy products to an unexpected level (such as spray dried milk products). Cow's milk has a primary role in the dairy industry today despite the growing trend towards buffalo, goat and sheep milk [11,12].

Since these traditional products have deep historical origins, they became part of culture; thus, they turned out to be an essential part of our everyday cuisine and a staple of most special occasions. As a result of welfare and increased population in many places worldwide, these products are now in greater demand than ever. Local manufacturers seek new and improved means of production and some have even taken the step to go from the indigenous and traditional artisanal production to an engineering project of complex science. While in some areas it started decades ago, this process still has barely begun in others.

Indian Khoa

Considering the manufacture of milk for all mammal species, India is at the top of rankings, consolidated by its buffalo milk producers. With respect to cows' milk alone, India is the second largest producer, after the USA, with 133.6 million tons between 2017 and 2018 [14]. Nine million tons of total milk produced in India are used in the production of Khoa; a traditional heat-dried Indian dairy product prepared by thermal concentration of milk in an open shallow pan with continuous stirring and scraping [9]. The majority of production is still artisanal in India, however substantial efforts were made to scientifically study the products and technologically enhance their production. Most of the Khoa produced in India belong to some small private merchants, whom have inherited this craft from ancestors and transmitted their traditional utensils of former times. Due to the small production scale (4-5 Liters of milk per batch) and the increasing demand on this type of product, different processes have been developed in order to improve the yield of Khoa's production [15]. In 1968, the first equipment to produce Khoa continuously with a capacity of 50 liters per hour has been created. The process included a steam jacketed cylinder fixed with rotational scrappers pursued by final concentration in an open flowing steam jacketed pan with mechanical scrapping agitators. Afterward, the process of Khoa making was optimized with many changes [16]. In 1968, Kumar et al.,[17] have created a machine in order to produce Khoa under rural conditions. In fact, the open shallow pan was semi jacketed and equipped with a mobile scraper in order to harvest the product during the heat treatment of milk [18]. National Dairy Development Board, Anand, India developed an Inclined Scraped Surface Heat Exchanger (ISSHE) for continuous khoa-making [17]. Currently, after 70 years of research and development,

70% of the volume is still produced by small, local producers and mostly with elementary technology.

Lebanese Qishta

In Lebanon, the situation is practically different. The lack of structured farms and the inconsistency in milk composition lead the producers of Qishta to use milk powder [19]. The historical origin of Qishta is not well known. In the Middle East different spellings are used for the Qishta such as Kishta, Kashta or Ghishta, in the present work the spelling Qishta will be used according to Kassaify et al., [8]. Today, Qishta is prepared from powdered or pasteurized liquid milk in either small dairy plants or in large-scale open shallow pan in order to be freshly consumed as a dessert. However, it will often be use in further reprocessing in order to produce typical oriental sweets. Milk is heated on one side in a large open shallow pan. Several minutes later and depending on the intensity of the flame, the phenomena of protein and fat denaturation will occur as the milk starts boiling, leading to the formation of a coagulum, called Qishta, at the surface. The boiling intensity pushes the Qishta formed to the opposite side (to the burner) of the pan. There has been no initiative to start the research and development of Qishta production up until the recent few years, except the work done by Kassaify et al., [8] on the chemical and microbiological characteristic. So far, the industry is practicing what can be classified as an artisanal approach of production (even though it is at factory scale), but a growing initiative to begin this process is now found. It is of huge interest to increase the volume and quality of Qishta production, mainly because of the increase demand of Qishta inside and outside Lebanon. The strategy they implemented so far was initially to contact the scientific community. From there on, they have to

allow researchers to start bigger projects concerning shelf life, compositional determination of products and by-products,

physicochemical mechanisms, as well as the industrial and standardized processing. This last part is an ultimate goal since recreating the same valued product, in all its features, but with an efficient industrial process instead of the kitchen-approach, is the crux of it all.

The success has been valuable so far, but there is a long way to go. Seeing how the Indians have worked for 70 years, it is observable that many years of strong effort and resources must be invested in order to reach even partial industrialization, but the situation might turn out completely different as a result of today's information availability.

Serbian Kajmak

In Serbia, the dairy sector depends on cow's milk processing. Milk production is stabilized at around 1.6 million tons liters per year. One half of the total quantity is purchased and processed in dairies while the other half is spent and/or processed in rural farms for cheese and cream and sold in markets [20]. According to the total cow's milk production and to the FAOSTAT data in 2008, Serbia took the 48th position worldwide [21]. Some of the cheeses which consist of 20% of the marketplace and regularly sold in green markets are: the special types of locally produced cheeses such as fresh or short shelf life cheese with geographic recognition and special cheeses like kajmak and sour cream [22]. The process of Kajmak formation is based on the top layer's surface activity of boiled milk. Hot milk is poured into the open shallow vessels where a sort of primary skin is formed on the top of the milk due to the evaporation and surface activity. The

kajmak created is collected, salted and placed layer by layer in suitable vessels where maturation takes place [10].

What motivated various investigators to search out for a suitable solution to industrialize kajmak production and improve its market position was the regional superiority of kajmak and its delicious organoleptic qualities. In order to induce a product with standard characteristics, the industrial production of kajmak needs to be standardized. It is important to have better information of processes concerning production and maturation stages of kajmak. It is also important to gain information regarding Kajmak composition and characteristics [6]. Polimark company has lately developed a new process for the production of kajmak that shows a promising solution. This process covers all steps comprised in traditional kajmak production. However, they are accomplished by strategies considered acceptable for industrial implementation, in such way that they eliminate safety risks and enable standardization of production [6]. Their process incorporates manifestly two significant steps: cold agglutination and hot incubation, that yield to the creation of both upper and lower kajmak layers. The situation in Serbia is to some extent in between India and Lebanon. There was a detailed research over virtually 20 years, and a summary of most of the work has been done.

Constituents of milk

Water

Water is the principal component of most dairy products. It varies from 2.5 to 94% (w/w) according to the product. In addition to pH and temperature, water plays a key role in food technology since it can impact directly the texture of the products and therefore their utilization. It is considered an

essential diluent in foodstuffs, since it can modify the chemical, physical and microbiological aspects of the final product [23].

Proteins

Milk and most of dairy product properties are influenced by their proteins content more than any other constituent. According to their properties, proteins are considered nowadays as one of the best characterized food systems. Research on milk proteins started since the early nineteenth century. Innovative work was accounted first by Schubler on the milk proteins and by Braconnot who was in charge of the appropriation of the word 'casein' in 1830. Bovine milk contains generally 2.5 to 3.5% of proteins. This amount depends on the breed, the individual variation of the animal, and to a lesser extent, on the stage of lactation, the nutritional status and the health of the animal [23,24]. Milk is considered as a good source of essential amino acids. In addition, it contains a wide array of proteins with biological activities, ranging from antimicrobial ones to those facilitating absorption of nutrients, as well as those acting as growth factors, hormones, enzymes, antibodies and immune stimulants [25,26].

Milk proteins are made out of whey proteins (20%) and caseins (80%). Caseins are recognized to convey phosphate and calcium, having numerous bioactive functions and contributing to proficient digestion [25]. Whey proteins have an assortment of dietetic and biological properties and consequently are extensively utilized in decreasing the possibility of diseases such as inflammation [27-28], cancer [29], human immune efficiency virus infection [30] and chronic stress-induced disease [31].

Casein

Caseins are defined as the milk fraction that precipitate by acidification to pH 4.6 at 20°C. Casein consists of four families: αS1-casein (α_{S1}-cn), αS2-casein (α_{S2}-cn), β-casein (β-cn) and κ-casein (κ-cn) representing 40%, 10%, 38%, 12%, of the total casein, respectively [32]. Caseins are characterized by different numbers of phosphorylated serine residues, which give them different properties [33]. α_{S1}-cn and β-cn do not contain any disulphide bonds however, α_{S2} and κ-casein own two. Due to their opened structure, caseins have a high surface hydrophobicity. This results in a fragile secondary structure and irregular coiling of the primary chain. Caseins are heat resistant due to their weak secondary and tertiary structures. This open structure similarly makes caseins sensitive against proteolysis within different enzymes, particularly pepsin [34].

The phosphate groups are situated in clusters bound to serine residues. Because of their negative charges, the phosphate groups have the capacity to bind ions, particularly Ca^{2+}. This ions binding is important in the transportation of phosphate and calcium to the neonate. Caseins are delicate to the variation in the calcium level of milk, and their precipitation can be induced when the calcium level is increased. The most sensitive type of calcium casein is the α_{S2}-cn, however κ-casein got the least amount of phosphate groups and is not influenced by the calcium concentration present in milk [35].

In order to stabilize their structure, caseins tend to connect to each other's, through hydrogen bonds, to form casein micelles with an average diameter of 200 nm. Almost 95% of the casein are consequently bounded in casein micelles. These micelles consist of 94% proteins, while the rest of the 6% are denoted as colloidal calcium phosphate that comprises phosphate, calcium, little amounts of magnesium, citrate and other components [36]. Different models have been established

in order to clarify the structure of casein micelle, such as the nanocluster model displayed by Holt and Horne [37] and the sub-micelle displayed by Walstra [38]. Until this moment, none of the models are totally checked. It is then known that the core of the casein micelle is the location of the most hydrophobic and calcium sensitive caseins, α-cn and β-cn, while the surface with its polar C-terminal outside the micelle core is the location of the most hydrophilic and calcium insensitive κ-cn which makes the casein soluble.

Whey Protein

Whey proteins or serum proteins constitute around 20% of the overall proteins of bovine milk. Whey or milk serum, defined as the remaining soluble fraction after casein precipitation at pH 4.6, comprises four main protein categories, namely β-lactoglobulin (β-Lg), bovine serum albumin (BSA), α-lactalbumin (α-La), and immunoglobulins [39]. whey proteins in general and α-La in particular, have high nutritional value, which leads to the fact that whey proteins derivatives are widely used in food industries. In bovine milk, almost 50% and 12% of whey proteins are represented by β-Lg and α-La respectively. Whey proteins are highly structured proteins with stable secondary and tertiary structures. Hydrophobic interactions, disulphide bonds, ion-pair interactions hydrogen bonding, and van der Waal's interactions are the major forces responsible for sustaining their globular structure [40]. Whey proteins are highly soluble in milk over a wide range of pH due to their native composition and to the huge amount of hydrophilic buildups on the surface of the globular structure and the high quantity of disulphide bonds [41]. The proteins are then resistant to proteolysis due to their globular structure.

Fat

Milk is an emulsion where fat, which represents around 3 to 5%, is presented as small globules or

droplets dispersed in the aqueous phase of the milk [42]. Their diameters vary from 0.1 to 20 µm. The stability of this emulsion is assured by a thin membrane called milk fat globule membrane (MFGM), which has an important role controlling the communication between the fat globule and the surrounding milk [43]. Triglycerides are the principal components of the milk fat. Moreover, we can find di- and monoglycerides, fatty acids, sterols, carotenoids, which give the yellow colour to the milk, in addition to the vitamins (A, D, E, and K), and all the other trace elements [44]. Milk fat globule membrane is composed of phospholipids, lipoproteins, cerebrosides, proteins, nucleic acids, enzymes, trace elements (metals) and bound water. The structure of this membrane is not fixed; however, it is dynamic due to the continuous exchange with the surrounding media; therefore, the thickness of this membrane varies from 5 till 10 nm. Due to their size and their low density, fat globule will migrate to the surface after a certain time if milk was left without any intervention [45]. Pasteurization has a slight effect on lipid composition and content. On the other hand, homogenization is a process that increases the number of lipid globules at least 100-fold and the surface area about 6 to 10 times, diminishes their diameter from around 3 to 0.8 µm and modifies the globule membrane composition and structure. The globule surface is mostly but not totally recoated with caseins.

Minerals

Table 2 shows the concentration range (expressed in mass and molar concentrations) of different minerals in cow milk. This composition is considered as generally steady yet slight deviations can be observed at different times. Thus, milk could be considered rich in proteins, containing high content of phosphate and calcium. Minerals concentration varies according to the lactation time period. The most significant variations in the composition occur at around parturition; in this

manner the concentration of the calcium in colostrum is much higher than that of normal milk and close to the end of lactation [46].

Table 2: Mineral composition of cow milk [45].

Mineral	Concentration ($mg.kg^{-1}$)	Concentration ($mmol.kg^{-1}$)
Calcium	1043-1283	26-32
Magnesium	97-146	4-6
Inorganic phosphate	1805-2185	19-23
Total phosphorus	930-992	30-32
Citrate	1323-2079	7-11
Sodium	391-644	17-28
Potassium	1212-1681	31-43
Chloride	772-1207	22-43

Calcium

Milk is a major source of calcium for human consumption. The colloidal phase contains 66% of the total calcium present as calcium phosphate while, the remaining calcium is present in the soluble phase [46]. Ionic calcium represents around 10% of total calcium [47]. At pH 5.2, inorganic calcium phosphate is completely destroyed, therefore all inorganic phosphate will be solubilized. The complete solubilization of calcium occurs at pH 3.5. A significant role on the kinetics of protein denaturation was shown when the amount of calcium increased. In fact, the unfolding and aggregation stages of β-lactoglobulin and α-La were affected with the increase in

Ca^{2+}concentration [48]. When there is an excess of calcium ions, the protein denaturation occurs at lower temperature than in standard case. Before heat treatment, the addition of $CaCl_2$ to skim milk provided a noticeable rise in the rates of whey proteins denaturation, apart from the immunoglobulins [49]. In order to understand the influence of increasing Ca^{2+} concentration on denaturation of whey proteins in milk, studies have been carried out. In 1984, Bernal and Jelen [50] demonstrated that when calcium ions are bounded to α-La, heat stability of this last is increased by promoting renaturation on cooling. Nevertheless, Li et al., [51] showed that Ca^{2+} stabilized the unfolded development of lactoglobulin and, consequently, advanced its denaturation.

Lactose

Lactose belongs to the group of organic chemical compounds called carbohydrates. Lactose is a disaccharide of glucose and galactose, and can be found only in milk [52]. Its hydrolysis occurs in the intestinal mucosal cells. In milk, lactose content varies from 4.5 to 5.5% [53]. Lactose is water soluble and has a low sweetness capacity evaluated as 16 comparing to that of sucrose estimated as 100 [52]. It plays a key role in fermentation process by impacting the amount of lactic acid produced during milk products fermentation. Human lactose intolerance arises from their incapacity to hydrolyse lactose due to the absence of lactase. This enzyme deficit is common in eastern Asia and African countries [54]. Lactose is the principal component of milk powder. It creates an impact matrix for the dispersion of fat and proteins. Lactose has a primordial role, with the participation of proteins, in emulsion stability. Maillard reaction, discussed here after, which involves the ε-amino group of lysine and the carbonyl function of a reducing sugar (lactose) is the

main reason along with fat oxidation behind the flavour, solubility and colour alteration during the storage of milk [55].

Heat treatment of milk

Milk stability is assured by the physiochemical properties of its components and by the equilibria resulted from the interactions between salts, proteins and fat. These equilibria are highly temperature-dependent. In the following chapter, the effect of heat treatment on pH, mineral balance, protein denaturation and the interactions between proteins and fat are studied.

Heat-induced changes in milk pH and mineral balance in milk

Ma and Barbano [56] demonstrated that upon heating milk at temperatures up to 80°C, the pH decreases from 6.8 to 6.2. In 1981, Fox [57] noted that milk may be heated for more than 3 hours at 140°C without any coagulation if the pH was adjusted continuously. According to Van Boekel [55], heat-induced acidification of milk is due to: 1) formation of organic acids and mostly formic acid, 2) insolubility of tertiary calcium phosphate and 3) casein's dephosphorylation. The continuous decrease in milk pH is due to the formation of organic acids, arising from the lactose degradation. Berg and Van-Boekel [58] noted that the formation of formic acids occurs through two ways. Lactose degradation is responsible of 80% and the rest is obtained as a result of Maillard reaction upon heating the milk at 110–150°C. The isomerization/degradation path is defined by the transformation of lactose to lactulose via the Lobry de Bruin-Alberda van Eckenstein transformation, then into galactose and other C5 and C6 compounds. The other 20% of formic acids are obtained upon the degradation of lactulosyllysine; an Amadori product obtained through the interactions of lactose with ε-amino group of lysine. Concerning the heat-induced dephosphorylation of casein, we distinguished two cases: the heat induced dephosphorylation of

sodium caseinate and calcium caseinate. Belec and Jenness [59] have demonstrated that 50% of the sodium caseinate dephosphorylation occurs within the first hour of heating at 120°C, however less than 80% of dephosphorylation occurs after 5h at 120°C.

Heat-induced dissociation of caseins

κ-casein has a primary role in maintaining the integrity of casein micelle. In addition to other factors, its dissociation contributes to the heat induced coagulation of milk. Singh and Fox [59–63] have demonstrated its role during the heat stability phenomenon. In fact, the dissociation of κ-casein from micelle leaves this latter in a depleted way, more sensitive to calcium binding and therefore to coagulate. In addition to the temperature, different factors have a big impact on the dissociation of κ-casein such as the amount of whey proteins, the minerals and the solid contents of milk. Concerning whey proteins, we distinguish two scenarios according to the pH. Below 6.7, the addition of β-Lg decreases the extent of heat-induced dissociation of κ-casein, while inversely at pH> 6.7, the heat-induced dissociation increases [64].

Heat-induced denaturation of whey proteins and casein- whey protein interactions

Due to their globular structure, whey proteins are thermolabile. While heating milk above 60°C, serum proteins unfold then denature (Figure 1).

Denaturation of the major whey protein β-Lg involves two different phases. The first phase exposes the hydrophobic residues and disulphide bonds due to the unfolding of the native globular structure. At this moment, in case the heat treatment is interrupted or limited, the unfolded whey protein will refold. At high temperature, the unfolded whey protein can interact with other molecules such as caseins mostly via covalent and disulphide bonds and therefore it will lead to

the formation of aggregates [39,65]. When heating milk at a temperature between 60 and 70°C, tertiary structure of the β-Lg will unfold, creating a sensitive monomer with free thiol group (Cysteine 121) and hydrophobic parts of the residues chain [66]. These monomers are able to interact with other monomers but also with caseins; therefore, forming protein aggregates [67].

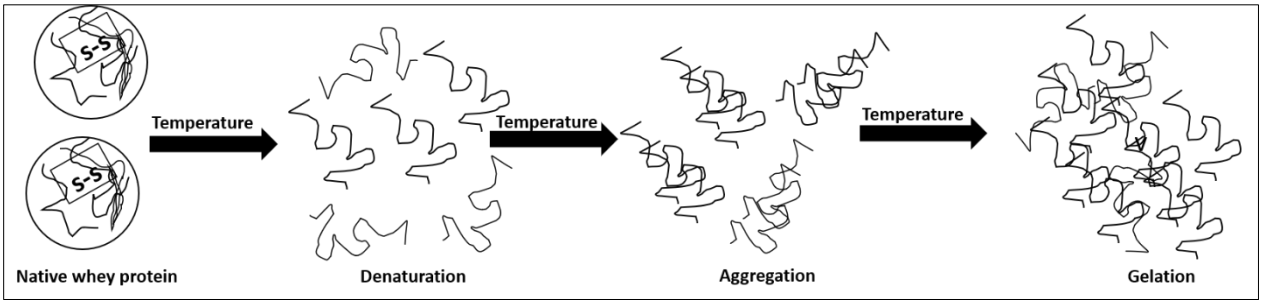

Figure 2: Stages of whey protein denaturation [66].

Temperature	Time	Structure/Reaction	Result
20°C	--	β-Lg (at pH<3.5 or pH>7.5 β-Lg (at 5.5<pH<7.5) or β-Lg (at 3.5<pH<5.5)	Native molecule
~ 40°C	--	↓ β-Lg	Dissociation/ Formation of monomers
~ 40-55°C	5-10 min	↓ β-Lg	Partial unfolding
~ 60-85°C	≥15 min	↓ -SH β-Lg → β-Lg $_{S-S}$ β-Lg/κ-cn α-La/β-Lg → α-La/β-Lg	Formation of complexes with other proteins and/or
~ 125°C	5-10 min	↓ β-Lg → $\beta\text{-Lg}_{S-S}$/ $\beta\text{-Lg}_{SH}$	Complete unfolding

Figure 3 : Effect of temperature on the interactions between β-lactoglobulin and other proteins. Mechanism of thermal denaturation of β-lactoglobulin in a neutral or slightly alkaline pH including the possible complexes with other milk proteins [66,67].

According to their structure and the strength of their intramolecular bonds, whey proteins do not have the same resistance to temperature. α-La is the least thermo-resistant, followed by β-Lg, BSA and immunoglobulin [68,69]. Depending on the temperature intensity, different aggregates can be formed during heat treatment of milk (Figure 2). These aggregates can be the results of complexes formed between denatured whey proteins or between caseins and whey proteins. Interactions occur between β-Lg and κ-casein if the temperature is above 70°C through the disulphide bonds. Below this temperature, interactions occur through hydrophobic links [68-70]. The position of κ-cn has a primordial role in the complex formation with the whey proteins. In fact, placed on the surface of casein micelles, it is easier for κ-casein to interact with the β-Lg then when it is dissolved in serum where the compact structure can reduce the association ability between casein and whey protein [71,72]. Heat treatment process has also an important role on promoting the interactions between proteins and hence the formation of complexes. In reality, a process which requires a slow heating rates or low temperature kinetics, fosters the interactions between β-Lg and the casein micelles. However, heating at high temperatures drives the β-Lg to refold in a non-native structure and therefore it forms aggregates with other whey proteins instead of being associated with κ-casein. In this case, the formation of complexes between β-Lg and α-La is promoting. Actually, α-La requires a continuous heating at high temperatures and it does not interact directly with the casein micelles [72-74].

Heat-induced protein- fat interactions and emulsion instability

Dickinson and Parkinson [75] defined emulsion stability as its resistance to change over time. In milk emulsions, the composition and the concentration of proteins present in the continuous phase has a primordial role on protein's absorption at the fat membrane. In fact, during heat treatment of whole milk, caseins and whey proteins are opponents, in terms of which is going to be absorbed first at fat droplet surface [75,76]. Whey proteins are highly sensitive to heat treatment and are responsible of the emulsion instability during the technological process. As discussed before, heating milk between 60 and 70°C changes the properties of whey proteins therefore they aggregate. The preference between whey protein and casein adsorption on fat membrane will be decided according to protein concentration. At low protein concentration, whey proteins adsorb in preference to caseins at the oil droplet surface due to their limited spreading at interface. However, at high protein concentration i.e. above 3 wt%, the caseins adsorb in preference to the whey proteins [75-78]. As individual molecules or as small aggregates, caseins have been used as emulsion stabilizing owing that to their flexibility and their high surface activity.

Once aggregated, caseins form loops and tails extending during the continuous phase. According to Hunt and Dalgleish [76], the formation of these tails leads to the formation of a secondary protein layer. However, Ye [79] indicated that increasing the ratio whey/casein resulted in the formation of a secondary layer at the fat droplet interface. Till today, the effect of heat treatment on the interaction between proteins and fat droplet and on the competition between whey proteins and caseins has not been well studied due to the complexity of the milk matrix.

At low protein concentration (below 3 wt%), a heat-induced flocculation of the oil droplets took place [75,80]. An intense heat treatment lead to the formation of a new interfacial layer thicker

than a monolayer, [81,82] indicating that the non-adsorbed proteins fraction are primordial and play a key role in the fat droplet's flocculation mechanism. In fact, removing this fraction from the emulsion decreases extremely the aggregation of the fat droplets. On the other hand, increasing the whey proteins concentration enhances the coalescence of the fat droplets and the emulsion viscosity until the critical concentration of gelation was reached. This later was estimated at around 3 wt% of non-adsorbed whey proteins [81]. Above 3% the heat-denatured whey proteins non-adsorbed will connect the different fat droplets in a continuous network. Euston et al., [82] compared the whey proteins to a glue that connects the fat droplets in the continuous phase. However, below the critical concentration, whey proteins are not able to play the glue role and therefore the gelation will not occur.

Qishta, Khoa and Kajmak: overview, process and final product composition

Qishta

Qishta is a traditional Lebanese dairy product, made through subjecting a small area of a large shallow stainless-steel pan, filled with whole milk, to high heat via flame (figure 3) according to "Hallab 1881" company process. It is a mixture of a thin skin layer formed on the surface of milk and of a coagulum formed in the heated zone of the pan. Milk fat aggregates contribute to give Qishta some characteristics of butter.

Qishta is related to cheese family (figure 4 a-b), especially to cream cheese varieties due to the presence of milk proteins as well as their particular coagulation process during Qishta development. This product is identified somehow between cheese and cream. However, according

to its composition, Qishta can be looked at as similar to Ricotta cheese with almost an equal amount of fat and protein of around 12% (Table 3). The final product has a sharp and white colour.

It is creamy, bulky with a smooth texture, and a sweet taste. Qishta can be consumed as it is or after further processing. It can be used as filler in different Lebanese desserts such as Knefe, Halewe eljeben, Mafrouke and many more. It has a shelf life of 4 hours at ambient temperature, and up to 4 days at 2-5°C. The pH of Qishta (6.5) is high comparing to other dairy products such as whipped creams, clotted creams and yoghurts. Such findings explain the short shelf-life of the product. Furthermore, combination of high moisture content with pH is a major factor in making the product susceptible to high microbial contamination and growth.

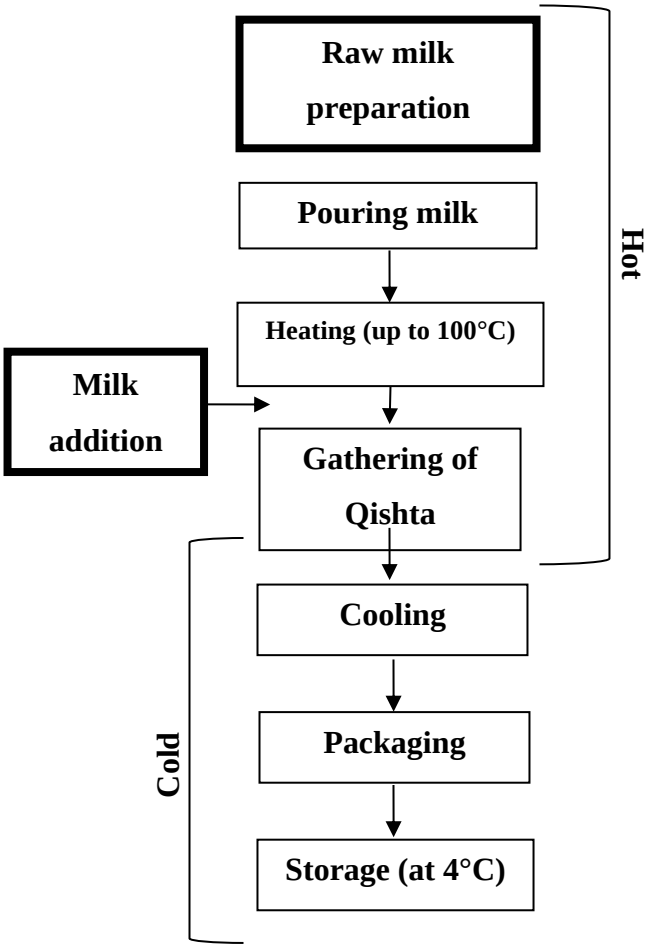

Figure 3: Major steps of Qishta's process according to the process used by "Hallab 1881" company Lebanon.

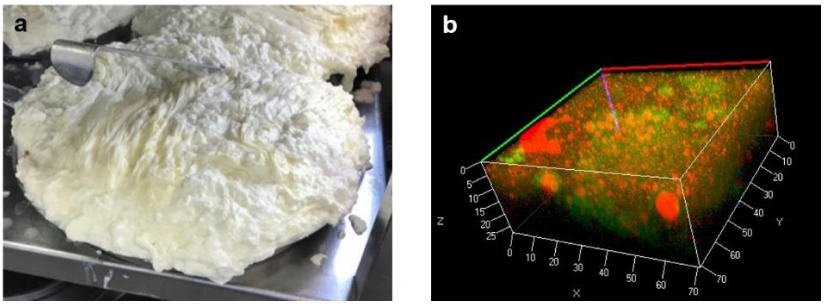

Figure 4: Qishta during draining step (a). Visualization of proteins (green) and fat (red) in Qishta (b) using confocal scanning laser microscopy. Nile red and fast green were used In order to stain fat globules and proteins.

Kajmak

Kajmak is a heat concentrated dairy product produced in regions of the Balkans, Turkey, Iran, Afghanistan and India [6]. In Serbia, the traditional process of Kajmak dominates almost all the production [10]. Regarding of its physio-chemical characteristics, Kajmak can be placed between cheese and butter [11]. The process of kajmak consists of pouring milk into an open shallow vessel where, due to both surface activity and evaporation, a skin is formed on the top of the milk (figure 5).

Table 3: The average composition (%) of butter, Qishta, cream cheese, kajmak and Ricotta cheese [5].

Parameter	Butter	Qishta	Cream cheese	Kajmak		Ricotta cheese
				Fresh	**Ripenned**	
Moisture	16	68	53-60	30-40	53-60	74.5
Fat	85	12	30-34	40-55	30-34	9
Fat in DM	>98	37.5	70	65-80	70	35.29
Protein	0.5	12	7-10	5-10	7-17	10.9
Protein in DM	0.6	37.5	20	7-17	20	42.7
Ash	0-1	1.6	0.5-0.8	0.5-2	0.5-0.8	0.6-4.5

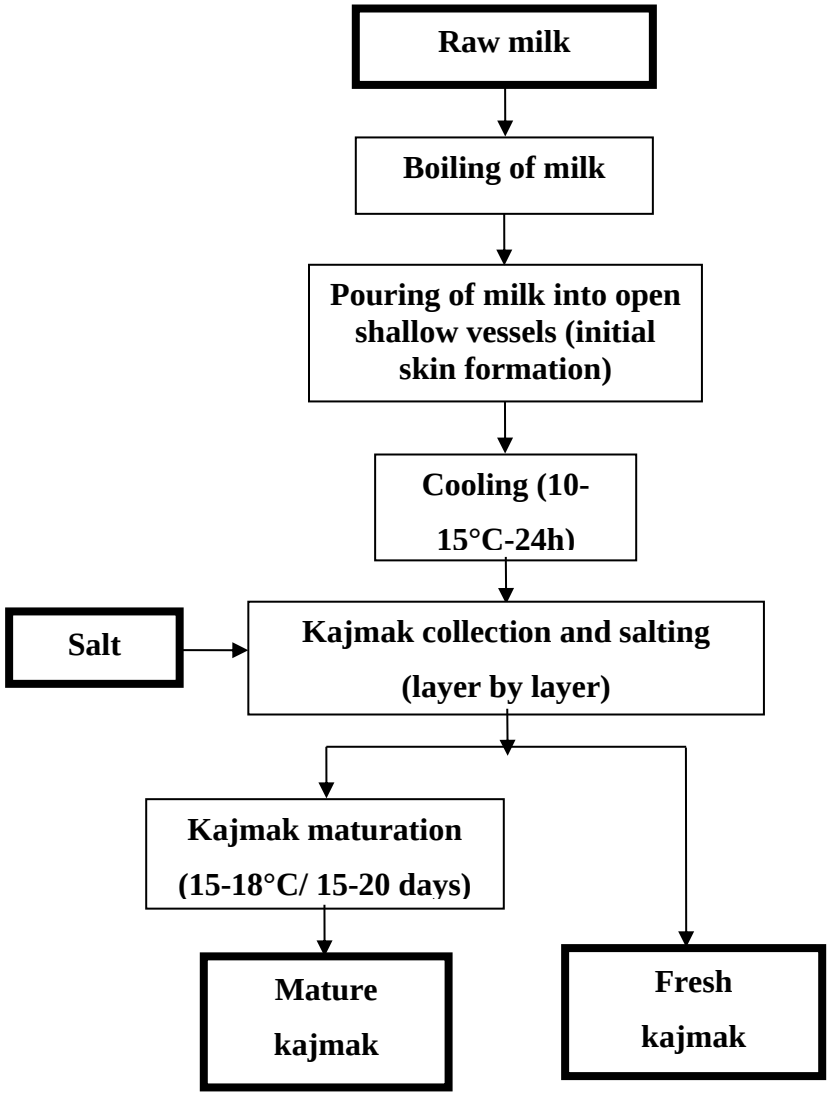

Figure 5: The procedure of traditional Kajmak production [5].

This process can be affected by different factors such as: initial milk temperature, milk composition, temperature difference between milk and air, temperature and humidity of the surrounding air and the type of raw milk used. In fact, even though cow milk is mostly used in the Kajmak's production, some regions in Serbia like Bosnia and Montenegro are now replacing cow

milk by ewe milk, or mixing the two types together. A gradual procedure of milk cooling occurs directly after initial skin formation and lasts for around 24 hours. Throughout the milk cooling procedure, fat globules arise from the most profound milk layers and join the recently formed initial skin. This action results in the development of a thin, yet smaller layer known as Kajmak. Kajmak formed on the top of milk is collected and salted then placed layer by layer in suitable vessels where maturation takes place. Kajmak's maturation occurs at 15 – 18°C, over 15 – 20 days. Matured kajmak, stored in cold conditions, below 8°C may last from 3 to 6 months, and sometimes even up to one year [11]. The yield of traditional kajmak production ranges from 4 to 5%. The residual milk that remains after Kajmak's collection is incompletely skimmed and has an average fat level of around 1.4 – 1.7%. This last is used in the production of some Serbian cheese [11]. The main components of Kajmak is milk's fat which constitutes around 45% of the fresh product and 60% of the ripened one. Protein part is less present with an average of 5 to 10% in the fresh product and 2 to 7% in the ripened one. According to Radovanovic et al., [10], the amounts of fat and protein are larger in the top layer than in the others. The difference between dry matter and protein contents matches with their textural appearances. Practically, lower layer is very viscous liquid similar to cream, with higher water content and much less proteins than the top layer which is similar to a crust. This considerable difference in protein content, as well as in overall appearance, results in the identification of different formation mechanisms of these two layers.

The colour of kajmak is mostly affected by the maturation period, amount of milk fat and milk type. During ripening period, the colour of Kajmak changes from white to yellow. The whiter colour of Kajmak in some regions is due to the usage of the ewe's milk in Kajmak preparation [11].

Khoa

Khoa or Mawa is a traditional Indian dairy product, generally utilized as a precursor for more complex desserts, yet it can be consumed alone. It is obtained by indirectly heating milk present in a stainless-steel pan. Milk is continuously stirred either mechanically or by hand, in order not to be burn. Water will then easily evaporate, leaving a semi-solid product having low moisture content of almost 30% in comparison to raw milk 90% [17]. Khoa is a traditional dehydrated dairy product prepared by thermal concentration of milk in an open shallow pan with continuous stirring and scraping [9]. The whole milk is heated and boiled in a stainless steel or an open pan and stirred continuously in order to avoid scorching. Due to the intense evaporation that occurs during the process of Khoa making, the thickness of milk increases as well as the concentration of the total solid's particles. Coagulation occurs when the concentration of milk reaches 2.5 and 2.8 times the initial milk's concentration in cow and buffalo milk respectively [15]. The Chemical composition varies considerably, with a moisture percentage ranging from 19.26 to 28.41. Khoa contained on average 27% fat, 19% protein, 25% lactose and no sucrose [83-84]. The type of milk used during the manufacturing of Khoa has a big impact on the final composition. It was reported that Khoa produced with cow milk appears more yellow that when produced with buffalo milk [15]. One kg of Khoa is produced within almost two hours by the traditional production, using 4 or 5 liters of buffalo milk. Recently, new developed techniques have expanded the yield to almost 1 kg/15 min/machine. The yield of Khoa is affected by several factors such as, quality of milk used, intensity and time of heating, processing, handling and lastly the moisture of the final product, which is considered as the main factor impacting the yield. Gupta and Gupta [85] showed that buffalo milk Khoa has more yield and better texture than cow milk Khoa. Depending on the

texture, composition and quality, three categories of Khoa exist, named Pindi, Dhap and Danedar. Khoa can be further processed into large variety of indigenous sweets such as Burfi, Pera, Gulab jamun etc. It can also be used as filling in many food items. Among cheese and butter products, Khoa is the most dairy expended product in India [86]. Khoa is a perishable food having a short shelf life of 3 days at room temperature and 2 weeks under refrigerated conditions [15]. Shelf life of Khoa has been accounted for exceptional changes due to the huge change in milk quality, climate, hygiene practice and accessible cooling technology between the producers; however, it is still prone for microbial contamination. In fact, *Staphylococcus aureus* and *Bacillus cereus* are the most contributable contaminating microorganisms in Khoa, contributing to a lot of food-borne diseases. Narang et al., [87] showed that 48 hours after its production, Khoa started to have a rancid flavour which deteriorates the sensory quality of this product. It has been subjected to an intensive work in order to increase its shelf life. Jha and Verma [88] showed that the addition of potassium sorbate increases the shelf life for more than 40 days. Rao and Singh [89] showed that the combination of potassium sorbate addition and the nitrogen injection can increase the shelf life up to 18 days at 5°C.

Conclusion

Qishta, Khoa, and Kajmak represent a family of dairy products which relies on heat treatment of milk during their processes. Despite the deep investigations held on milk, these products still need a lot of research in order to understand the mechanism of their formation. These three products share almost the same process steps, with a slight difference applied in each country. However, their composition is widely different due to raw material used that differs from a region to another. In India, in spite of the efforts done on understanding and industrializing the products, almost all

of the quantity is produced following the traditional method. In Serbia and Lebanon, more research has to be accomplished in order to understand the mechanisms involved in the Qishta and Kajmak formation before trying to industrialize products. However, the essential question which should be asked is the following: does the use of machines allows to conserve the traditional aspects of these kind of products?

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CHAPTER : II

ARTICLE II - RESULTS

Introduction

Despite the high demand on Qishta in Lebanon, no scientific researches were done in order to characterize the chemical composition and the mechanism involved in the formation of this product except one article that describes the chemical and microbiological profile of the Lebanese Qishta (Kassaify, Najjar, Toufeili, & Malek, 2010). The process of Qishta consists of heating milk from one side in an open shallow vessel. This results in a heterogenous temperature distribution that has a significant impact on the final product. The addition of milk during the process, at different time and at different quantities, results in a non-consistent product. Thus, the Qishta composition varies according to the employee's performance, hydration process, milk addition, ambient temperature, and heating temperature intensity.

It is of high importance to characterize the chemical composition and the Qishta formation process as well as the study of the effect of temperature distribution and the interactions that occur between milk components during the heat treatment. Such study will help to master the process and understand the role of each component on the final product. Understanding the mechanism involved in the Qishta formation such as the interactions between casein and whey proteins and between proteins and fat globules. This will help to improve and understand the clue behind this unique process and whether we are capable to obtain the same product with the same texture and same organoleptic characteristics but with a different technique.

In this context, the first part of this thesis has focused on the characterization of the process of Qishta formation, the chemical composition of this product and the chemical mechanism involved in the coagulum formation. The capability of making Qishta without fat or by variating its amount was investigated. The identification and the quantification of the individual proteins present in Qishta were done through traditional and mass spectrometry analysis. This last, coupled with

electrophoresis analysis and confocal microscopic images, were used in order to explain the molecular interactions that occur between milk components during the process.

Article II

Qishta - A Lebanese Heat Concentrated Dairy Product Characteristics and Production Procedures

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ARTICLE II **CHAPTER II** **RESULTS**

Abstract

This study aims at exploring the chemical composition of a traditional Lebanese dairy product known as Qishta, describing the process of how to prepare it and understanding the mechanisms leading to its formation. The process of making Qishta can be divided into two phases: a hot phase during which milk is heated in a stainless-steel large shallow vessel, and a cold phase consisting of draining, cooling and packaging. According to milk temperature, two reaction zones were identified: zone A with an average temperature of 100 °C, and zone B with an average temperature of 60 °C. The results showed that Qishta had a moisture, fat, protein, lactose and ash content of 68%, 11.7%, 12.1%, 5.4% and 1.6%, respectively. Our findings showed that Qishta is a lipoprotein product having an equal amount of fat and proteins (≈12%); this composition is almost similar to that of Ricotta cheese made from whole milk. In addition, our results assert that the interactions between caseins and whey proteins lead to gel formation. Milk initial fat percentage had a significant effect on Qishta production. The highest yields were obtained when the initial fat percentage was 3.6% (182.5 g of Qishta).

Keywords: Qishta, heat treatment, milk, casein, whey protein

1. Introduction

During the last decade, Lebanese dairy manufacturing occupied an important role in the agro-industrial field. The local production of milk and dairy products covers only a quarter of the need [1]. Hallab 1881 company, one of the oldest family enterprises located in the north of Lebanon, is known for their sweets and dairy products such as Qishta. Hallab company is the pioneer of oriental sweets in Lebanon, producing more than 200 t of Qishta per year. Although the historical origin of this unique product is not really known, it began as a homemade product. In the Middle Eastern region, different spellings are used for Qishta such as Kishta, Kashta or Ghishta. In our study, the spelling Qishta will be used according to Kassaify et al. [2]. During the Othman empire, the product spread in the region and similar products existed in Turkey (Kaymak) [3] and in Serbia (Kajmak) [4].

Within the same context, Qishta is a popular Middle Eastern dairy product prepared using traditional heating and skimming processes [5]. The lack of milk collection industries in Lebanon has led the producers of Qishta to use milk powder as the raw material instead of fresh milk. Consequently, milk powders are imported from France, Denmark, the Netherlands, and Eastern Europe (particularly from the Czech Republic) [1]. The process of making Qishta can be divided into two phases: (i) the hot phase and (ii) the cold phase (**Figure 1**). The hot phase consists of heating acidified milk for two to three hours (performed by pouring and boiling acidified milk into open shallow vessels). Approximately 10 min after the beginning of heating, a skin layer (**Figure 2**a) appears on the milk surface (Qishta skin) as a result of the combined influence of: (i) protein denaturation and fat coalescence on the milk surface leading to a concentrated layer of both components; and (ii) intense evaporation of water from the surface due to the increase in milk temperature. The skin formed will be broken, creating a pathway for the aggregates (second layer)

formed in the heated zone and then gathered at the opposite side of the flame (**Figure 2**b). These aggregates will be gathered with the skin and drained in order to form Qishta (**Figure 2**c). The cold phase includes draining, cooling and Qishta packaging. The product has a chemical composition and texture comparable to those of Ricotta cheese prepared from whole milk, with a slightly higher amount of fat and protein. In Lebanon, the process of making Qishta varies according to the region, however the final product is almost the same. The process described in this article is in accordance with that reported by Kassaify et al. [2].

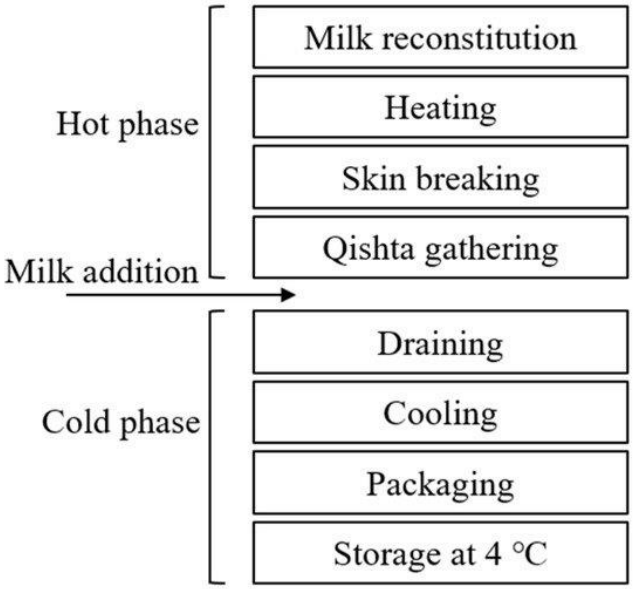

Figure 1. Key steps of Qishta process as observed at Hallab 1881 s.a.l. (Limited Anonymous Society).

Figure 2. (**a**) Skin formation (**b**) Aggregates gathering (**c**) Qishta drainage.

Qishta can be related to cheese family (especially to cream cheese varieties due to the presence of milk proteins and their particular coagulation during the Qishta making process). Qishta is usually not consumed fresh; however, it is further processed in order to prepare large oriental sweet varieties such as Knefe, Mafrouke and so forth. Knefe is made by filling Qishta between 2 layers of roasted semolina, poured with cane syrup and sprinkled with ground pistachio (**Figure 3**a), while Mafrouke is a mixture of roasted semolina dough and cane syrup topped with Qishta (**Figure 3**b) and fried nuts. Overall, Hallab 1881 company offers more than 50 products that contain Qishta.

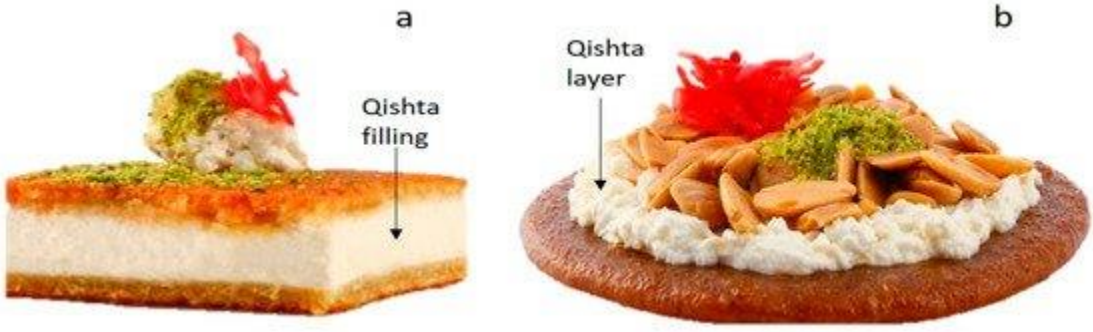

Figure 3. (**a**) Knefe, (**b**) Mafrouke prepared at Hallab 1881 company.

Despite the high consumption of Qishta in Lebanon, only one article has described the microbiological and chemical profile of this product [2]. The present work aims to determine the chemical composition of Qishta, to monitor the temperature profile of milk during the heating process and to understand the mechanism of Qishta formation.

2. Materials and Methods

2.1. Materials

Milk was provided by Lactel (Laval, France). It is an ultra-heat treated (UHT) whole milk with the following composition: 3.6% fat, 3.2% protein, 4.8% lactose and 1% ash. Lactic acid was purchased from Fischer Scientific (Loughborough, UK). The plate (stainless-steel, 316 L) used in Qishta preparation was provided by Nafco (Baouchriyeh, Lebanon) and the burner was provided by Brûleur AEM (Chelles, France) equipped with a pressure regulator.

2.2. Qishta Preparation Procedure

The plate (shallow vessel) used in Lebanon for Qishta production has a diameter of 1 m, a capacity of 9 L and a thickness of 2 mm. A new plate, with a 0.5 m diameter, 3 L capacity and a 2 mm thickness was adapted during our study by Nafco, in order to produce Qishta at a smaller scale. Lactic acid (1.5 mL) was added to 4 L of UHT milk in order to decrease the pH from 6.7 to 6.4 (the same as the procedure used at Hallab 1881 company). The traditional preparation process consists of heating milk for 2 h and simmering the aggregates formed at the surface. During the heating process and depending on the evaporation rate, milk is added in order to readjust its level in the plate. In our experiments, milk was not added in order to keep the milk's composition well known, and therefore the process was interrupted after 25 min of heating. The remaining milk at

the end of process was collected for further analysis. In Lebanon, this milk known as red milk and is revalorized into traditional dairy desserts.

2.3. Temperature Distribution

During heat treatment, the temperature distribution profile was monitored using 5 probes provided by ATC Mesures (Tourcoing, France). During the Qishta making process, the probes were immerged in the milk and their positions are indicated in **Figure 4**.

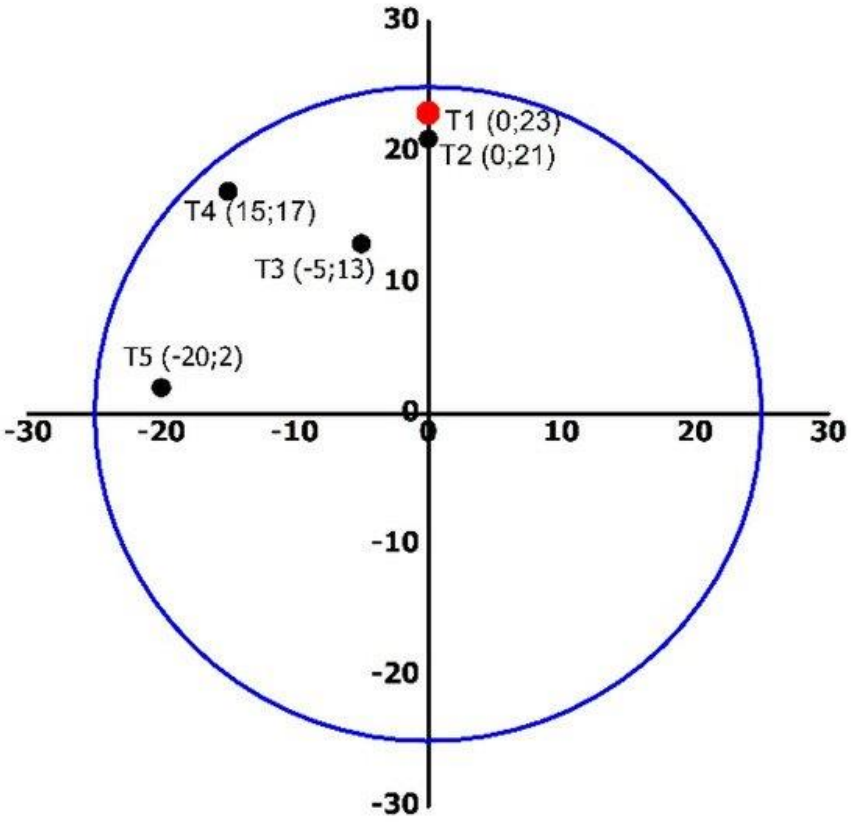

Figure 4. Position of the probes in the plate (cm). Probes are indicated by the letter T and are positioned in the plate according to the axes X and Y drawn on the plate. T1 is positioned above

the burner. T2, T3, T4 and T5 have the following coordinates respectively (0; 21), (−5; 13), (−15; 17) and (−20; −2). Probes were immerged in milk without touching the plate.

2.4. Physicochemical Analysis for Qishta

Qishta and red milk (residual milk at the end of the production according to the nomination used in Lebanon) were analyzed in triplicate for their compositions. Total nitrogen (expressed as protein equivalents) was quantified by the Kjeldahl method (International Dairy Federation, 2002). Ash content was determined by standard 27/1964 IDF, dry matter (DM) by adapting the oven drying method at 102 ± 2 °C (International Dairy Federation, 1982), and fat by applying the butirometric method (International Dairy Federation, 1986). Lactose content was determined using 3,5-dinitrosalicylic acid (DNS) [6]. Evaporation rate was monitored using a scale located under the plate during the heating process. The remaining milk in the plate was weighed every 2 min; therefore, the amount of water evaporated was calculated.

2.5. Static Light Scattering

The granulometric distribution was determined by laser light scattering using a MasterSizer 2000 (Malvern Instruments, Malvern, Worcestershire, UK) equipped with a 5 mW He–Ne laser operating at a wavelength of 633 nm. Samples were diluted into the Malvern cell (volume: 100 mL) with distilled water to reach appropriate obscuration (25%). Refractive indexes for solvent, particle and adsorption were 1.33, 1.3 and 0.1, respectively. These indexes were recommended by the manufacturer (Malvern).

2.6. SDS-PAGE Analysis

Samples of UHT milk (Lactel), red milk and Qishta were analyzed by SDS-PAGE electrophoresis under reducing (SDS-R) and non-reducing (SDSNR) conditions using the method described by Anema [7]. The resolving and stacking gel contained 15%, 12% and 4% acrylamide, respectively. Electrophoresis was performed using a vertical electrophoretic unit type TV200YK twin-plate, associated with the source voltage EV202. SDS-PAGE was executed at 30 V until the samples had completely left the stacking gel, then the voltage was increased to 90 V until the tracking dye reached 80% of the gel. Staining of the gel was performed in a 0.23% solution of Coomassie Blue R-250, containing 3.9% (*w*/*v*) Trichloroacetic acid, 6% (*v*/*v*) acetic acid and 17% (*v*/*v*) methanol for 90 min.

2.7. Milk Fat Effect

Bottled UHT skimmed milk (Lactel, Laval, France) was used in order to test the feasibility of producing Qishta without fat. Five milk samples with five different fat concentrations (3.6%, 2.6%, 1.6%, 0.6%, 0%) were prepared by mixing whole milk (3.6% of fat) with skimmed milk in order to test the effect of fat concentration on Qishta yield.

2.8. Statistical Analysis

One-way analysis of variance (ANOVA) was conducted using the Statistical Package for Social Sciences (SPSS) software for Windows (version 13.0, SPSS). A Duncan test was carried out to assess any significant differences between the means. Differences were considered statistically significant when $p \leq 0.05$.

3. Results and Discussion

3.1. Chemical Composition of Qishta

The results obtained in Table 1 show the chemical composition of Qishta, UHT and red milk. Qishta has the following composition: moisture (68 ± 2%), fat (11.7 ± 0.6%), protein (12.1 ± 0.7%), lactose (5.4 ± 0.2%) and ash (1.6 ± 0.2%). These results were no different from those obtained in Lebanon (Hallab 1881 company). The concentration of protein and fat were much higher in Qishta than in Lactel milk, indicating a migration from milk to the gel during heat treatment. At the end of the process, the remaining milk was rich in protein and fat; however, this milk cannot be compared to that obtained in Lebanon, since the process was stopped after 25 min of heating.

Table 1. Chemical composition of raw milk, Qishta and red milk.

Sample.	***Moisture %***	***Fat%***	***FDM%[1]***	***Protein%***	***PDM%[2]***	***Lactose%***	***Ash%***
Raw	87.4	3.6	31	3.2	27.5	4.8	1
Qishta	68 ± 2	11.7 ± 0.6	36.6 ± 2.1	12.1 ± 0.7	37.2 ± 1.5	5.4 ± 0.2	1.6 ± 0.2
RM*	82.0 ± 0.1	4.2 ± 0.1	21 ± 1	4.0 ± 0.2	20.0 ± 1.7	7.0 ± 0.2	0.8 ± 0.1

*RM: Red Milk, FDM: Fat dry matter, PDM: Protein dry matter

Our findings asserted that Qishta is a partially dehydrated product with a composition similar to that of Ricotta cheese prepared from whole milk, with almost the same amount of fat and protein (Table 1) [8]. Our results were in accordance with those reported by Kassaify et al., [2], who found almost the same composition. In fact, the composition of the final product would vary according to the milk composition, hydration process, heating time, amount of milk added and intensity of

heating. The high value of the solid content in the remaining milk can be explained by the high evaporation rate taking place during the heat treatment of milk (**Table 1**). During Qishta preparation, the concentration of milk increased proportionally with water evaporation. This latter is the main reason behind the addition of milk during the process; a low level of milk in the plate leads to an undesired burnt taste in the final product.

3.2. Temperature Distribution Profile

Temperature distribution was investigated during 25 min of heating. **Figure 5** shows the milk temperature distribution according to the different positions of probes on the plate. The general trend of the temperature profile monitored by the five probes show some similarities. At first glance, two behaviors were observed according to the position of the probes, resulting in two reaction zones: A and B. In zone A, which is closer than zone B to the flame and where Qishta is produced, the temperature increased by 25 °C/min. Two minutes later, the rate became 3.24 °C/min and reached a stable temperature of 100 °C after 15 min of heating. In zone B, the kinetics of the temperature increase were slower than that obtained in the first zone with 2.9 °C/min in the first 3 min of heating. The temperature became stable at around 65 °C after 15 min of heating. The heterogeneity in the temperature distribution can be explained by the position of the burner on the edge of the plate.

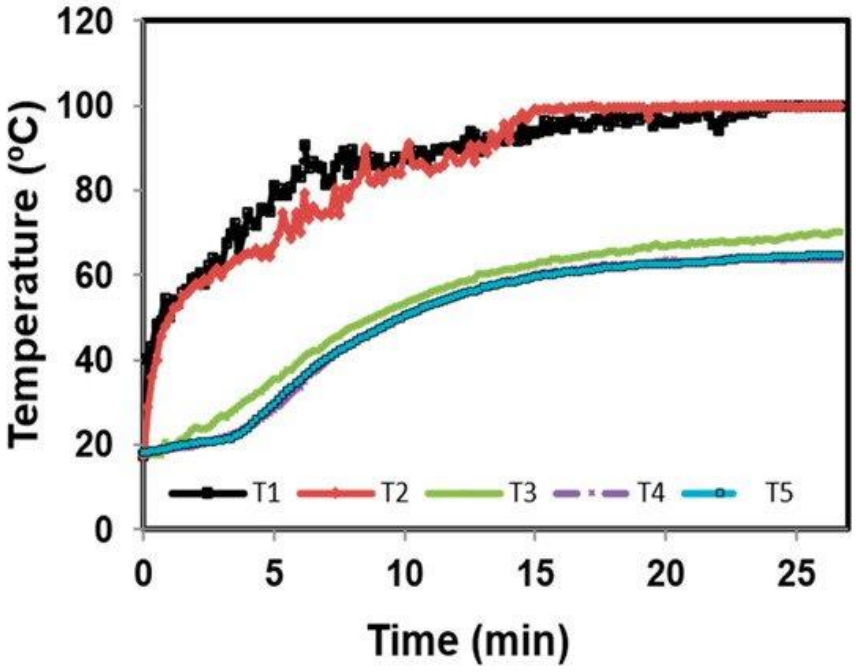

Figure 5. Temperature distribution profile during the Qishta formation: T1: black, T2: red, T3: green, T4: purple, T5: blue.

It has been reported that heat treatment induces many detrimental effects in emulsions [9,10]. During Qishta production, the heat treatment leads to fat coalescence, protein denaturation and finally to gel formation. Protein aggregation is considered as a key point during the process of making Qishta. Considering their solid primary chains, caseins are known to be heat resistant; they can withstand an intense heating of 140 °C for 15–20 min. When heated at 100 °C, the size of casein micelles decreases because of both kappa-casein (κ-casein) detachment from the micelle surface and colloidal phosphate liberation [11]. These phenomena probably took place in zone A.

Due to their globular structure, whey proteins (WP) are more sensitive to heat than caseins. Above 60 °C, they lose their tertiary structure and denature. This new unfolded structure exposes the amino acid groups and allows them to interact with other proteins through disulfide bonds

[12,13]. In Qishta production, this is likely to happen in both zones A and B. Increasing the WP concentration leads to an enhancement of fat droplet coalescence as well as emulsion viscosity until the critical concentration of gelation is reached. This was later estimated at around 3 wt% of non-adsorbed WP [9]. Above 3%, the non-adsorbed denatured WP will connect the different fat droplets in a continuous network. Euston et al. [12] compared WP to a glue that connects the fat droplets in the continuous phase. However, below the critical concentration, WP are not able to play the glue role and therefore the gelation does not occur.

The aggregates or the gel formed on top of the milk contain fat, casein, lactose and WP. During heat treatment, β-lactoglobulin (β-lg) binds to κ-casein on the surface of casein micelles through disulfide bonds and hydrophobic interactions [14,15]. In fact, due to the severe temperature applied, β-lg dissociates and loses its tertiary and part of its secondary structure, leaving the free thiol group (Cys121) exposed to the interactions with κ-casein through disulfide bridges [16].

The intensity and the period of heating have a significant impact on the process undergone by the milk. The process of making Qishta induced an increase in milk concentration due to water evaporation. **Figure 6** shows that the evaporation rate followed an exponential increase estimated at 0.6 mL/min. After 20 min of heating, around 400 mL of water (≈13%) had evaporated from 3 L of milk, leading to an increase in milk concentration from 10% to 14%.

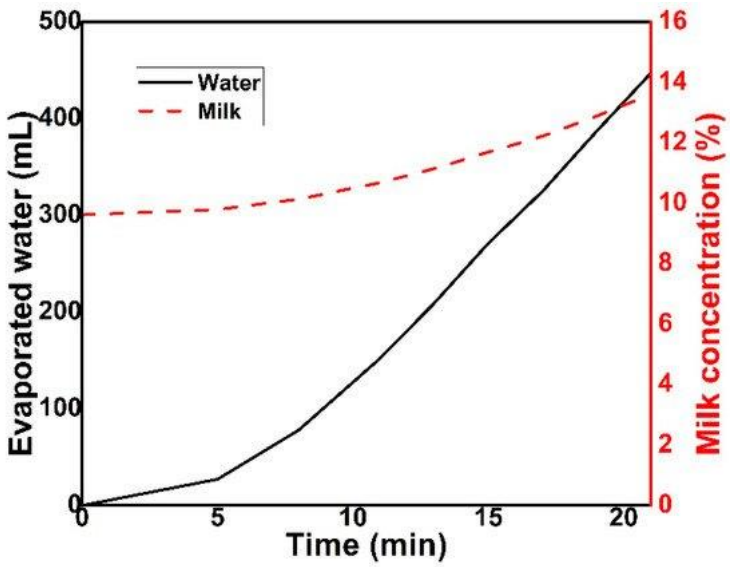

Figure 6. Relationship between the water evaporated and the concentration of milk during the process of Qishta production. Measurements were made in triplicate.

3.3. Emulsion Stability to Heat Treatment

Static Light Scattering (SLS)

Figure 7 shows the changes in particle size distribution of milk samples taken from the heated zone induced by the thermal treatment. Before heating, the average size of the particles used in this study was about 1.1 µm. When the milk was heated, the particle size distribution changed: larger particles (mostly aggregates) were observed after 5 min, with 5% of the total particles having a diameter between 258 µm and 750 µm. The volume weighted particle size distributions (D90) at this moment was 0.63 µm (90% of the particles had a diameter less than 0.63 µm). The increase in the droplet size could be explained by the fat coalescence phenomena or by the interactions that occur between fat globules and proteins during the heat treatment, or by a combination of both phenomena. Raikos [17] has noticed an increase in the particle size to 1−10 µm when milk was

heated at 140 °C for 80 s. This increase was attributed to the interactions that occurred between non-adsorbed protein molecules in the serum phase and proteins adsorbed at the interface of fat globules. A trimodal distribution was observed after 10 min of heating with a continuous increase of the particle size where (D90) reached 1.94 µm. The heterogeneous temperature distribution and the convection forces existing throughout the plate led to the formation of different aggregate sizes represented by the trimodal distribution.

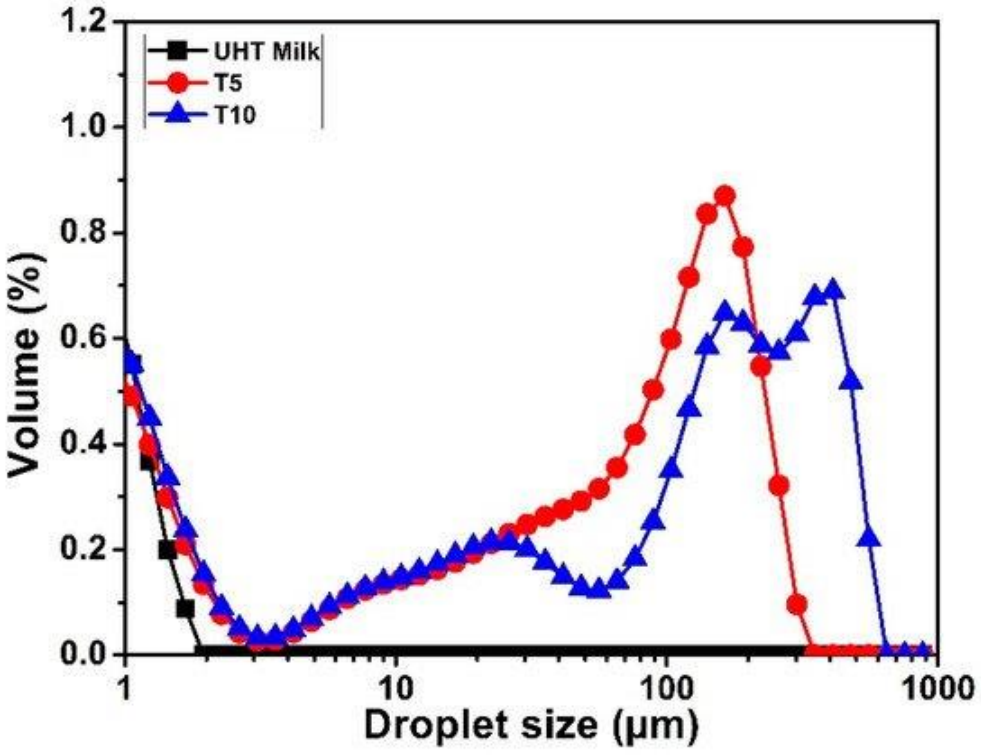

Figure 7. Follow-up of the particle size distribution using static light scattering (SLS) analysis during the first 10 minutes of heating. UHT milk before heating (control) ■ after 5 min of heating ● and after 10 min of heating ▲.

Dickinson [18] defined emulsion stability by its resistance to change over time. Our results indicated that protein aggregation took place when the milk was heated. This protein aggregation

might be involved with fat participation in Qishta formation. In milk emulsions, the composition and concentration of proteins present in the continuous phase have a primordial effect on the protein adsorption at the fat membrane. In fact, during heat treatment, caseins and WP are in competition to be adsorbed on the surface of fat droplets [19,20]. The particle size distribution is an important feature of many products, ranging from powder suspensions to emulsions, determining not only the physical properties such as flowability, but also the visual aspect and sensorial properties [21].

3.4. Identification of Major Proteins in Qishta

The electrophoresis analysis of UHT milk, Qishta and the remaining milk (red milk) was performed under reducing and non-reducing conditions (**Figure 8**). Under non-reducing conditions, the results showed that the major whey protein β-lg was absent in the studied samples. Similar protein patterns of high molecular weight (HMW) fractions (50 kDa to 150 kDa) were identified in all samples under both different conditions. However, the intensity of these bonds was higher in non-reducing conditions. Between 50 kDa and 75 kDa, one of these patterns could be attributed to bovine serum albumin BSA (66 kDa). Concerning the UHT milk, the high molecular fraction of 150 kDa, which appeared under non-reducing conditions, was almost absent under reducing ones. Nevertheless, it can be clearly observed that the stacking gel obtained under the non-reducing condition contained some protein fractions. These fractions, with a very high molecular weight, were lost for analyses. Under reducing conditions, the most relevant fraction was that attributed to β-lg, with a much higher intensity than in the non-reducing gel; thus, the intensity of the casein patterns had also increased. Concerning the HMW patterns, the intensity

obtained under reducing conditions decreased differently in each of the three samples. In fact, the reducing agent (β-mercaptoethanol) was not able to break all links between the aggregates which probably indicates the presence of new bonds, other than disulfide bridges between proteins. The analysis of the composition of the HMW patterns has revealed the presence of lysinoalanine and lanthionine crosslinks (data not shown). Results showed that the heating process during Qishta production induced the formation of HMW proteins as a result of the interactions between WP and caseins via disulfide bridges, as reported elsewhere [14,15].

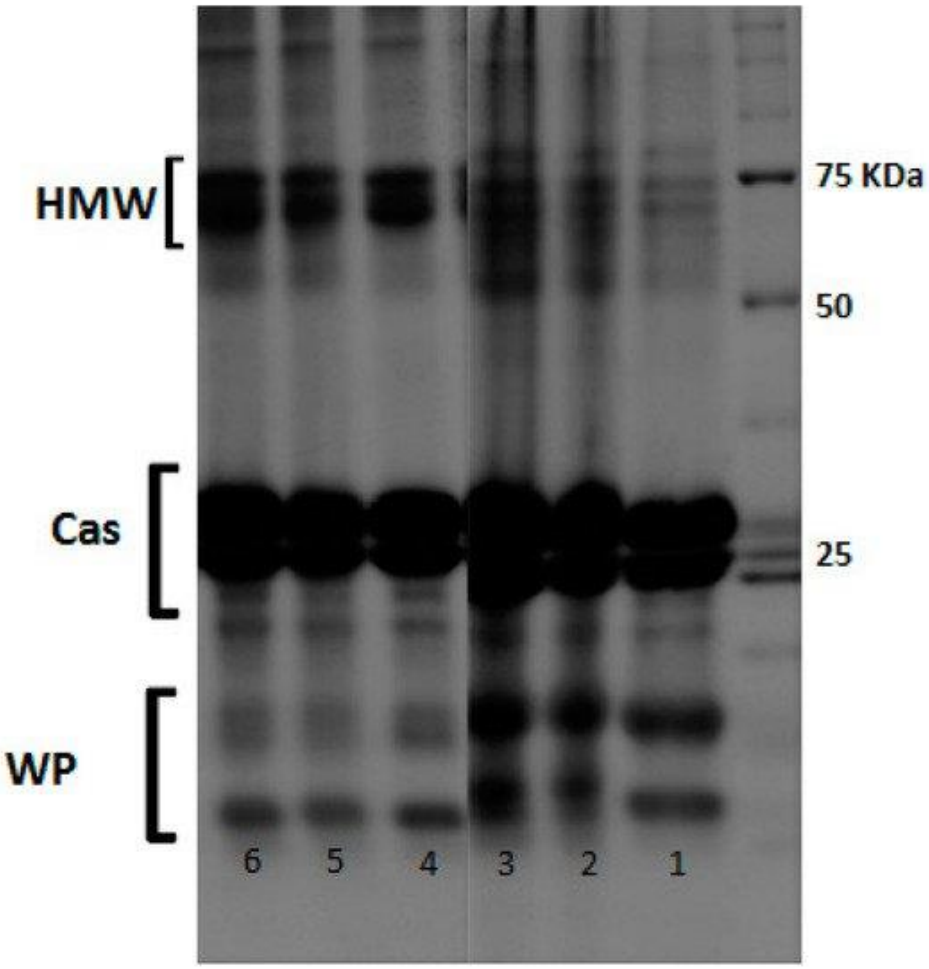

Figure 8. SDS electropherogram under reducing (1−3) and non-reducing conditions (4−6). 1,4: UHT milk; 2,5: red milk; 3,6: Qishta. Cas: casein, WP: whey protein, HMW: high molecular weight.

3.5. Effect of Temperature on Milk Fats

Milk fat content influences the physicochemical, sensorial and quality characteristics of Qishta. Milk heat treatment induces numerous changes in the milk fat globule membrane (MFGM), whose role is to protect the fat globule from coalescence, denaturation and interactions with serum proteins via sulfhydryl–disulfide interchange reactions [22]. **Figure 9** shows a supposed model of the interaction mechanisms that occur during the heat treatment of milk.

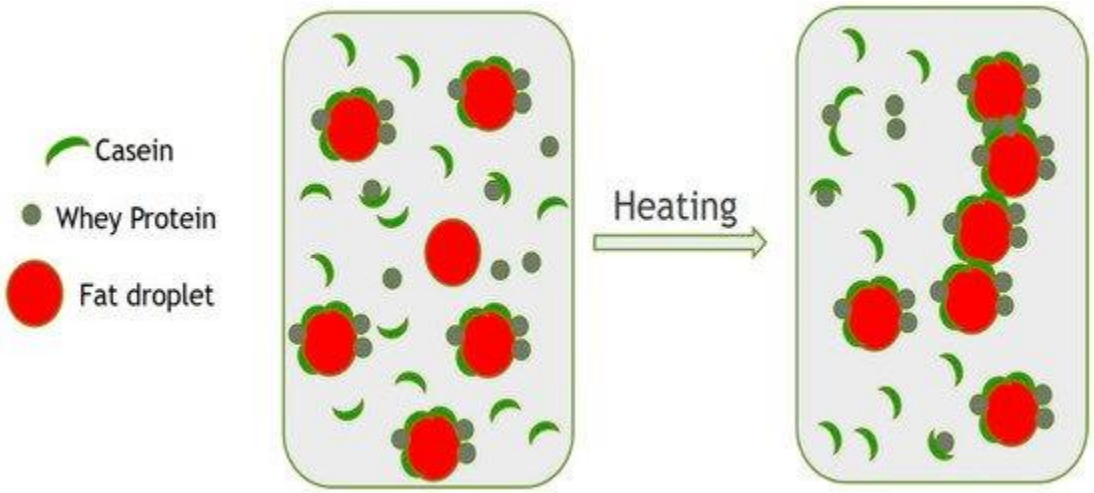

Figure 9. Interactions between different milk components during heat treatment.

Upon heat treatment, β-lg and α-lactalbumin (α-la) bind to the fat globule [23,24]. However, the mechanism by which these proteins interact with the fat globule is still not evident.

When milk is heated above 70 °C, denaturation of MFGM proteins and exposure of various amino acid residues, particularly cysteine, take place. Thus, H_2S is released (which results in the

development of an off-flavor) and disulfide interchange reactions occur with WP. At high temperature (>100 °C), this will lead to the formation of a denatured WP layer that will adsorb on the MFGM. This adsorbed layer, in the presence of lactose, will participate in a Maillard reaction, while the cysteine could be involved in dehydroalanine formation by β-elimination and can react with lysine or cysteine and form lysinoalanine and lanthionine, respectively [25].

The extent heat treatment applied during the process of Qishta preparation results in the destruction of the MFGM, therefore increasing the susceptibility of fat to oxidation.

Houlihan et al. [26] stated that with the increase in the heating time (from 2.5–20 min at 80 °C) the amounts of β-Lg and α-La adsorbed on the MFGM increased, however the amounts of phospholipids and triacylglycerols decreased. In addition, a small amount of κ-casein was also present, and this component increased during heating. These results indicate that MFGM is involved in heat-induced interactions with milk proteins, in particular β-Lg and κ-casein, and that the amount of these components that adsorb onto the membrane depends on the extent of the heat treatments. κ-casein can either interact directly with MFGM components, or with β-Lg through disulfide interchange during heating.

3.6. Effect of Fat on Qishta Formation

Skim Milk Experiment

Lactic acid (1.5 mL) was added to skimmed milk in order to decrease its pH. Ten minutes after heating, the milk started to burn. There was no aggregate formation except a skin at the milk surface. **Figure 10** shows that the milk initial fat percentage has a significant effect on Qishta production. The highest yield was obtained when the initial fat percentage was 3.6% (182.5 g).

However, when the initial fat percentage was 1.6%, Qishta yield decreased from 182.5 g to 171 g. **Figure 10** also shows that when the fat percentage increased in milk from 0.6% to 1.6%, the yield increased from 118 g to 134 g. A significant difference ($p \leq 0.05$) was observed between the yields, indicating that the milk fat concentration has a significant impact on the yield. Increasing the fat percentage from 0.6% to 3.6% has been shown to have a positive effect on the production yield of Qishta.

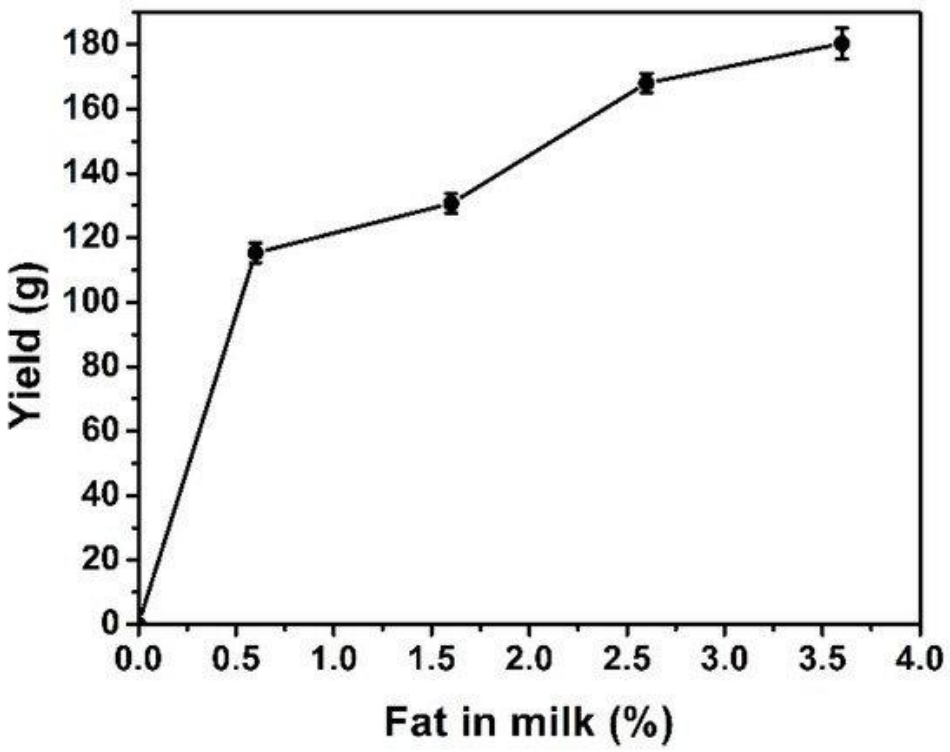

Figure 10. Yield average of Qishta as determined depending on the initial fat concentration in milk (0.6%, 1.6%, 2.6%, and 3.6%). Measurements were made in triplicate.

4. Conclusions

Qishta is a specific dairy product with a non-consistent composition that varies depending on handlers, raw material, and the process applied to its production. Different Lebanese dairy

industries have not succeeded to replace the traditional process by an advanced one in order to resolve the problem of the consistency and the limited quantity produced. Thus, in order to improve the process of the production of Qishta, the composition and the mechanism must be well studied. Despite the beliefs that Qishta contains a high fat content, we demonstrated that it actually holds an equal amount of fat and protein and a texture closer to cheese rather than cream products. Increasing the milk fat concentration has proven to have a positive impact on the yield. Further analysis could be focused on the impact of increasing the fat concentration above 3.6%. This study must be coupled with an analysis of the chemical composition and the organoleptic properties of Qishta.

Further research could also be focused on investigating the structural differences between the two Qishta layers and the interactions between the proteins and fat forming the gel network.

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CHAPTER : III

ARTICLE III - RESULTS

ARTICLE III

Effect of Milk Heat Treatment on the Molecular Interactions during the Process of Qishta-a Lebanese dairy product

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Abstract

Protein-protein cross-linking and protein-fat interactions were investigated in this study. Liquid chromatography coupled to mass spectrometry (LC-MS/MS) was used for the detection of lysinoalanine and lanthionine during the process of Qishta formation; a Lebanese dairy product obtained by heating whole milk in an open shallow vessel for more than 2 hours and harvesting the aggregates formed at surface. The presence of these two cross-links in Qishta points out their importance in the gel formation and therefore their impact on Qishta's texture. Our findings showed that disulphide bridges are also involved in Qishta formation. The amino acid residues (cysteine, serine or threonine) involved in the β-elimination and in the Dehydroalanine formation were identified. Our finding also showed that fat globules interacted with the proteins during milk heat treatment and contributed in the gel structuring.

Key words: Casein, Whey protein, Fat, Cross-links, LC-MS/MS, Qishta, Lysinoalanine, Lanthionnine.

INTRODUCTION

Heat treatment of milk during the process of dairying either for cheese and cream making or any milk byproduct formation, results in a number of interactions involving all milk components such as proteins, minerals and fat. According to the process and the severity of heat treatment applied, a large number of chemical, physical and biochemical reactions occurs in milk [1]. These reactions are of high importance, since they determined both the texture and the organoleptic properties of the final product. The major reactions undergone during milk heat treatment are denaturation and aggregation of proteins, fat coalescence, interactions between fat globules and proteins, and Maillard reactions [2]. Qishta is a heat-treated dairy product widely consumed in Lebanon [3]. In the Middle East, different spellings are used for Qishta such as Kishta, Kashta or Ghishta [4]. Qishta is obtained by heating whole milk in an open shallow vessel for 2 to 3 hours. At the end of the process, the coagulum formed at the milk surface will be gathered and drained in order to form Qishta. Qishta is defined as a lipo-protein product containing equal amount of fat and proteins. Protein cross-linking has been studied during the preparation of some food products and antibiotics. In fact, Al-saadi et al[1]., define cross-linking as a covalent interaction within the same protein (intramolecular) or between two different proteins (intermolecular). It was demonstrated that protein cross-linking can improve gel network of yoghurt [5] and can prohibit age gelation in UHT milk 6. Disulphide bonds were the first type of cross-links detected in food 7. The sulfhydryl-disulfides linkage was also reported to have an impact on the protein-fat interactions. Ye et al., (2004) showed that disulfides bridge associated β-lactoglobulin (β-lg) and α-lactalbumin (α-la) to the milk fat globule membrane (MFGM) during heat treatment of milk. Xenobiotic cross-links such as lysinoalanine (LAL), lanthionine (LAN) and Histidinoalanine (HAL) constitute new type

of cross-links found in food matrix and dairy products [10–16]. Their formation involved two steps: β-elimination yielding dehydroalanine (DHA) and condensation reactions [17]. The presence of LAL has been confirmed in a large variety of milk products such as pasteurized milk, UHT milk and whey protein concentrate but to different ranges [17–19]. For a long time, the presence of LAL has been considered as an undesirable and its detection had been suggested to have a toxic effect. In fact, histopathological changes in renal cells, also known as nephrocytomegaly, have been observed in rat experiments after consuming soy protein containing LAL [20]. However, Sieber et al., [16] reported that the presence of cross-links connecting the proteins, such as LAL, does not have a toxic effect on human health. In addition, LAL has been identified in a large variety of food products such as legume protein [17], meat protein [21], fish protein (Miller et al., 1983) and infant formulas (Pompei et al., 1988).The aim of this study was to understand the effect of milk heat treatment during Qishta process on the molecular interactions between milk proteins on one hand and between MFGM and protein on the other hand.

Materials and Method

Materials

The plate (stainless-steel, 316 L) used in Qishta production was provided by Nafco (Nafco, Lebanon) and the burner from AEM (AEM, Chelles, France). UHT milk was purchased from Lactel (Lactel, France). Lactic acid was purchased from Fischer Scientific (Fischer Scientific, UK).

Qishta Preparation Procedure

The plate (shallow vessel) used for Qishta production has a diameter of 0.5 meter, a capacity of 3 liters and a thickness of 2mm. This plate was adapted in order to produce Qishta at smaller scale. 3 liters of milk were prepared by adding 1ml of lactic acid, which is the amount needed in order to decrease the pH from 6.7 to 6.4 (procedure applied at "Hallab 1881" company). The process consists of heating milk for 2 to 3 hours and gathering the aggregates formed at the milk's surface. During the heat treatment and depending on the evaporation rate, milk is added in order to readjust its level in the plate.

SDS-PAGE Analysis

Samples of UHT milk (Lactel) and Qishta were analyzed by SDS-PAGE according to the method described by Anema (2000). The resolving and stacking gel contained 15 %, 12 % and 4 % acrylamide respectively. Electrophoresis was performed using vertical electrophoretic unit type TV200YK twin-plate, associated with the source voltage EV202. SDS PAGE was executed at 30 V until the samples had completely left the stacking gel, then the voltage was increased to 90 V until the tracking dye reached 80% of the gel. Staining of gel was performed in 0.23% solution of Coomassie Blue R-250, containing 3.9% (w/v) TCA, 6% (v/v) acetic acid and 17%

(v/v) methanol, during 90 minutes. The electropherogram obtained from SDS-PAGE was converted by image converter software and then analyzed using the ImageJ 1:46 software. The identification of each protein was realized by comparing the migration distance of each band to that of a protein marker having known molecular weights. The intensity of the patterns corresponds to a gray value that varies between 0 and 250.

Sample preparation for quantification and cross-links analysis

1 mL of milk sample and 10 mg of Qishta were added separately into 15 mL tubes. To denature the samples, 1 mL of 8 M urea, 150 mM NaCl and 100 mM Ammonium bicarbonate (ABC) was added. The mixtures were then vortexed for 30 min at 4°C with Vortex-Genie 2 ™ (Scientific Industries, Bohemia, USA). 8 mL of chloroform methanol (1:2) (v:v) were added to the samples and the mixture was vortexed for 30 min. A triphasic solution with protein interphase was produced. To maximize the separation phase, the tubes were centrifuged at 4032 *g*, 4°C for 30 min using Centrifuge Allegra® 64R (Beckman Coulter, Brea, USA). Both upper and lower phases were carefully discarded. The interphase was dried under air vacuum using a sample concentrator for 60 min (SBH130, Stuart, Staffordshire, UK). The dry interphase was resuspended by adding 1 mL of 6 M urea, 150 mM NaCl, 100 mM ABC and the mixture was vortexed overnight at 4°C. Protein extracts were stored at -80 °C until their usage.

Digestion of samples

Samples were prepared using a modified enhanced Filter Aided Sample Preparation (eFASP). eFASP was used in order to increase proteome coverage and sample recovery for quantitative proteomic experiments [24] . Before their use, 0.5 mL Amicon® ultra centrifugal filters equipped

with a cut-off of 10 kDa (EMD Millipore, Darmstadt, Germany) were incubated overnight with a passivation solution containing 5% (v/v) Tween®-20, then rinsed with ultrapure water. 100 µg of protein were transferred to an Amicon® filter, followed by 100 µL of exchange buffer (8 M urea, 0.2% DCA, 100 mM ammonium bicarbonate pH 8.8). The filtrate was removed after a centrifugation step for 30 minutes at 10,000 *g*. 200 µL of exchange buffer were then added to the Amicon® filter, which was again centrifuged. This operation was repeated twice. The proteins were alkylated for 1 hour at room temperature (20°C) in the dark using 100 µL of alkylation buffer (8 M urea, 50 mM iodoacetamide, and 100 mM ammonium bicarbonate, pH 8.8). The Amicon® filter was centrifuged again for 30 minutes at 10,000 g and the filtrate was discarded. After this alkylation step, 200 µL of exchange buffer were added to the Amicon® filter, which was again centrifuged for 30 min at 10,000 *g*, and the filtrate discarded. 200 µL of digestion buffer (0.2% DCA, 50 mM ammonium bicarbonate pH 8.8) were added to the Amicon® filter, before another centrifugation step (30 min at 10,000 *g*). This operation was repeated twice; the filtrate being removed and discarded. The Amicon® filter was transferred to a new 2 mL concentrator collection tube. 100 µL of digestion buffer with 40 µL of trypsin/LysC (Promega, Madison, USA) were added and incubated in the Amicon® filter while being shaken in a heating block tube (MHR23, Hettich, Netherlands) overnight at 37°C. Thereafter, the peptides present in the Amicon® filter were recovered in the tube by centrifugation for 15 minutes at 10,000 *g*. To maximize the peptide recovery, two washing steps were implemented using 50 µL of ammonium bicarbonate solution (50 mM pH 8.8). The filtrate containing all peptides was then transferred to a 1.5 mL Eppendorf® microtube (Eppendorf, Hamburg, Germany). 200 µL ethyl acetate and 2.5 µL of Trifluoroacetic acid (TFA) were added, inducing the peptide precipitation (white color). 800 µL of ethyl acetate

were added again. The resulting solution was centrifuged for 10 minutes at 10,000 *g* and the organic phase was eliminated. This operation was repeated twice. The Eppendorf® microtube was placed for 5 minutes at 60°C in a heating block (SBH130, Stuart, Staffordshire, UK) leading to the evaporation of the remaining ethyl acetate. The samples were dried at room temperature in a SpeedVac™ Concentrator (Eppendorf™ Concentrator Plus, Eppendorf). Later, 100 µL of a methanol/water (50/50) mixture were added to the resulting solid phase and left until evaporation. For Mass Spectrometry (MS) analysis, the samples were dissolved in 10 µL of ultrapure water supplemented with 0.1% of formic acid (FA). The sample concentration was estimated by measuring the optical density (OD) at 215 nm using a droplet UV spectrometer (DS-11+, Denovix, Wilmington, USA). Before analysis, the concentration of the sample was adjusted to 1 µg/µL by dilution with ultrapure water containing 0.1% FA. Each sample was analyzed in triplicate.

LC-MS/MS Orbitrap eFASP

LC-MS/MS protein analysis was performed using Orbitrap Q Exactive plus Mass Spectrometer hyphenated to a U3000 RSLC Microfluidic HPLC System (ThermoFisher Scientific). 1 µL of the peptide mixture having a concentration of 1 µg/µL was injected to solution A (5% v/v acetonitrile and 0.1% formic acid) for 3 minutes at 5 µL/min using an Acclaim PepMap100 C18 pre-column (5 µm, 300 µm i.d.×5 mm) (ThermoFisher Scientific). The peptides were next separated on a C18 (Acclaim PepMap100 C18) reversed phase column (3 µm, 75 mm i.d. × 500 mm) (ThermoFisher Scientific), using a linear gradient (5-40%) of solution B (75% ACN and 0.1% formic acid) and a flow-rate of 250 mL/min in 160 min followed by 100% solution B for 5 minutes. The column was regenerated by washing it for 5 minutes with solution B and then re-equilibrated with solution A

during 10 minutes. The column and the pre-column were placed in an oven at 45°C. The total duration of the analysis was 180 min. The liquid chromatography (LC) runs were acquired in positive ion mode with MS scans from *m/z* 350 to 1,500 in the Orbitrap mass analyzer having 70,000 resolution for MS and MS/MS. The automatic gain control was set at 1×10^6 for MS. MS/MS scans were sequentially acquired in the high-energy collision dissociation (HCD) cell for the 10 most-intense ions detected in the full MS survey scan. Automatic gain control was set at 5×10^5, and the normalized collision energy was set at 28 eV. Dynamic exclusion was set at 90 s and ions with 1 and more than 8 charges were excluded.

Quantification MaxQuant

Analysis of Lactel milk using LC-MS/MS from eFASP digestion data was performed using MaxQuant [25]. MaxQuant enables high peptide identification rates, ppb-range mass accuracies individualization and proteome-wide protein quantification. Nature biotechnology, 26(12), and Andromeda search engine were used for database searching against the UniProtKB/Swiss-Prot Bovine database containing forward and reversed sequences (*Bos taurus*, January 2018, Sequences: 32,513) [25]. MaxQuant also contains common contaminated proteins identified in proteomics analysis. MaxQuant analysis included an initial search with a precursor mass tolerance of 20 ppm, a main search precursor mass tolerance of 6 ppm and a fragment mass tolerance of 20 ppm. Trypsin was used with variable modifications such as methionine and proline oxidation, lysine acetylation, deamidation on asparagine or glutamine (NQ) and serine, threonine phosphorylation and with the fixed modification carbamidomethyl cysteine. The minimal peptide length was set to six amino acids and the maximum number of missed cleavages was set to three.

The match-between-runs function was used (match time window = 2 minutes, alignment time window = 20). The False Discovery Rate (FDR) was set to 0.01 for both peptide and protein identifications. The proteins identified by the same sets of peptides were grouped and reported as one protein group.

Identification of cross-links

Raw files from eFASP digestion were analyzed using Mass Spec Studio v 2.1.2.3107 [26]. LAL Cross-links between serine and lysine were examined using a mass shift of −18.01056 Da due to the elimination of H_2O, while LAN cross-links between 2 cysteine as well as LAL between cysteine and lysine were searched using a mass shift of - 33.9877 Da due to the elimination of H_2S. Carbamidomethyl cysteine and methionine oxidation was set as dynamic modification. Trypsin was selected as an enzyme with 3 missed cleavages. Error of MS and MSMS precursor was set to 10 ppm. The minimum charge of peptides was set to 3 and the maximum was set to 8. Peptide cross-link with a score higher than 18 was considered for further analysis.

Microstructure characterization using confocal laser scanning microscope (CLSM)

Samples were labeled with Nile Red and Fast Green in order to stain the fat globules and the proteins, respectively. Nile Red (10 µL) and Fast Green (5 µL) were added to 1 mL of milk or 0.8g of Qishta. Samples were gently mixed to avoid tridimensional structure degradation. Samples were kept at room temperature (20°C) for, at least, 15 minutes before observations and then 100 µL of milk and 0.1g of Qishta were placed in Lab-Tek chamber (Nunc™ Lab-Tek™ II Chambered Cover glass). Confocal laser scanning microscope (ZEISS LSM 780, Carl Zeiss Micro Imaging GmbH) was used to characterize emulsions microstructures. Images were acquired with a Plan

Apochromat 40x /1.3 numerical aperture oil immersion objective, using Zen Software (Carl Zeiss Micro Imaging GmbH). Fluorophores excitations were performed using 561 nm laser line for Nile Red imaging and 633 nm laser line for Fast Green.

Statistical analysis

One-way analysis of variance (ANOVA) was conducted using the SPSS software for Windows (version 13.0, SPSS). A Duncan test was carried out to assess any significant differences between the means. Difference was considered statistically significant when $P \leq 0.05$.

Results and Discussion

Chemical composition of Qishta and UHT milk

Protein identification and quantification by mass spectrometry

174 proteins were identified and quantified in milk and Qishta using Label free method. Holland et al., (2004) have succeeded in identifying more than 150 proteins in whole bovine milk while Vincent et al., (2015) have identified 186 proteins in Holstein and Jersey cow's milk. The identified proteins number depends on the technique used [29]. Table 1 represented the average of relative abundance of the major proteins quantified in both milk and Qishta samples. Our findings showed that caseins and whey proteins are the most abundant proteins present in both milk and Qishta, and represent more than 97 % of total protein present in the samples. Among the 6 major proteins, beta-casein (382518) and alpha-S1-casein (274999) were the most abundant while alpha-lactalbumin was the less abundant one. Lactadherin, Serum albumin and Lactoferrin were present as traces. Our results were in line with Abd El-Salam, (2014) who found that casein and whey proteins represent almost 95 % of total proteins in milk.

Table 2: Average relative abundance of the proteins present in milk and Qishta.

T: Protein name	*Lactel UHT milk*		*Qishta*	
Beta-casein (β-cn)	382518	±27334	348378	±96824
Alpha-S1-casein (αs1-cn)	274999	±58068	186559	±5682
Kappa casein (κ-cn)	178523	±8670	198968	±46452
Beta-lactoglobulin (β-lg)	96122	±11083	123990	±38322
Alpha-S2-casein (αs2-cn)	52432	±21994	76506	±26965
Alpha-lactalbumin (α-la)	41804	±3573	39050	±9497
Lactadherin	863	±27	948	±231
Serum albumin	394	±14	450	±70
Lactoferrin	364	±29	435	±123

Table 2 showed the percentages of the four main caseins (αs_1-cn, αs_2-cn, β-cn and κ-cn) and the two major serum proteins (β-lg) and (α-la) present in fresh raw milk, Swedish UHT milk and Lactel milk used during Qishta production. Concerning fresh cow milk, the amount of proteins varies between 2.5 to 3.5%. This variation could be attributed to breeding, individual variation and nutrition status of the animals. The ratio casein/whey in fresh milk is approximately 80/20 [31,32].

Karlsson et al., (2017) showed that the amounts of caseins and whey proteins present in UHT milk were 87 and 13 % respectively. These results are in line with our findings; however, the percentages of the individual caseins were different. In fact, the significant difference occurred in κ-cn which represented 16 % and 4.2 % in the French and the Swedish milk respectively [31,33,34].

Table 2: Casein and whey protein distribution in Lactel, Swedish UHT and fresh raw milk [31,33,34].

Protein	*Lactel UHT (%)*	*Swedish UHT (%)*	*Fresh raw milk (%)*
Casein	86.4	87.4	83.0
β-cn	37.0	44.1	27.1
αS1-cn	27.5	30.5	26.0
κ-cn	15.9	4.2	10.0
αS2-cn	6.0	8.6	10.0
Whey protein	13.6	12.6	17.0
β-Lg	9.2	9.4	11.0
α-La	4.4	3.2	4.3

The relatively high amount of κ-cn (15.9%) present in Lactel milk could be explained by an overestimation resulted from the interaction with α_{S2} through disulphide bonds. In fact, κ-cn owns two cysteines residues able to interact with the thiol group of α_{S2}-cn through disulphide bridge as reported by Eigel et al., (1984). Figure 1 showed the relative abundance of major proteins present in UHT Lactel milk and Qishta. No significant difference ($P<0.05$) was observed between the percentage of each protein in milk and Qishta indicating that the heat treatment applied did not promote the denaturation of a specific proteins to the detriment of other. Concerning UHT milk,

β-cn (37%) and α_{S1}-cn (27.5%) constitute the major parts (approximately 65%) of the casein while α-La represents the minor percentage (≈ 5%). Regarding Qishta, the gap between the highest and the lowest percentage decreased and reached 18% (31% in UHT milk). β-cn represented the major protein present in Qishta, however α_{S1}, κ-cn and β-lg have almost similar percentages (≈ 18%). The comparison between milk and Qishta showed that the percentages of β-cn and α_{S1}-cn in the Qishta decreased while those of β-lg and α_{S2}-cn increased.

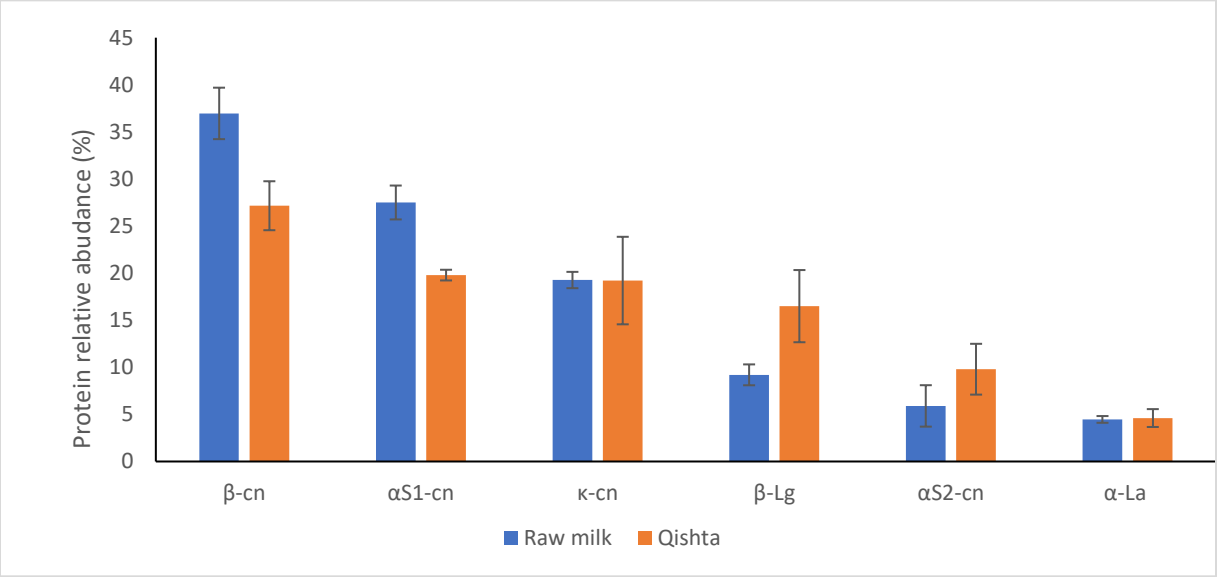

Figure 1: Individual percentages of the 6 major proteins present in UHT milk and Qishta obtained by dividing the individual abundance by the total abundance.

The high rate of β-lg migration from milk to Qishta could be attributed to the high thermal sensitivity of this protein. In fact, when heating milk above 65°C, β-lg denatures [36]. β-lg owns a free thiol group allowing the interaction with casein micelles [35]. A complex between β-lg and can be formed through disulphide bridge during milk heat treatment having a significant impact on Qishta's texture. The position of κ-cn on the surface of the micelles makes it more accessible for interaction with β-lg [36].

Protein-Protein Interaction

Identification of S-S crosslinks in Qishta by SDS-PAGE

Figure 2 showed a quantitative comparison between Qishta proteins under SDS-R and SDS-NR. The electropherogram was converted by image converter software and then analyzed using the ImageJ software. Our results showed that the intensity of the peak corresponding to α-la increased from ≈ 215 under SDS-NR to ≈ 245 under SDS-R. Same trend was observed for β-lg and κ-cn. The major increase was observed for β-lg with ≈ 40% from SDS-NR to the SDS-R (185 to 255). The intensity of high molecular weight aggregates (HMW) decreased form 250 under SDS-NR conditions to 240 under SDS-R. The addition of β-Mercaptoethanol under SDS-R resulted in breaking the disulfide bonds between proteins. This change has led to an increase in the intensity of individual proteins (α-la, β-lg and κ-cn) with a major change occurring especially in β-lg. The decrease of the aggregate's intensity indicates that these proteins were interacted through disulfide bridges. Heating milk above 70 °C causes the denaturation of whey proteins. According to the temperature intensity, time of heating, pH and protein concentration, the major denatured whey protein (β-lg) will form a complex with casein micelles which will directly impact the characteristics of the dairy product formed [10,37,38].

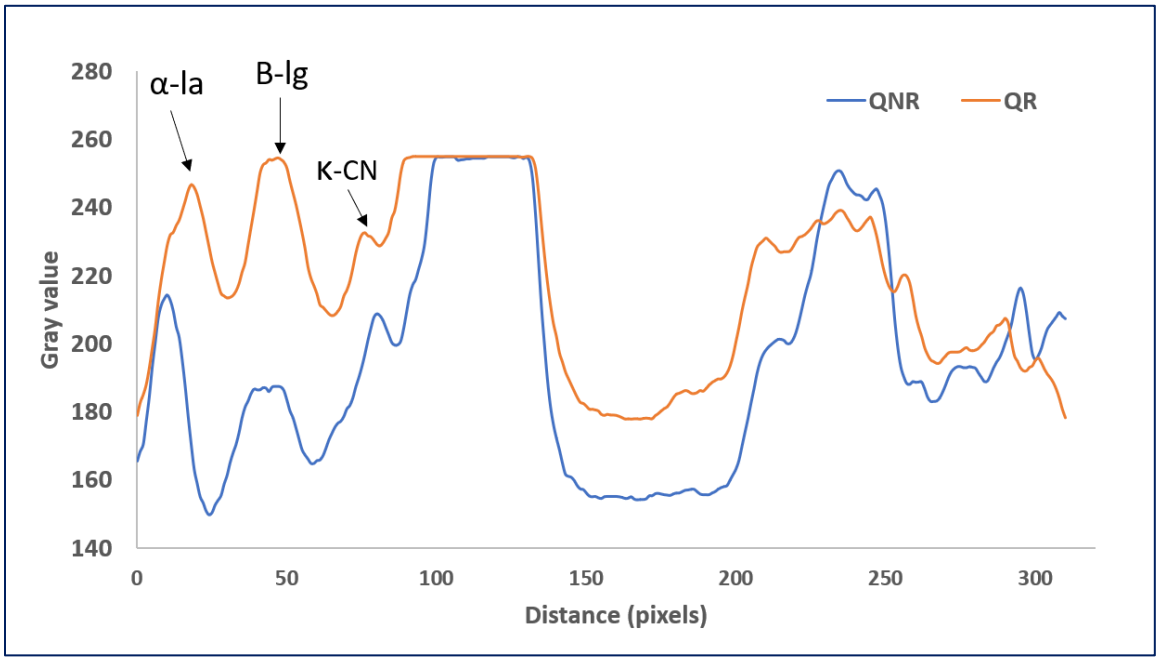

Figure 2: The difference in protein intensity (Gray value) of Qishta analyzed under reducing conditions (R) in Orange and under non-reducing conditions (NR) in blue. The electropherogram was converted by image converter software and then analyzed using the ImageJ software.

Anema and Li, (2003) reported that during skim milk heat treatment, the size of the casein micelles increased with the rate of denatured whey proteins suggesting ,therefore, the probable association between β-lg and κ-cn. Cho et al., (2003) reported that the heat treatment exposed the hidden cysteine residue in β-lg, consequently, creating a reactive sulfhydryl group which is able to interact through thiol-disulphide exchange reaction with κ-cn. This hypothesis was confirmed by the addition of some thiol-blocking agent that prevent the interaction between these two proteins [39]. In addition to the disulfide bonds, it was reported that hydrophobic and ionic interactions could play an important role in the complex formation between β-lg and κ-cn. α-la does not associate with casein micelles on its own like β-lg; instead, it has to form complexes with β-lg to be later associated with casein micelle whenever a prolonged heating period is provided (Oldfield, Singh, Taylor, & Pearce, 2000). At this level, it can be concluded that disulphide bonds are involved in

the coagulum formation. However, the presence of HMW aggregates after the addition of β-Mercaptoethanol suggests the involvement of other type of cross-links in Qishta formation.

The analysis of these HMW patterns specifically those having a molecular weight between 50-60 kDa has revealed the presence of α_{S2}-cn, α_{S1}-cn, α-la and β-lg. α_{S2}-cn and α_{S1}-cn were the 2 major proteins present in Qishta and milk samples analyzed under the 2 conditions. However, the intensity of these proteins was higher under NR conditions confirming the hypothesis that disulphide bonds are not the only cross-link that exists between the proteins forming the coagulum (Data not shown).

Identification of LAL and LAN present in milk and Qishta

The mechanism of LAL and LAN formation consists of two steps (Fig 3) : i) DHA formation resulting from β-elimination of cysteine, serine or threonine and ii) Michael addition of lysine or cysteine to the DHA formed leading to the formation of LAL or LAN respectively [14,15,17,19,42,43]. The objective of our study was to investigate the presence of LAL and LAN in Qishta then to locate the peptide sequences involved in the cross-link's formation (β-elimination of cysteine, serine or threonine). This will assist in explaining the mechanism of Qishta formation and therefore proving the impact of cross-links on the network structure formed during heat treatment of milk. The *m/z* ratio of the crosslinking peptides detected were compared to the theoretical ones. The high

accuracy of Q-Exactive plus mass spectrometer (less than 5 ppm) allowed to identify the crosslinks. Finally, the confirmation was done by the MS/MS spectra.

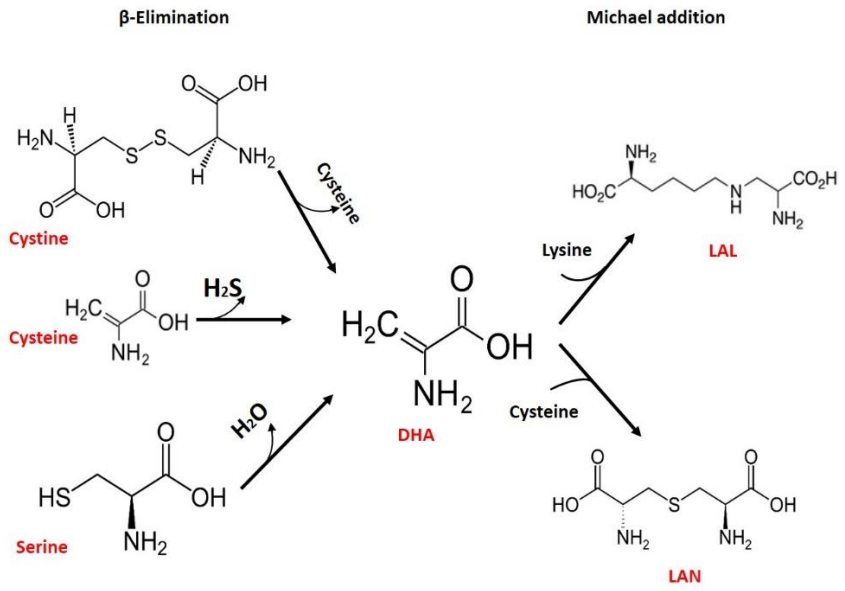

Figure 3: The mechanism of LAL and LAN formation. β-elimination of cystine, cysteine or serine resulting from dehydroalanine formation. Michael addition of lysine or cysteine yielding LAL or LAN respectively.

Six *m/z* values of compounds present in milk or Qishta matched with the theoretical *m/z* values of two peptide chains linked with LAL between serine and lysine residues. Four *m/z* values (925.8921 677.3094 648.5323 465.9223) were present in both milk and Qishta (Table 3, peptides 1, 4, 5 and 6). However, two *m/z* values (921.8939, 957.9172) were only present in Qishta samples (Table 3, peptides 2 and 3). Basic Local Alignment Search Tool (BLAST) was used in order to identify the peptide sequences involved in LAL cross-link formation and confirmed that the sequences were presented exclusively in bovine protein (α-La, α_{S2}-cn and β-cn). Four of these inter-chain cross-links connected 2 α-La proteins, one connected 2 α_{S2}-cn and the last one connected α_{S2}-cn with β-cn. Peptide 1, 2, 3 and 5 engaged an identical α peptide sequence (DDQNPH**SS**NI**C**NI**S**CDK). It was not possible to identify which serine residue was involved in cross-link formation, since α protein sequence owns three serine residues. Figure 4 showed the MS/MS spectra of quadruple charged double-chain peptide having a theoretical Mr of 3683.5756, originating from two peptides

sequence linked with LAL residue between one of the three serine residues and lysine. The fragmentation of the parent ion yielded 16 y-fragments, 16 b-fragments, and 4 internal fragments.

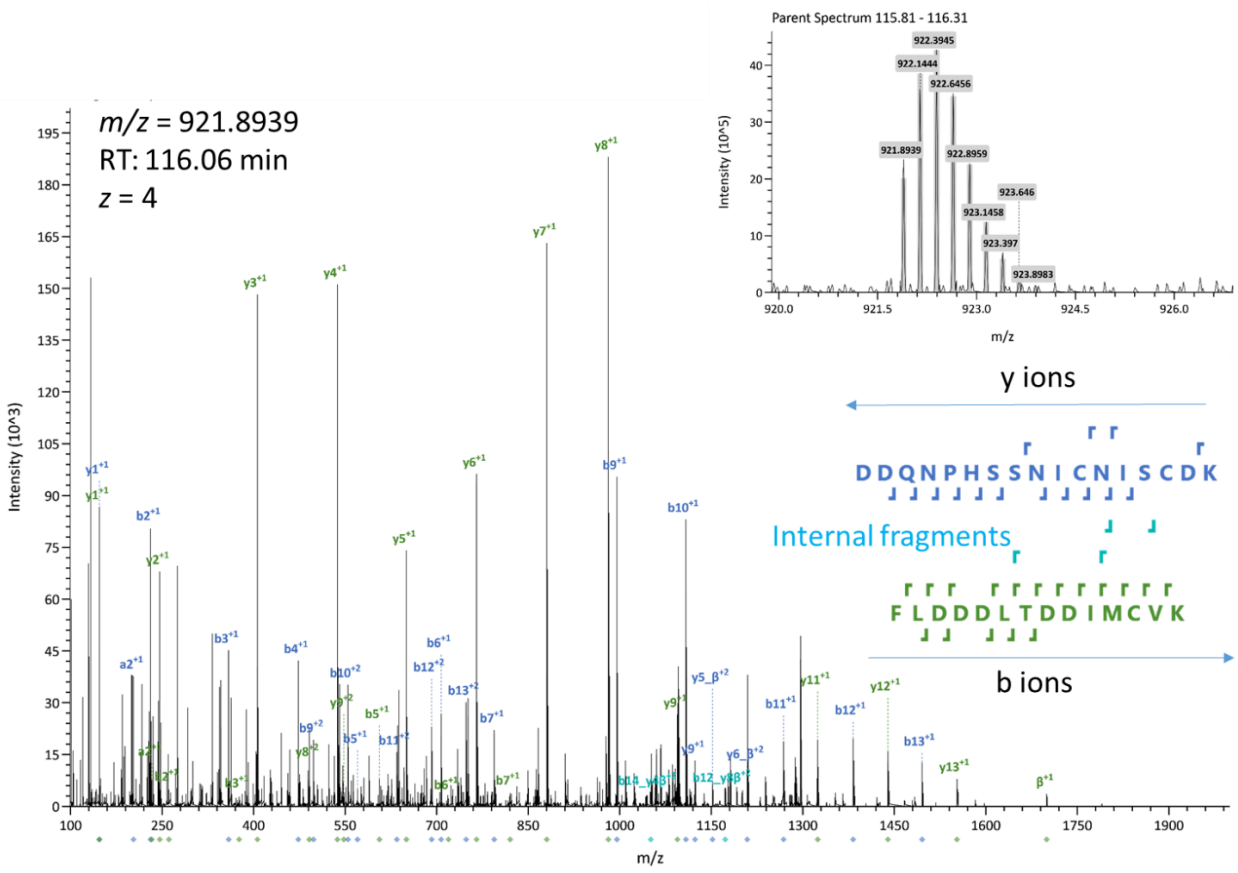

Figure 4: MS/MS spectrum of double peptide sequence DDQNPHSSNICNISCDK and FLDDDLTDDIMCVK linked by LAL derivative from Serine and Lysine interaction. These peptide sequences were found in bovine whey protein alpha lactalbumin of Qishta. Amino acids are referred with their abbreviation code.

Two detected *m/z* values of compounds detected only in UHT milk and Qishta (498.5855 and 619.2674) matched with the theoretical *m/z* values of two peptide chains linked by LAN between two cysteine residues (Table 3, peptides 7 and 8). BLAST confirmed that the two sequences were present only in milk protein (α_{S2}-cn and α-La). These two intra-chain cross-links occurred in both casein and whey protein (α_{S2}-cn and α-La respectively). No lanthionine was found due to the interaction between cysteine and DHA resulted from the *β*-elimination of serine residue. The

theoretical fragmentation patterns comparison with the MS/MS spectra obtained, allowed the affirmation of the cross-links identified. Figure 5 showed the MS/MS spectra of the triply charged intra-chain peptide having a theoretical Mr of 1492.7565, and originating from LAN linkage between one of the three serine residues and lysine in the ENLCSTFCKEVVR sequence. The fragmentation of the parent ion yielded 11 y-fragments and 2 b-fragments.

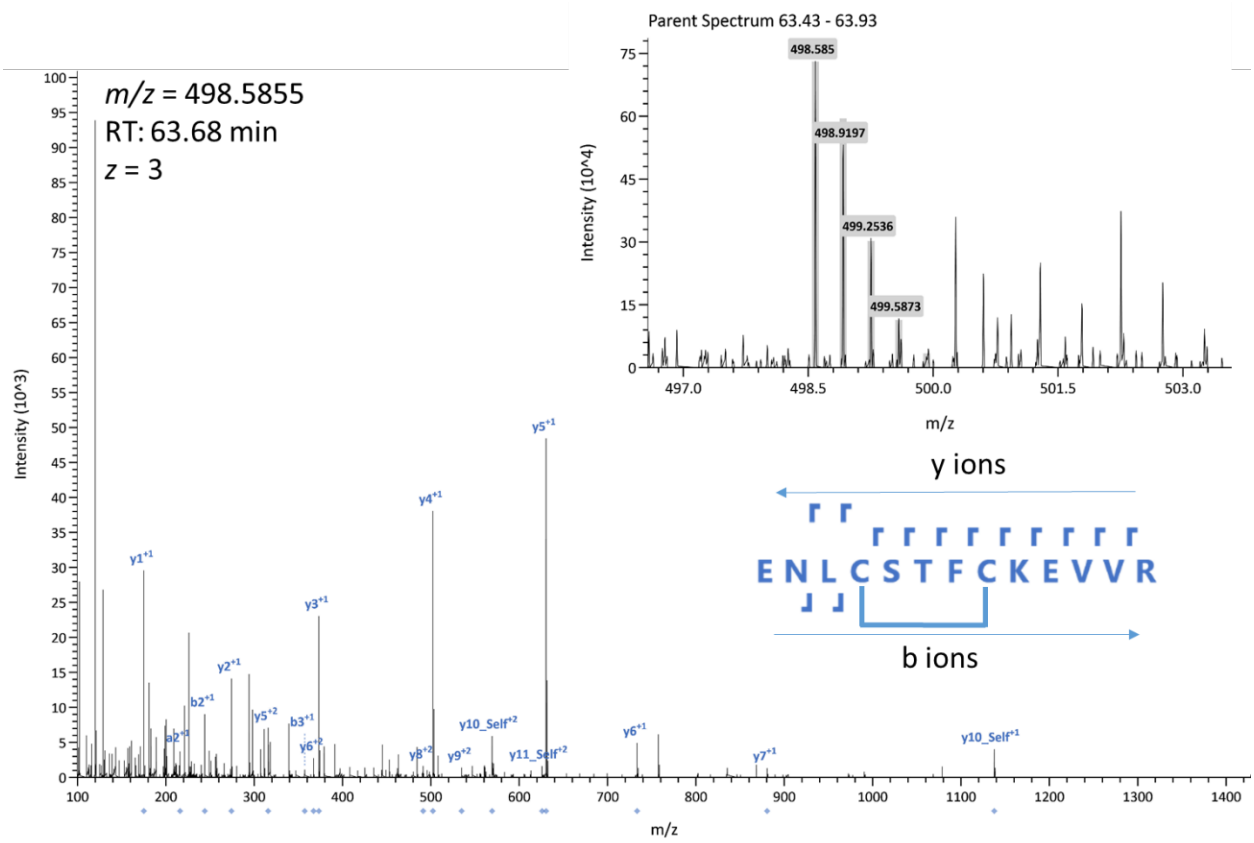

Figure 5: MS/MS spectrum of the intra chain LAN cross-link between two cysteines in the peptide sequence. This peptide sequence was found in α_{S2} casein of the Qishta protein. Amino acids are referred with their abbreviation code.

Table (4) showed the intensity of 6 LAL (serine-lysine) cross-links found in UHT milk and Qishta. The average intensity of LAL detected was higher in Qishta than in milk. In fact, the average intensities of LAL 1 and 4 in Qishta were 4.00E±06 and 4.32E±06 respectively, while these values were 3.35E±06 and 5.22E±05 in milk, respectively. LAL number 2 and 3 could not be detected in

milk. It has been used as an indicator of the severity of heat treatment applied during milk processing (Faist et al., 2000; Sieber et al., 2007). Our results were in line with Faist et al., (2000); and Hasegawa et al., (1987) who succeeded in detecting the presence of LAL in UHT milk but at different concentrations. Sieber et al., (2007) did not find LAL peptide in UHT milk containing 2.7% of fat. However, they found LAL in two cheese samples. Faist et al., (2000) demonstrated that the amount of LAL increased progressively with the temperature applied during milk processing. In fact, the amount of LAL was the highest in sterilized milk and the lowest in raw cow's milk. Their hypothesis was confirmed with the analysis held on cheese samples, where they found that the amount of LAL increased according to the severity of the process applied during cheese manufacturing. The use of mass spectrometry analysis, in order to detect the presence of cross-links and to locate the peptides involved in their formation, is considered as new tool. To the best of our knowledge, this analysis has not been applied in milk products except for the study conducted by Rombouts et al., (2015) who has succeed in quantifying and locating LAL in BSA by using tandem mass spectrometry coupled with higher energy collisional dissociation. As discussed above, two types of LAL were detected in Qishta, however, they were absent in UHT milk. LC-ESI-MS/MS analysis succeeded in identifying peptide residue (serine) involved in the β-elimination prior to the Michael addition with lysine. These identifications were impossible to be done with the previous studies which applied high-performance chromatography methods in order to quantify the amount of cross-links present in the samples tested. The analysis held on the residual milk during Qishta production (results not shown) were in line with the previous study suggesting that LAL can be used as in indicator of the heat treatment application during milk processing. In Fact, the intensity of LAL and LAN detected in heated milk were much higher than

that present in UHT milk used for Qishta production. At this step, it was confirmed that LAL and LAN are involved with the participation of disulphide cross-links in the Qishta structure formation.

m/z	Number	Qishta intensity	Average	St dev	Milk intensity	Average	St dev
925.8931	1	7.95E+06	4.00E+06	2.64E+06	3.08E+06	3.35E+06	5.78E+05
		1.83E+06			2.76E+06		
		2.21E+06			4.22E+06		
921.8943	2	4.60E+06	3.60E+06	1.28E+06	0.00	0.00E+00	0.00E+00
		1.68E+06			0.00		
		4.53E+06			0.00		
957.9168	3	2.27E+06	1.12E+06	7.69E+05	0.00	0.00E+00	0.00E+00
		0.00			0.00		
		1.08E+06			0.00		
677.3095	4	8.72E+06	4.32E+06	2.93E+06	5.18E+05	5.22E+05	6.47E+04
		6.83E+05			6.19E+05		
		3.56E+06			4.29E+05		
648.5322	5	8.54E+06	3.21E+06	3.55E+06	1.32E+06	1.20E+06	2.68E+05
		4.25E+05			7.98E+05		
		6.58E+05			1.48E+06		
465.9223	6	3.68E+06	2.49E+06	7.96E+05	8.42E+05	1.30E+06	4.04E+05
		2.14E+06			1.91E+06		
		1.64E+06			1.16E+06		

Table 4: Intensity of the LAL cross-links, resulted from the β-elimination of serine and condensation with lysine, present in Lactel milk and Qishta. Analysis were done in triplicate.

The last cross-link peptide identified was LAL resulting from β-elimination of cysteine residue. Its *m/z* value (587.3213) matched with the theoretical *m/z* values of two peptide chains linked by LAL between two cysteine and lysine residues. BLAST search confirmed that the two sequences were present only in milk bovine serum albumin (BSA). This interchain cross-link connected two BSA. Figure 6 showed the MS/MS spectra of the triply charged double-chain peptide having a theoretical Mr of 1758.9639, originating from two peptides sequence linked with LAL residue

between cysteine residue and lysine residues. The fragmentation of the parent ion yielded 3 y-fragments and 3 b-fragments.

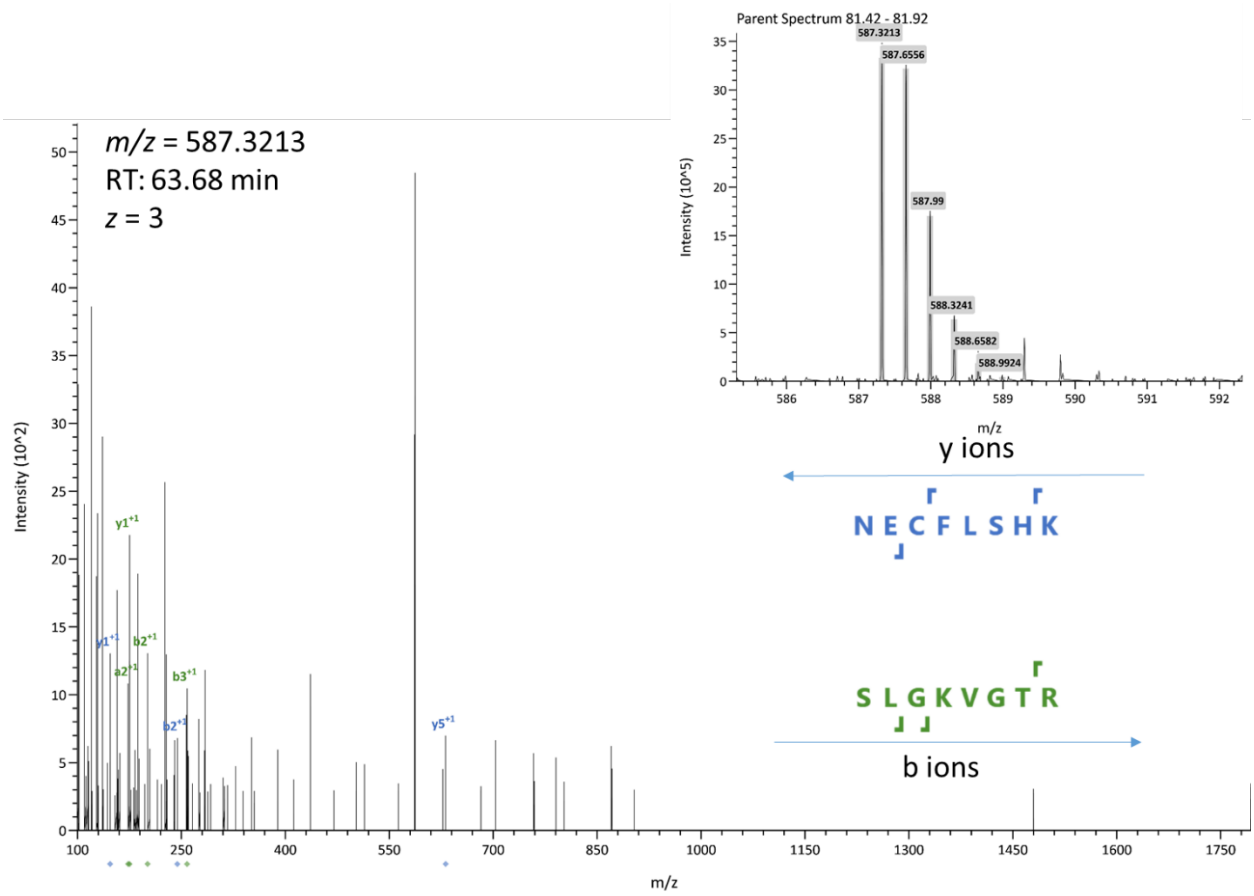

Figure 6:MS/MS spectrum of the double peptide sequence NECFLSHK and SLGKVGTR linked by LAL derivative from Cysteine and Lysine interaction. These peptide sequences were found in the bovine serum albumin whey of the Qishta. Amino acids are referred with their abbreviation code.

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Sample	Theorical Mr and charge state	Detected *m/z*	Elution time	Experimental Charge state	Error (ppm)	α protein	α peptide sequence	Theorical Mr monoisotopic	β peptide	β protein sequence	Theorical Mr monoisotopic
LAL between Serine and Lysine											
Q1-1	(1) 925.8921 (*z*=4)	925.8931	105.48	4	-1.08	sp|P00711|20-142|LALBA_BOVIN	DDQNPH**S** **S**NI**C**NI**S**C DK	2002.8108	sp|P00711|20-142|LALBA_BOVIN	FLDDDLTDDI**M** **C** V**K**	1714.7429
Q1-2		925.8931	105.66		-1.08						
Q1-3		925.8929	105.66		-0.86						
LF-1		925.8934	105.31		-1.40						
LF-2		925.8931	106.36		-1.08						
LF-3		925.8932	106.56		-1.19						
Q1-1	(2) 921.8939 (*z*=4)	921.8944	115.92	4	-0.54	sp|P00711|20-142|LALBA_BOVIN	DDQNPH**S** **S**NI**C**NI**S**C DK	2002.8108	sp|P00711|20-142|LALBA_BOVIN	FLDDDLTDDIM **C** V**K**	1698.7480
Q1-2		921.8942	116.39		-0.33						
Q1-3		921.8943	116.31		-0.43						
LF-1		x	x		x						
LF-2		x	x		x						
LF-3		x	x		x						
Q1-1	(3) 957.9172 (*z*=4)	957.9168	95.45	4	0.42	sp|P00711|20-142|LALBA_BOVIN	DDQNPH**S** **S**NI**C**NI**S**C DK	2002.8108	sp|P00711|20-142|LALBA_BOVIN	FLDDDLTDDI**M** **C** V**K**K	1842.8379
Q1-2		x	x		X						
Q1-3		957.9168	95.04		0.42						
LF-1		x	x		X						
LF-2		x	x		X						
LF-3		x	x		X						
Q1-1	(4)	677.3095	59.92		-0.15						
Q1-2		677.3099	60.39		-0.74						

Q1-3	677.3094 (z=3)	677.3095	59.45	3	-0.15	sp|P02663|16-222|CASA2_BOVIN	N**M**AINP**S**K	889.4327	Isp|P02663|16-222|CASA2_BOVN	ENL**C**STF**C**K	1157.4844
LF-1		677.3094	59.47		0						
LF-2		677.3094	60.07		0						
LF-3		677.3094	60.06		0						
Q1-1	(5) 648.5323 (z=4)	648.5322	50.86	4	0.15	sp|P00711|20-142|LALBA_BOVIN	DDQNPH**S** **S**NI**C**NI**S**C DK	2002.8108	sp|P00711|20-142|LALBA_BOVIN	IW**C**K	605.2995
Q1-2		648.5322	51.25		0.15						
Q1-3		648.5322	50.29		0.15						
LF-1		648.5322	50.32		0.15						
LF-2		648.5322	50.91		0.15						
LF-3		648.5322	50.97		0.15						
Q1-1	(6) 465.9223 (z=3)	465.9223	30.54	3	0	sp|P02663|16-222|CASA2_BOVIN	I**S**QR	502.28639	sp|P02666|16-224|CASB_BOVIN	EAMAP**K**HK	910.4695
Q1-2		465.9223	30.65		0						
Q1-3		465.9224	30.26		-0.21						
LF-1		465.9223	29.54		0						
LF-2		465.9223	29.60		0						
LF-3		465.9223	30.30		0						
LAN between two cysteine											
Q1-1	(7) 498.5855 (z=3)	498.5854	63.81	3	0.20	sp|P02663|16-222|CASA2_BOVIN	ENL**C**STF **C**KEVVR	1526.7221			
Q1-2		498.5854	63.92		0.20						
Q1-3		498.5855	63.67		0						
LF-1		498.5854	63.67		0.20						
LF-2		498.5854	64.11		0.20						
LF-3		498.5854	64.08		0.20						
Q1-1	(8)	619.2674	37.66	3	0			1888.7680			

Q1-2	619.2674 (*z*=3)	619.2675	37.74		-0.16	sp|P00711|20-142|LALBA_BOVIN	DDQNPHSSNI**C**NIS**C**DK				
Q1-3		619.2673	37.32		0.16						
LF-1		619.2673	37.12		0.16						
LF-2		619.2673	37.49		0.16						
LF-3		619.2673	37.40		0.16						
LAL cysteine and Lysine											
Q1-1	(9) 587.3213 (*z*=3)	587.3198	80.80	3	2.55	sp|P02769|25-607|ALBU_BOVIN	NE**C**FLSHK	976.4436	sp|P02769|25-607|ALBU_BOVIN	SLG**K**VGTR	816.4817
Q1-2		587.3198	81.75		2.55						
Q1-3		587.3199	81.43		2.38						
LF-1		587.3198	80.75		2.55						
LF-2		587.3199	81.41		2.38						
LF-3		587.3201	81.60		2.04						

Table 3: Double chain peptides containing LAL and LAN

**The error was calculated from the absolute difference between the theoretical and detected molecular masses (Mr) divided by the theoretical Mr and multiplied by 106*

Red and bold amino acids corresponding to amino acids involved in cross-links. Post translational modifications were highlighted with black and bold amino acids.

Cross-link amino acids (CLAA) formation depends on the heat treatment, the pH, and the processing time applied (Friedman et al., 1981). Nisin and duramycin are examples of protein antibiotics where LAN, LAL and HAL can be found naturally [12]. In food matrix, and specially in milk and dairy products, the presence of DHA resulting from β-elimination of cysteine, serine and threonine and the extent use of heat treatment, have led to the increase of the amount of cross-links present in these kind of products. Friedman, (1999) has noticed the presence of LAL in different types of milk, and succeeded in detecting of this cross-link in raw milk but at low concentration (15 µg/g protein). The influence of the presence of cross-links has not been well studied, especially in dairy field. Disulphide bonds either present as inter or intra molecular scale have been proved to have an important impact on the gel strength, firmness, viscosity and elasticity. Consequently, it has a significant impact on the texture of the final product [11]. Gerrard et al., (1998) have studied the effect of transglutaminase on casein cross-linking, therefore, on the gel strength during yogurt preparation. Lauber et al., (2000) has also studied the effect of this enzyme on the dough of white pan bread.

Identifying the presence of cross-links such as LAL and LAN and then locating the amino acids and the peptide sequences involved in such links, are of high importance since it allows the understanding of the mechanism of Qishta formation and confirms the presence of links other than the disulphide bridges leading to the formation of this product. Further studies should be held in order to study the effect of cross-links on the digestibility of food product since it has been reported that increasing the protein network has a negative effect on protein digestibility [47]. This report represents the first work demonstrating the presence of the cross-links in Qishta and also allowing the identification of the amino acid sequences involved in β-elimination and Michael addition.

Rombouts et al (2015) have succeeded to demonstrate and locate these cross-links in two different matrixes: Wheat gliadin and bovine serum albumin, without determining the sequences involved. LC MS/MS coupled with HCD got the advantage to locate the amino acids involved in the cross-link's formation, allowing us to distinguish between the different sources of DHA and between the amino acids involved in β-elimination. This technique has been demonstrated to allow a maximum number of cross-links identification comparing to collision-induced dissociation (CID), and electron-transfer dissociation (ETD) methods. However, it results in the lowest coverage distribution for the αpeptide (≈ 50%). This issue can be solved by combining HCD with ETD which has been proved to give the highest sequence coverage.

Protein-Fat interaction

Milk characterization before and after heat treatment.

Changes in the microstructure of milk during Qishta process were monitored by confocal microscopy in order to review the effect of temperature on the interactions between proteins and fat globules. In order to achieve that, samples of milk were taken every 2 minutes, until the apparition of Qishta. It is important to mention that the samples of milk were taken from the same area located near the flame where temperature varied between 90 and 99 °C (known as its highest). The kinetic of aggregates formation is showed in figure 7. The CLSM image of UHT milk showed a homogeneous repartition of both proteins and fat globules. Since UHT milk was used for Qishta production, small amounts of large fat globule were observed. After 2 minutes of heating and at 52°C, the apparition of new structures was noticed as a result of protein and fat interaction. At this stage, fat globules had a bigger diameter than that in milk and a spherical regular shape (figure

7B). After 4 minutes of heating, the amount of aggregates increased and fat globules had bigger and more regular spherical shape (figure 7C). 6 minutes of heating, temperature reached 80 °C and the size of the complexes kept on increasing (figure 7D).

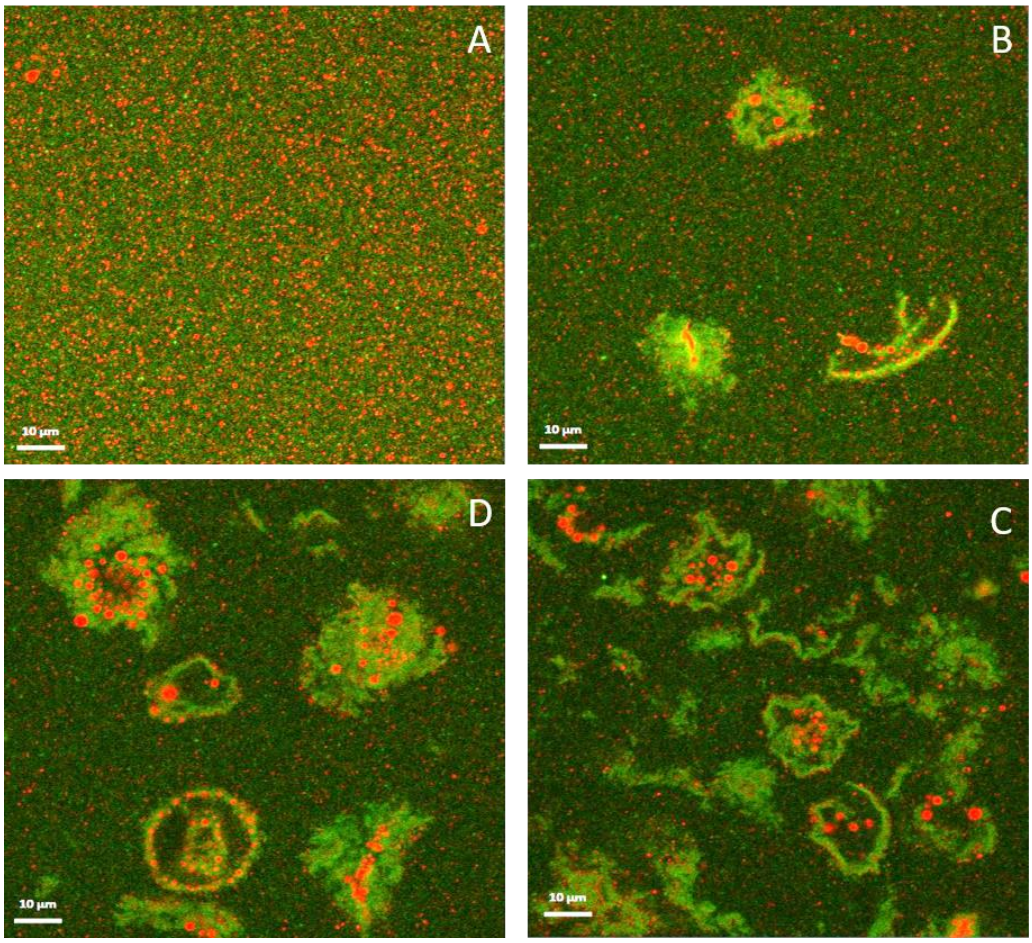

Figure 7: Visualization of milk fat globules and proteins with confocal microscopy. Before heat treatment (A), after 2, 4, 6 minutes of heating (B, C and D). Fat globules appears in red while protein appears in green.

Since Fast Green dye labelled both casein and whey protein, it was not possible to distinguish between these two types of proteins. Globular proteins are known to be more sensible to temperature than caseins [48] . Heating above 60 °C, whey proteins will unfold and denature according to the heat treatment applied. Caseins are more resistant since they can withstand a

temperature of 140°C for more than 20 minutes [49]. Emulsions containing whey protein and casein, such as milk, have not been well studied. In fact, during the heat treatment of whole milk, casein and whey proteins are in competition in order to adhere on the milk fat globule membrane [50–52]. According to the protein concentration, the adhesion reaction between fat globules and milk proteins will occur. At low protein concentration, whey proteins will be the preferable to adsorb, however at higher protein concentration (above than 3%), casein will adhere [51,52]. The milk used in our study during Qishta formation contains 3.2 % of proteins which probably means that caseins adsorption to the fat globule will be more pronounced.

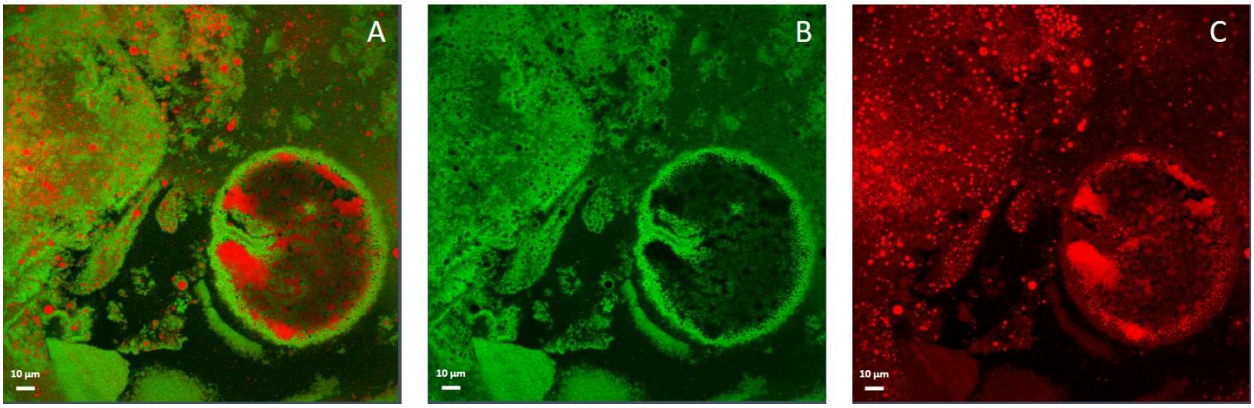

Figure 8: Visualization of proteins (green) and fat (red) in Qishta (A). Protein repartition (B) and fat droplets repartition (C).

Milk characterization

After 8 minutes of heating, the aggregates forming Qishta became visible. The CLSM images showed a large compact gel of proteins exhibiting an irregular form with a large fat droplet indicating the coalescence phenomena (Figure 8A). The individual distribution of proteins showed that they form a glue connecting and trapping fat globules (Figure B and C).

The kinetic of Qishta formation can be summarized as follows: an intense evaporation of water due to the increase in milk temperature, followed by the formation of aggregates resulted from the interaction between denatured proteins and coalesced fat globules. The CLSM images have showed that the aggregates size increased with time, and that the maximum size was reached after 8 minutes heating which corresponds to either a visible gel or to Qishta formation. At this level, Qishta can be defined as a dehydrated gel consisting of a complex of proteins trapping the fat globules. In addition to these complexes which represent the majority of Qishta, the CLSM images showed that the structures obtained at 0, 2, 4 and 6 minutes were present as well. These observations could be explained by the heterogenous temperature distribution and by the specific techniques of heating and skimming used during Qishta process.

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Conclusion

In this chapter we demonstrated that Qishta is a lipo-protein product with a composition and texture similar to those of Ricotta cheese (Maubois & Kosikowski, 1978). Qishta consists of almost the same amount of fat and protein estimated at 12%. The heat treatment used in the process of Qishta making, in addition to the stainless-steel plate, create a heterogenous temperature distribution profile, leading to divide the plate into 2 zones with different range of temperature. A coagulum is formed at the top of the milk in the heated zone and will be harvested at the opposite side of the plate due to the convection forces. The yield of Qishta was directly correlated to amount of fat present in the milk. Increasing the fat concentration to 3.2 % has been demonstrated to give the highest yield of Qishta. Skim milk was not able to produce Qishta. Mass spectrometry analysis has showed that whey proteins and caseins were present in Qishta at almost 20 and 80 % respectively. The electrophoresis analysis, under reducing and non-reducing conditions, has demonstrated that whey protein and casein were interacted in the gel forming the Qishta, via disulphide bonds. In addition to the s-s bonds, the electropherograms has revealed the presence of a high molecular weight aggregates interacted through cross-links other than the disulphide bridges. We demonstrated, through mass spectrometry analysis coupled with HCD, that Lysinoalanine and Lanthionine were present in the gel forming the Qishta and that these cross-links could be used as an indicator to the severity of the heat treatment applied since they were absent in the milk used in the Qishta production which is a UHT milk. Finally, Confocal laser scanning microscopy have visualized the proteins and fat globules interacted in the coagulum and has showed that proteins form a glue that entrap the coalesced fat globules .

CHAPTER : IV

ARTICLE IV - RESULTS

Introduction

The shelf life of any dairy product is defined by its capacity to maintain its basic sensorial quality and therefore to meet consumer expectations for a given period (Lukač-Havranek & Hadžiosmanović, 1996). Different factors can impact dairy product's shelf life such as the quality of milk used, the process applied, the packaging, the storage conditions and so on. These factors can be classified into three categories : physical, chemical and microbial (Muir, 2011). Microbial transformations include bacterial growth, while physical changes concern minerals crystallization, syneresis and protein gelation. Chemical changes concern fat oxidation and non-enzymic browning. Despite the intense heat treatment applied during the process of Qishta formation, the product is considered as an easy perishable food and has a short shelf life, comparing to other dairy products, estimated at 4-5 days when stored at 4 °C. Post microbial contamination, bad work environments (temperature can reach 45 °C), bad storage conditions, lipolysis, a relative low pH estimated at 6.4 and fat oxidation could explain the short shelf life of Qishta. In this chapter, we aim to study the effect of the storage conditions (4 °C) on the fat oxidation and therefore on the shelf life of Qishta. In order to do that, two strategies were adopted: first we determined the primary and secondary indicators of fat oxidation and, in parallel, we investigated the efficiency of the front face fluorescence spectroscopy coupled with chemometric tools as a rapid, non-destructive method in order to distinguish between Qishta samples stored for 20 days at 4 °C.

Article IV

Utilization of front-face fluorescence spectroscopy for monitoring lipid oxidation during Lebanese Qishta aging

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Abstract

Front-face fluorescence spectroscopy technique coupled with chemometric tools was used for predicting the freshness state of a Lebanese dairy product called Qishta, stored up to 20 days. Acid, peroxide and thiobarbituric acid reactive substances (TBARS) values reached no more than 0.93 mg NaOH g^{-1} fat, 6.22 meq O_2 Kg^{-1} fat, and 0.0313 mg malonaldehyde (MA) kg^{-1} sample, respectively, throughout the investigated storage time. In parallel, fluorescence emission spectra of tryptophan and riboflavin, and fluorescence excitation spectra of vitamin A were recorded and showed the highest fluorescence intensity for the Qishta samples aged of 20 days and the lowest intensity for the fresh ones. The primary and secondary indicators of lipid oxidation showed that Qishta can be stored for 20 days without any alteration despite the increase in the TBARS after 16 days of storage. Principal component analysis (PCA) applied on riboflavin emission spectra allowed better discrimination between Qishta samples with a clear distinction of those aged 20 days while some overlapping was noticed between samples aged below 16 days. A high correlation (R^2 = 0.92) was observed between the peroxide value and the intensity of the riboflavin fluorescence recorded at 460 nm.

Keywords: Qishta, Fat Oxidation, Aging, Fluorescence spectroscopy, PCA

Introduction

Heat treatment is used as an efficient processing tool to increase the shelf life of food products such as dairy products. However, the heat treatment applied can generate undesirable effects such as fat oxidation and proteolysis (Ajmal et al., 2018; Vazquez-Landaverde, Torres & Qian, 2006). Nowadays, consumers are more conscientious and aim a category of fresh food free of artificial additives and being less processed.

Jensen, Ferris & Lammi-Keefe (1991) define the milk fat as an exceptional medium where different systems exist. Milk fat, which represents around 3 to 5 % in cow milk, is presented as small globules or droplets and dispersed in the aqueous phase of milk. Their diameters vary from 0.1 to 20 µm. Triglycerides are the principal components of milk fat. Moreover, di- and monoglycerides, fatty acids, sterols, carotenoids are present in milk giving it a yellow color (Jensen, 2002, Jensen, Ferris & Lammi-Keefe, 1991; Jensen et al., 1990).

In food products, the lipid may undergo autoxidation, photo-oxidation, thermal oxidation and enzymatic oxidation that differ in the type of free radical produced or oxygen species. Autoxidation is the most common oxidation process leading to oxidative deterioration, which can alter the texture and the flavor, generating some undesirable volatile products such as aldehydes, ketones, alcohols, esters, lactones, and hydrocarbons. The unsaturated aldehydes and ketones formed are mainly responsible for undesirable smells and taste in the dairy products known as rancidity (O'Brien & O'Connor, 2011).

Qishta is a Lebanese heated dairy product consumed as a dessert and used as a filler in some Oriental sweet production such as Knefeh, Mafrouke, etc. It can be defined as a lipo-protein product containing almost an equal amount of fat and protein (≈ 12%), pH quite similar to milk (≈ 6.4), high a_w (≈ 0.98) and high moisture content (≈ 70 %). Due to these characteristics, Qishta is

considered as highly perishable items and has a shelf life of 5 days when stored at 4 °C (Kassaify et al., 2010). Despite the wide consumption of Qishta in Lebanon and Middle East countries, no bibliography was found investigating the effect of the fat oxidation on its shelf life.

Primary and secondary indicators of fat oxidation have been used as indicators of food quality (Barriuso, Astiasarán & Ansorena, 2013; Botosoa, Chèné & Karoui, 2013; O'Brien & O'Connor, 2011; Al-Rowaily, 2008). Al-Rowaily (2008) has studied the effect of heat treatment on the chemical variations of lipids in some local dairy products using peroxide, p-anisidine, TBARS, acid and TOTOX values. Even though the physico-chemical analyses had been proved as efficient methods, they are considered as time-consuming and require the use of a lot of chemical products.

Rapid screening techniques are mainly used nowadays to predict the fat oxidation level instead of the laborious and destructive methods mentioned above. Front-face fluorescence spectroscopy (FFFS) is considered today as a rapid, non-destructive, and relatively cheap technique, for measuring quality parameters especially in the dairy field (Kamal & Karoui, 2017; Botosoa, Chèné & Karoui, 2013; Karoui, Dufour & De Baerdemaeker, 2007; Karoui et al., 2006a; 2006b; Miquel Becker et al., 2003). This technique was used to: i) predict the fat oxidation of semi-hard cheeses during ripening (Karoui, Dufour & De Baerdemaeker, 2007); ii) monitor the light-induced changes in plain yogurt and the effect of light on the oxidation of cheese products during storage (Miquel Becker et al., 2003; Christensen, Povlsen & Sørensen, 2003; Mortensen et al., 2003; Wold, Jørgensen & Lundby, 2002). Finally, Karoui, Martin & Dufour (2005) have utilized the FFFS technique to discriminate milk samples according to their geographic origins. The high abundance of fluorophores in milk products such as vitamin A, tryptophan, riboflavin

and nicotinamide adenine dinucleotide (NADH), among several other fluorescent compounds, gave this technique an added value in the dairy field (Karoui et al., 2006b).

Our study aims to monitor fat oxidation of Qishta during its storage for up to 20 days at 4 °C using: i) primary and secondary indicators of oxidation; and ii) FFFS as a rapid and non-destructive technique to predict the chemical changes, especially lipid oxidation. To the best of our knowledge, this study is the first to aim in investigating the effect of storage on the lipid oxidation of Qishta.

Materials and Methods

Production of Qishta

According to Hallab 1881 company (Lebanon), the process of Qishta consists of heating whole milk in a large open shallow plate (diameter of 1 m and capacity of 12 L) from one side for 3 hours. Fifteen minutes after the beginning of heating, aggregates appear on top of the milk in the heated zone, where the temperature reaches 100 °C. These aggregates, defined as a mixture of protein and fat globule, are the main components of Qishta. Milk is added continuously in order to readjust its level in the plate due to the evaporation of water. The aggregates and/or the gel formed were gathered at the opposite side of the flame until a precise amount was obtained (usually between 2 and 3 Kg). Qishta was drained at 4 °C for one hour and then distributed in 14 plastic containers with a capacity of 150 g each, and stored at 4 °C during 0, 3, 6, 9, 16 and 20 day(s).

Milk was provided from Lactel (Lactel, France). It is an UHT whole milk having 3.6 % fat and 3.2 % protein. The plate (stainless-steel, 316 L) was provided from Nafco (Nafco, Lebanon) and the burner was provided from AEM (AEM, Chelles, France) equipped with a pressure regulator.

Physicochemical analysis

Acetic acid glacial 100 %, dichloromethane, n-hexane, Celite® 545, sodium sulphate anhydrous, ethanol denatured 95 % volume and propan-2-ol were purchased from VWR (France), while Para-anisidine and 2-thiobarbituric acid reagent were provided from MERCK (Germany). Roquette (France) and VWR (France) provided starch and chloroform respectively. Potassium iodide 99 %, phenolphthalein solution (1 % ethanol) and pure sodium hydroxide (97 %) were purchased from VWR (France) and LABOGROS (France). Finally, Isooctane (UV-IR-HPLC) and sodium thiosulphate were provided from VWR (France) and Panreac Quimica (Spain), respectively.

Extraction of fat

Fat extraction was held at the same day of the analyses and according to the Association Françoise de Normalisation (AFNOR (1991). A volume of 400 mL of hexane/ethanol (3/1) was added to 135 g of Qishta. The mixture was stirred at ambient temperature for 90 minutes (10^3 rpm). The extracted fat was filtered by Whatman No. 1 filter paper. It was then eluted and separated from the mixture by passing it through a 2 cm high column of Celite® and sodium sulphate laid on the bottom of a Büchner filter. The filtrate was dried in a rotary evaporator (Büchi, Rotavap R-3) at speed 4. Bath temperature was previously equilibrated at 40 °C. All the physico-chemical analyses were performed in triplicate.

Measurement of acid value

Acid value of Qishta was determined according to AOCS (Cd 6d-63, 1997) method with a slight modification since only 2.5 g of the extracted fat were mixed with 15 mL of a solution of ethanol/dichloromethane (1/1; v/v) (Botosoa, Chèné & Karoui, 2013).

Measurement of peroxide values

Peroxide value of Qishta was analyzed according to AOCS (Cd 8-53, 1997) method with a slight modification, which corresponded to the fact that only 20 mL of a mixture of acetic acid / chloroform (3/2; v/v) and 15 mL of ultrapure water were necessary for 2.5 grams of extracted fat. The titration was done using sodium thiosulfate ($Na_2S_4O_6$) (0.005 mol L^{-1}) (Botosoa, Chèné & Karoui, 2013).

Measurement of thiobarbituric acid

Thiobarbituric acid value of Qishta, during storage was determined using the direct method described by Pokorny and Dieffenbacher (1989) with a slight modification. 0.05 g of extracted fat was mixed with 5 mL of TBA solution (0.02 mol L^{-1}) and 5 ml of butanol and maintained in a heated bath (Büchi, France) equilibrated at 95 °C for 2 hours. The solution was cooled with tap water for 10 minutes before analyzing the absorbance values. TBA reacting with malonaldehyde (MA) forms a pink MA-TBA complex at 530–535 nm, which was measured using a spectrophotometer (UV 2600, Shimadzu, Noisiel, France).

Fluorescence spectroscopy measurements

Fluoromax-4 spectrofluorimeter (Jobin Yvon, Horiba, NJ, USA) was used in order to record the fluorescence spectra. The incidence angle of the excitation radiation was set at 60° in order to minimize the reflected light, the scattered radiation and the depolarization phenomena. The spectrofluorimeter was supplied with a thermostated cell and Haake A25, AC 200 temperature controller (Thermo-Scientific, France). A quartz cuvette filled with three grams of Qishta was used for the analysis. Spectra of Qishta were recorded in duplicate. The sample was illuminated by the photons of excitation (light beam: ~3 mm wide and ~0.3 mm high) at its center for 3 min, limiting sample dehydration. Emission spectra of tryptophan ($305 < \lambda em < 450$ nm) and riboflavin ($405 < \lambda em < 650$ nm) were recorded after excitation at 290 nm and 380 nm respectively. Excitation spectra of vitamin A ($252 < \lambda ex < 390$ nm) were acquired with the emission wavelength set at 410 nm. A rhodamine cell in the reference channel was used in order to correct the recorded spectra.

Mathematical analyses of data

All spectra recorded were normalized by reducing the area under each spectrum to a value of 1 in order to reduce the scattering effects (Karoui et al., 2007; 2008). Principal component analysis (PCA) was applied on the normalized spectra to visualize the variations between Qishta samples during storage. PCA is a descriptive, representative and exploratory method aiming to reduce the variables into a lower number. It allows the visualization of correlations among the original variables by finding a combination between them that describe the major trends in the data. Variables are transformed into new orthogonal axes called principal components (PCs). In addition, the eigenvectors are similar to spectra and are nominated spectral patterns. The interpretation of the positive and negative peaks allows a better characterization of the emission

and excitation spectra. The correlation between the spectral patterns and the PCs allows the characterization of the potential wavelengths used to discriminate between spectra.

PCA and spectral patterns were performed using MATLAB version R 2013b and 2014a (The MathWorks Natick, MA, USA) and PLS Toolbox 7.9 and 8.0 (Eigenvector Research Inc., Wenatchee, WA, USA).

One-way analysis of variance (ANOVA) was conducted using the SPSS software for Windows (version 13.0, SPSS). A Duncan test was carried out to assess any significant differences between the means. The difference was considered statistically significant when $P \leq 0.05$.

Results and discussion

Evolution of the physico-chemical parameters during aging

Evolution of acid value during Qishta aging

Table 1 showed the variation of acid value during 20 days of Qishta storage at 4°C. Acid value reflects the quantity of acids resulting from the hydrolysis of triacylglycerols. In fact, the term free fatty acid refers to a fatty acid without glycerol (Mannion, Furey & Kilcawley, 2016). Acid values varied between 0.8 and 0.9 mg NaOH g^{-1} fat. The minimum value (0.8) was obtained for Qishta samples aged 20 days, while the highest one (0.93) was observed for those kept for 16 days at 4 °C. Fresh Qishta (0 day) exhibited an acid value of 0.89 mg NaOH g^{-1} fat, which is significantly higher compared with values usually reported for UHT milk. This significant increase ($P<0.05$) may be due to the impact of heat treatment (100 °C) during the production of Qishta related to lactose degradation and formations of acids such as formic acid. In addition, it was reported that

half of this increase of acid value is due to the formation of organic acids from lactose; the remainder is due to the precipitation of calcium phosphate and dephosphorylation of casein (Fox et al., 2015).

The significant increase ($P<0.05$) of acid value observed between days 9 and 16 could be ascribed to the development of microorganisms presenting lipolytic activity during storage at 4 °C. Thereby, psychrotrophic bacteria, yeasts and molds are usually present in refrigerated products.

Evolution of primary oxidation products during Qishta aging

Hydroperoxides formation, as a result of primary lipid oxidation, is responsible of undesired reactions, giving rise to complexes of saturated and unsaturated ketones and aldehydes. These molecules can negatively alter the flavor and the color of food products (Ramis-Ramos, 2003).

Table 1 showed the variation of primary parameters of oxidation values. Concerning the evolution of peroxide, the average value decreased from 6.22 to 3.47 meq O_2 kg^{-1} fat after 20 days of storage. Fatty products having a peroxide value higher than 20 meq O_2 kg^{-1} fat are considered rancid and non-edible, while a value between 0 and 5 meq O_2 kg^{-1} fat corresponds to a fresh high-quality product (O'Keefe & Pike, 2010). Fresh Qishta has a peroxide value of 6.22 meq O_2 kg^{-1} fat which reflects a medium level of oxidation. The sudden decrease between day 16 and 20 could be explained by the formation of the secondary products of oxidation. Rehman & Salariya (2006) found an amount of 0.38 and 17.8 meq O_2 kg^{-1} fat for fresh and aged Khoa stored for 10 days at 25 °C. Khoa is an Indian dairy product obtained by a similar process to that of Qishta. Al-Rowaily (2008) noted an amount of 0.155 meq O_2 kg^{-1} fat for raw cow milk. The high peroxide value

obtained in fresh Qishta could be due to the use of UHT milk and the process applied which consists of boiling milk for more than 3 hours.

Considering the primary indicators of fat oxidation, a decrease in both acid and peroxide values was observed after 20 days of storage; Qishta samples can be considered acceptable since the values are within the acceptable range.

Table 1: Primary and secondary indicators of oxidation of Qishta stored at 4 °C for 20 days.

	Primary lipid products		Secondary lipid products
Aging time (days)	Acid value (mg NaOH g^{-1} fat)	Peroxide value (meq O_2 kg^{-1} fat)	TBARS (532 nm)
0	$0.89^{b,c}$	6.22^{c}	0.0076^{a}
3	0.86^{b}	6.06^{c}	0.0108^{b}
6	$0.84^{a,b}$	5.37^{b}	0.0110^{b}
9	0.86^{b}	5.56^{b}	0.0167^{b}
16	0.93^{c}	5.29^{b}	0.0175^{b}
20	0.80^{a}	3.47^{a}	0.0313^{c}

Means values within a column sharing a common alphabet do not differ significantly ($p < 0.05$); values presented are mean values for three samples ($n = 3$).

Evolution of secondary products during Qishta aging

The oxidation level of Qishta was better represented by TBARS than by peroxide value since the noticeable increase in the TBARS of Qishta aged of 20 days was not reflected in peroxide value. Al-Rowaily (2008) reported a similar tendency while measuring the TBA value of microwave heated milk. TBARS measures mainly malonaldehydes (O'Keefe & Pike, 2010) but at those levels of oxidation TBARS could be more representative for oxidation status than peroxide value (Al-Rowaily, 2008). During 20 days of storage, the TBARS values increased from 0.0076 to 0.0313 (**Table 1**). Qishta exhibited the highest value of 0.0313 at 20 days of storage. The most important increase of TBARS (0.0175 to 0.0313) was observed between the 16^{th} and 20^{th} day of storage with a difference of 0,0138. Despite this increase, the values obtained reflected an acceptable quality of Qishta even at 20 days of storage at 4 °C. Indeed, these values, particularly TBARS of Qishta aged 16 days (0.0175), are significantly lower when compared to those obtained by Al-Rowaily, (2008), who reported TBARS value of 0.086 for yogurt and 0.021 for Labaneh produced by conventional method after 15 days of refrigerated storage. Ishak & Abdullah (2011) considered cakes as non-rancid if the TBARS value was less than 0.576 mg MA kg^{-1}. The TBARS values of raw cow milk and UHT milk were 0.014 and 0.027 mg MA kg^{-1} respectively (Al-Rowaily, 2008).

Based on these results (primary and secondary indicators of lipid oxidation), Qishta can be considered as acceptable and non-rancid and can be stored in the same conditions without affecting the lipid oxidation. These results must be coupled with microbiological and sensory analysis in order to conclude on the shelf life of Qishta.

Evolution of fluorescence spectra during Qishta aging

The abundance of intrinsic fluorophores in dairy products, such as vitamin A, riboflavin, tryptophan, NADH and so on, has promoted the development of fluorescence spectroscopy methods. These techniques can provide, coupled with the chemometric tools, some chemical and physical properties of food products. FFFS is considered as a cheap, rapid and non-destructive method that could replace the physico-chemical analyses (Andersen & Mortensen, 2008; Karoui & De Baerdemaeker, 2007). The fluorescence spectra of vitamin A was reported to provide information related to: i) the interaction between proteins and lipids; and ii) the physical state of the triglycerides (Andersen & Mortensen, 2008); while the fluorescence spectra of riboflavin was linked with protein and fat oxidation (Karoui et al., 2006; Becker et al., 2003).

Fluorescence spectra of tryptophan acquired after excitation at 290 nm on Qishta samples during aging

In dairy products analysis, tryptophan emission spectra was considered as an indicator of the protein structure (Andersen and Mortensen, 2008). **Figure 1a** showed the normalized emission spectra acquired after excitation wavelength set at 290 nm. The emission spectra of all samples exhibited a maximum at around 375 nm. Except for the 20 days aged Qishta that had the highest fluorescence intensity and the largest width, all Qishta samples exhibited almost the same emission spectra. The observed shift from the maximum emission wavelength to the larger wavelength range can be explained by exposing more tryptophan residues to the aqueous phase of aged Qishta samples, in agreement with previous findings of Karoui et al.(2008) who observed a red shift of tryptophan emission spectra acquired on aged egg albumen.

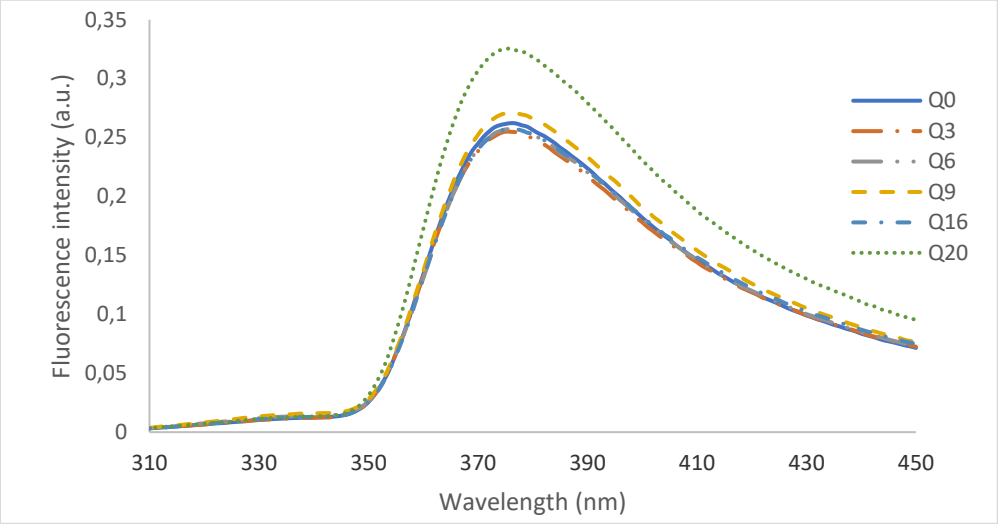

Figure 1a: Normalized emission fluorescence spectra recorded after excitation wavelength set at 290 nm on Qishta sample aged 0 (-), 3(– . -), 6(- . .), 9 (- - -), 16 (- . -) and 20 days (…).

Additionally, the exposed tryptophan residues in Qishta could be shielded from the aqueous phase by other protein molecules as a result of protein–protein interactions, the rate of which increases with protein unfolding. The high fluorescence intensity observed for Qishta aged 20 days could be due to protein aggregation that impact significantly the fluorescence intensity compared to the storage time-induced protein unfolding, which causes more tryptophan residues to become exposed to the aqueous phase of Qishta samples.

Fluorescence properties of vitamin A acquired after emission at 410 nm on Qishta samples during aging

Figure 1b showed the excitation fluorescence spectra recorded after emission wavelength set at 410 nm. These excitation spectra exhibited a maximum peak located at ~320 nm and two other minors located at 292 and 305 nm. These observations are in agreement with previous findings of Karoui and Dufour (2003) reporting that the maximum fluorescence intensity of vitamin A excitation spectra scanned on different varieties of soft cheese, after emission at 410 nm, was

located at 322 and 305 nm. Again, as observed for tryptophan spectra, vitamin A spectra acquired on Qishta samples aged 20 days presented the highest fluorescence intensity.

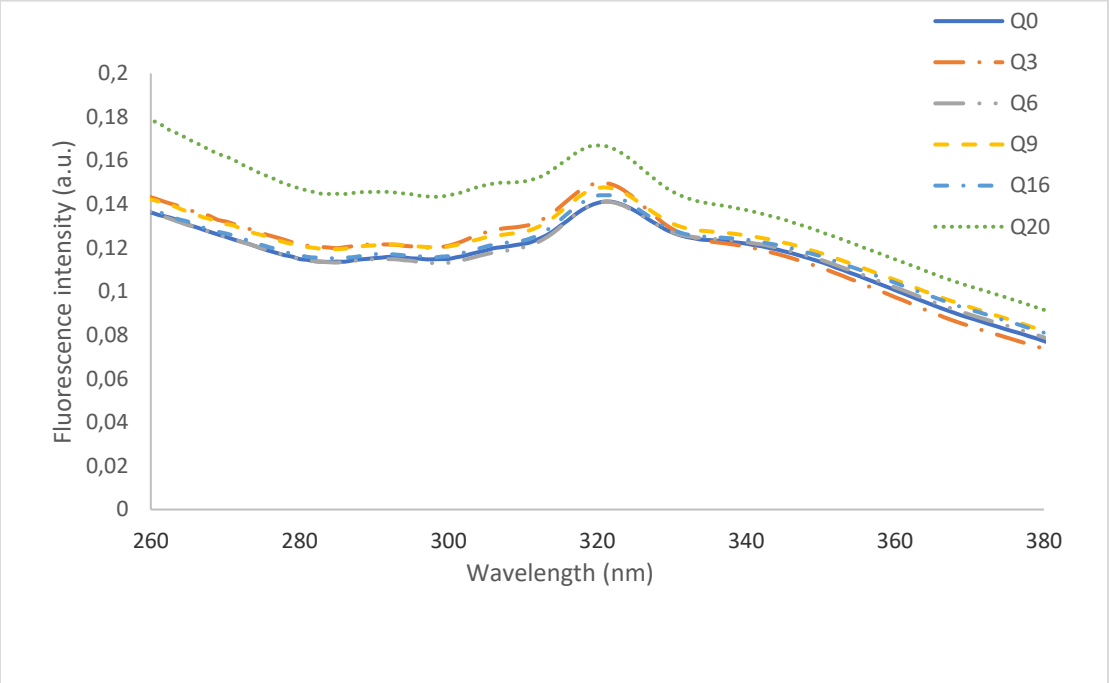

Figure 1b: Normalized emission fluorescence spectra recorded after excitation wavelength set at 410 nm on Qishta sample aged 0 (-), 3(– . -), 6(- . .), 9 (- - -), 16 (- . -) and 20 days (…).

Indeed, Qishta samples aged 0 day had the lowest fluorescence intensity at 320 nm, while those kept up to 20 days had the highest one. It has been reported that the shape of the vitamin A excitation spectrum is correlated with the physical state of the triglycerides in the fat globules (Karoui et al., 2006a). The ratio of fluorescence intensity at 320nm/292 nm increase with the increase of storage time which could be explained by the increase of the viscosity of triglycerides. This could be attributed to cristallization of triglyceride during storage, in agreement with previous findings (Andersen & Mortensen, 2008; Karoui et al., 2006a). Similar trend was observed during the ripening of semi-hard cheeses since changes in the fluorescence intensity ratios at 322 nm/295 nm were noted and ascribed to the crystallization of triglycerides between 1 day and 81 days of

ripening (Dufour et al., 2000). Finally, the changes in the shapes of vitamin A spectra may also result from fluorescence transfer between tryptophan residues of proteins and vitamin A located in the fat globule membrane.

Fluorescence properties of riboflavin acquired after excitation at 380 nm on Qishta samples during aging

Considering the riboflavin fluorescence spectra (**Figure 1c**), the emission spectra exhibited 2 maxima located at 455 and 530 nm. Except for Qishta spectra acquired on day 0, the fluorescence intensity increased with the storage time. As observed for vitamin A and tryptophan spectra, the 20 days aged Qishta exhibited the highest fluorescence intensity.

An increase in the fluorescence intensity in the region located between 405 and 480 nm was noticed during the storage period of Qishta. This region was reported to reflect the oxidation resulted from the products formed by aldehydes and amino acids. In the same region, lumichrome, a photo breakdown product from riboflavin, exhibits fluorescence between 444–479 nm. In addition, β-carotene absorbs in the region located between 400–500 nm. β-carotene can also undergo photodegradation, which may influence the shape of riboflavin fluorescence spectra. The obtained results are in line with the findings of Karoui et al., (2007; 2006a) who observed an increase of the fluorescence intensity of spectra acquired after excitation set at 380 nm for both egg and cheese. Surprisingly, we noticed an increase of the fluorescence intensity at 530 nm, which is in discordance with the findings of Wold et al., (2002). An explanation could arise from the transfer of energy that occurs between fluorescent compounds allowing an increase of fluorescence intensity at 530 nm and/or from the presence of other fluorophores in Qishta having maximum excitation at 530 nm.

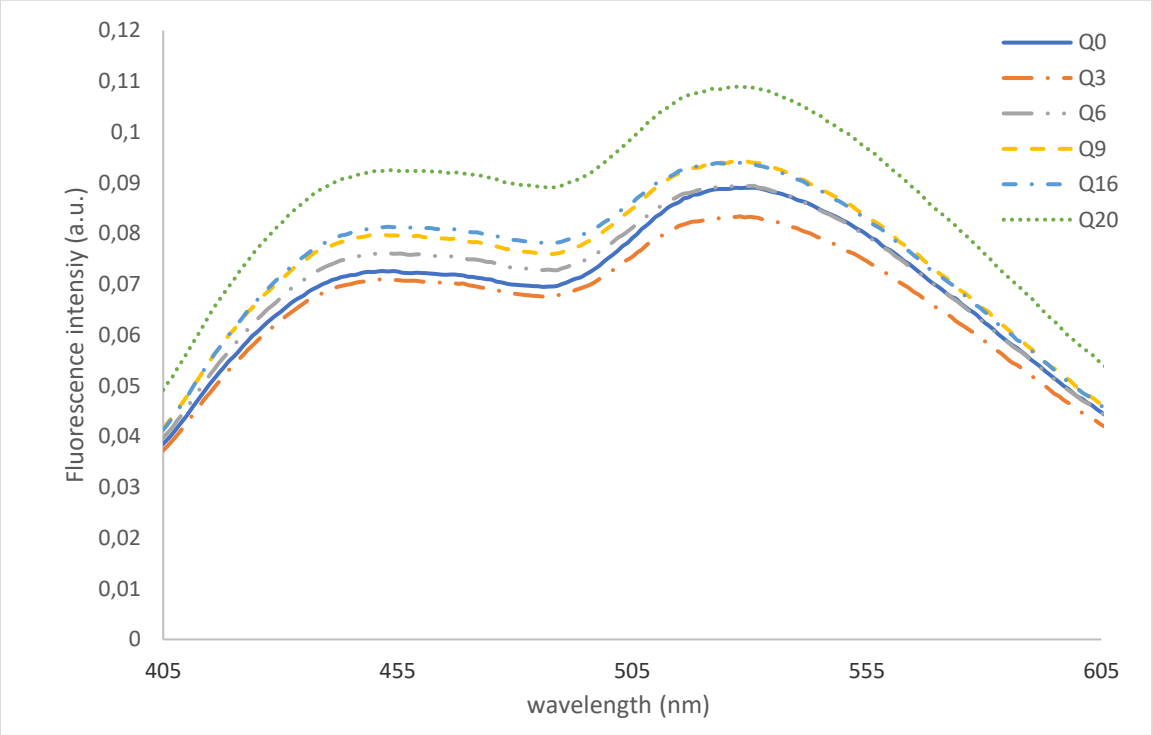

Figure 1b: Normalized emission fluorescence spectra recorded after excitation wavelength set at 380 nm on Qishta sample aged 0 (-), 3(– . -), 6(- . .), 9 (- - -), 16 (- . -) and 20 days (…).

Discrimination based on fluorescence spectra recorded on Qishta samples

Evaluation of the discriminant ability of fluorescence spectra of tryptophan acquired after excitation wavelength set at 290 nm on Qishta samples during 20 days of storage.

Most of the investigated spectra presented similar shapes, therefore it was of high importance to find a mean in order to distinguish between the samples studied. Thus, PCA was used to extract information from the data tables. This multidimensional statistical technique was applied to the 12 spectra collected on Qishta at different storage times (**Figure 2**). The map defined by PCs 1 and 2 (69.74 and 27.79 % of the total variance, respectively) of the PCA performed on spectra acquired after excitation at 290 nm (corresponding to tryptophan) divided the samples into 2 groups. Group

1 consisted mostly of the samples aged from 0 to 16 days, while group 2 comprised the samples aged 20 days. A clear differentiation was shown between these 2 groups since the former group is located mostly on the negative side of PC1, while the latter one is positioned on the positive side. However, the distinction inside group 1 was not feasible since Qishta samples were overlapped.

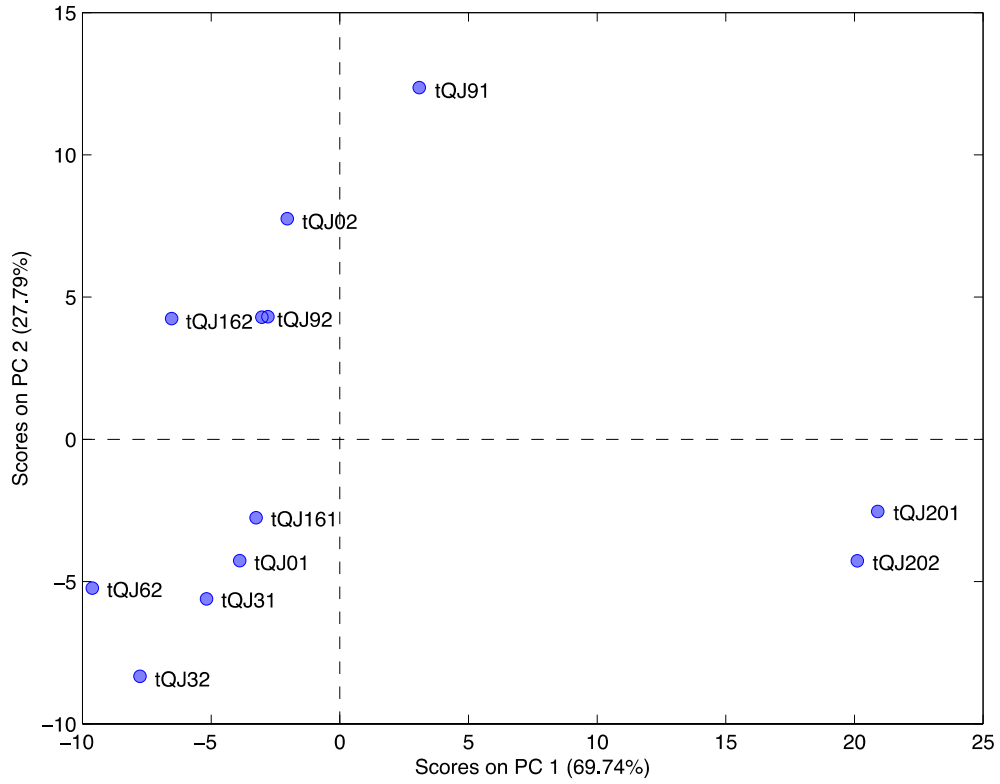

Figure 2: PCA similarity map defined by the principal components 1 and 2 after excitation wavelength set at 290 nm on Qishta samples during 20 days of storage.

Evaluation of the discriminant ability of fluorescence spectra acquired on Qishta excitation during storage after emission wavelength at 410 nm on Qishta

Concerning PCA applied to the excitation spectra recorded after emission at 410 nm (excitation spectra of vitamin A), a better discrimination was obtained than that observed with the emission

tryptophan spectra. In fact, the map defined by PCs 1 and 2 (93.42 % and 5.44 % of the total variance, respectively) showed some clear discrimination of Qishta samples according to their storage time (**Figure 3a**). Qishta samples aged 20 days were always distinguishable from all the other samples. Regarding PC1, all samples had negative score values except those aged 20 days and one sample aged 9 days.

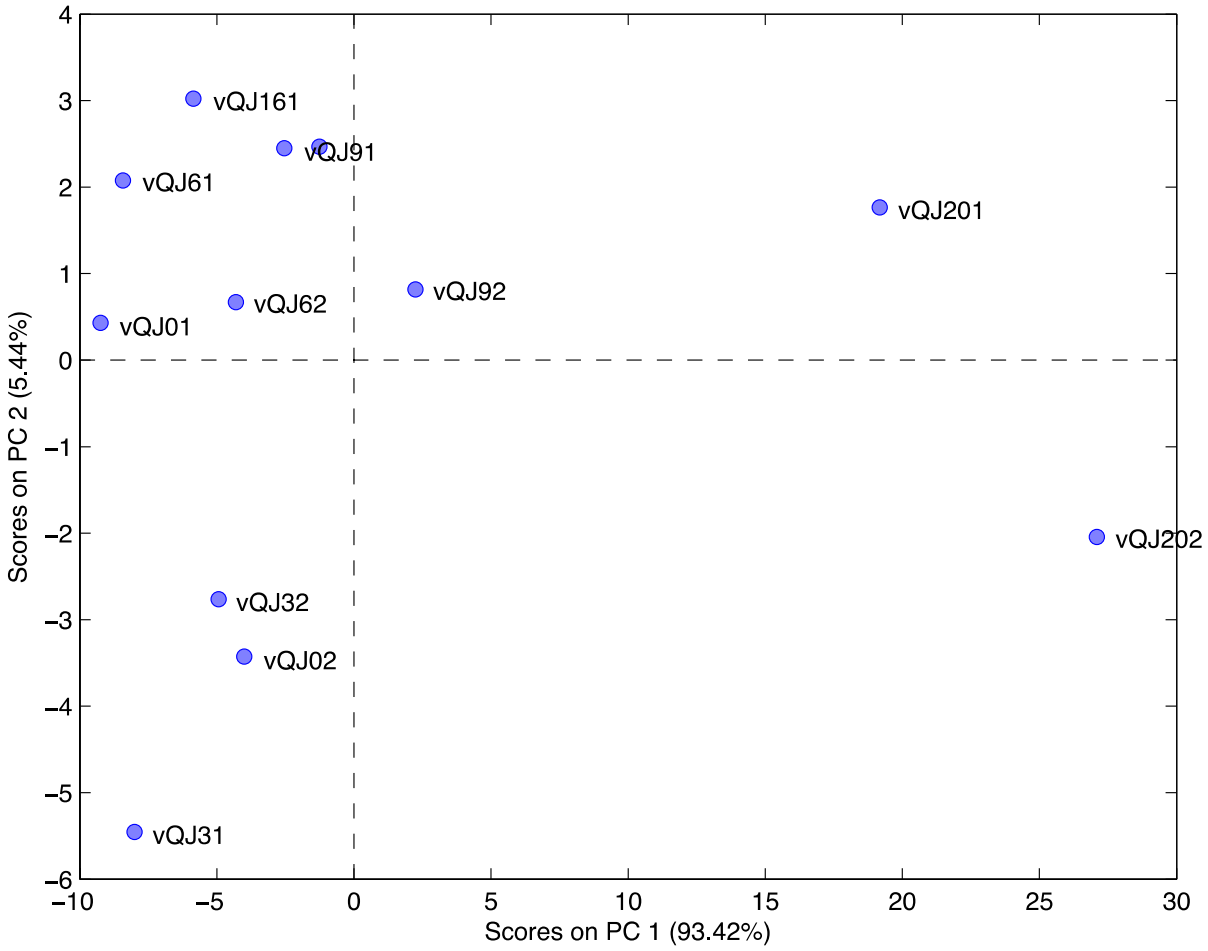

Figure 3a: PCA similarity map defined by the principal components 1 and 2 after emission wavelength set at 410 nm on Qishta samples during 20 days of storage.

The spectral pattern 2 showed an opposition between a negative peak located at 310 nm and a positive one at 375 nm (**Figure 3b**) indicating major changes at the molecular level between samples aged 0 and 3 days from the others. Karoui & Dufour (2003) have already obtained this spectral pattern while they were comparing the difference between the centers and the surfaces of ripened soft cheeses. They suggested that the shape of the spectral pattern of vitamin A reflects the variation occurred in the triglyceride molecules, as well as the interaction between proteins and fat globules during cheese ripening and storage. Botosoa et al., (2013) have used the spectral pattern of vitamin A in order to discriminate between cake samples during aging. According to the results obtained on the emission and excitation spectra of tryptophan and vitamin A respectively, it can be concluded that Qishta samples aged 20 days can be discriminated from other samples. The distinction between Qishta samples aged 0, 3, 6, 9 and 16 was not so clear due to the overlapping observed. The differences detected could be due to the interaction developed between protein and fat globule during the storage, aggravated by the high moisture content which has been suggested to increase the molecular interactions in the food matrix (Botosoa, Chèné & Karoui, 2013; Karoui and Dufour, 2003).

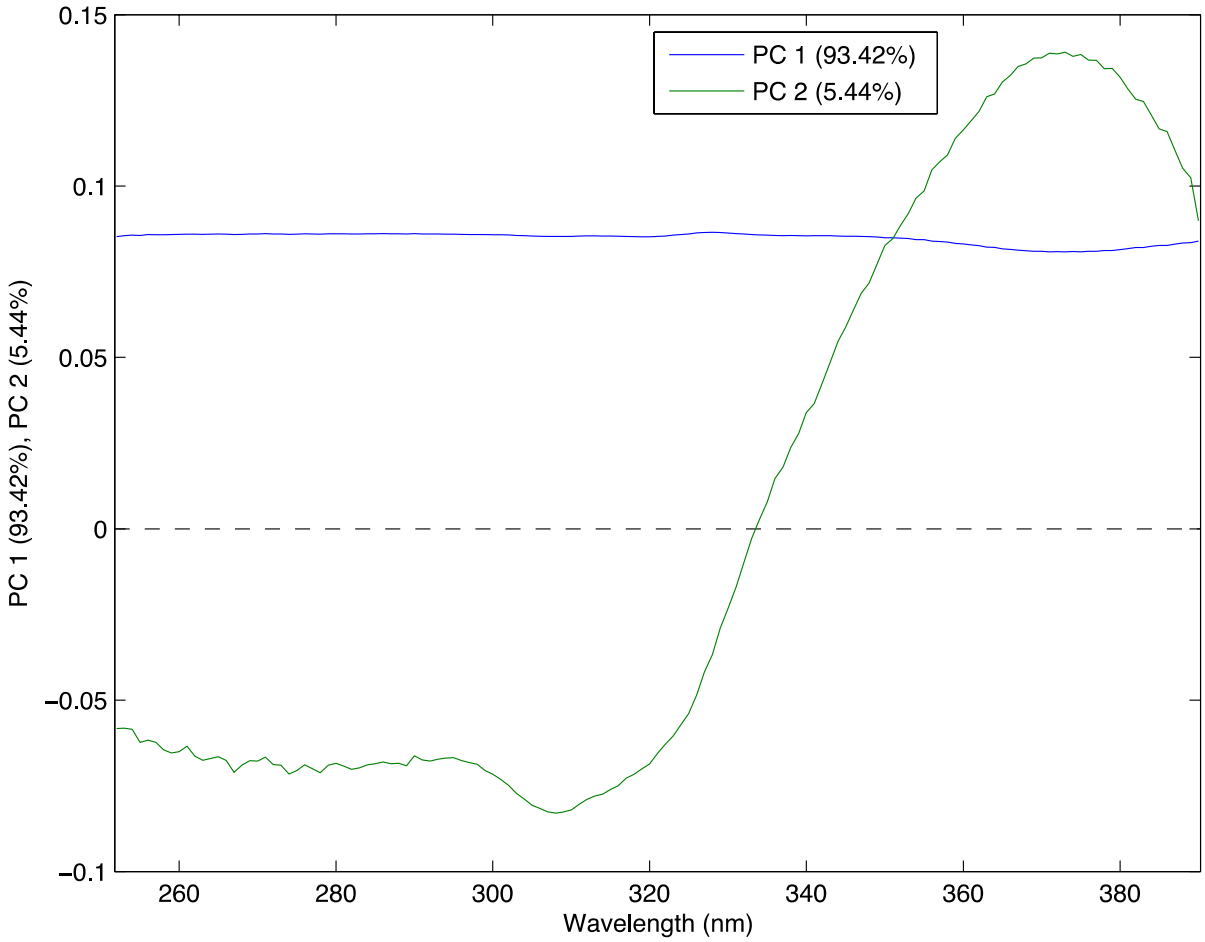

Figure 3b: Spectral pattern corresponding toe principal components 1 and 2 after emission wavelength set at 410 nm on Qishta samples during 20 days of storage.

Evaluation of the discriminant ability Fluorescence spectra of riboflavin acquired after excitation at 380 nm on Qishta samples

The map defined by PCs 1 and 2 (97.13 % and 2.57 % of the total variance, respectively) showed always a clear discrimination between samples aged 20 days and other samples (**Figure 4a**). Concerning PC1, Qishta samples aged 0, 3 and 6 days had negative values while almost all the other samples (age > 6 days) exhibited positive values. It can be concluded that PC1 divided the

samples according to their ages, and contrary to the previous spectra, the discrimination between all Qishta samples was better with less overlapping. These results were in accordance with the TBARS analysis since the map defined by PCs 1 and 2 divided the samples into 3 groups: the first one consists of Qishta aged 0 day, the second one belongs to Qishta aged 3, 6, 9 and 16 days and the last one contains Qishta aged 20 days. The ANOVA test held on the TBARS values has also divided the Qishta samples into 3 groups significantly different and consisting of the same Qishta samples.

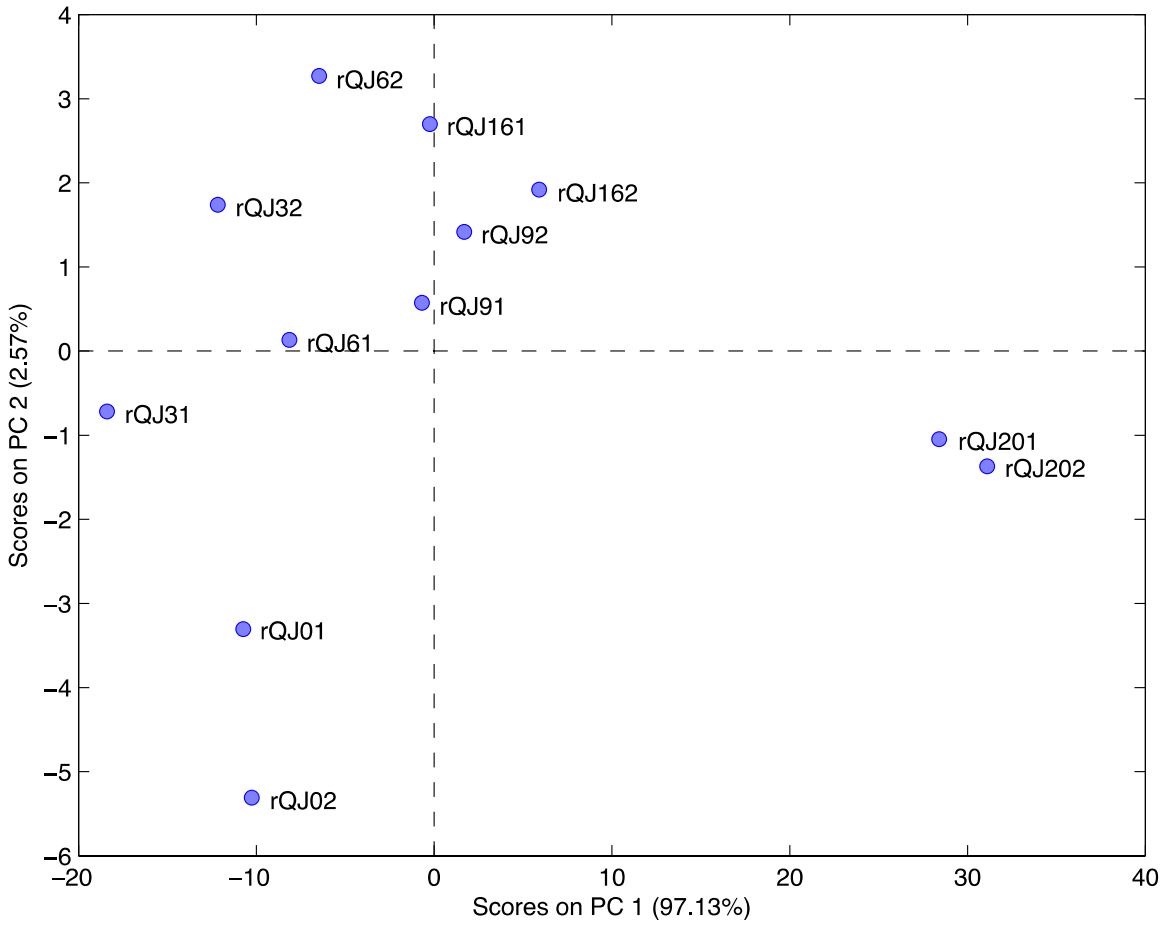

Figure 4a: PCA similarity map defined by the principal components 1 and 2 after excitation wavelength set at 380 nm on Qishta samples during 20 days of storage.

The spectral pattern 2 (**Figure 4b**) showed a positive peak located around 460 nm suggesting the formation of photo break down products from Qishta samples stored for 3, 6, 9 and 16 days.

The correlation between primary indicators of oxidation and high-intensity bands obtained at 460 nm of the spectra scanned after excitation set at 380 nm was investigated. A high correlation (R^2 = 0.92) was found between peroxide values and the fluorescence intensity. A negative correlation was noticed between peroxide value and normalized fluorescence intensity at 460 nm suggesting probably that this could be used as an indicator of the transformation of primary products to secondary ones.

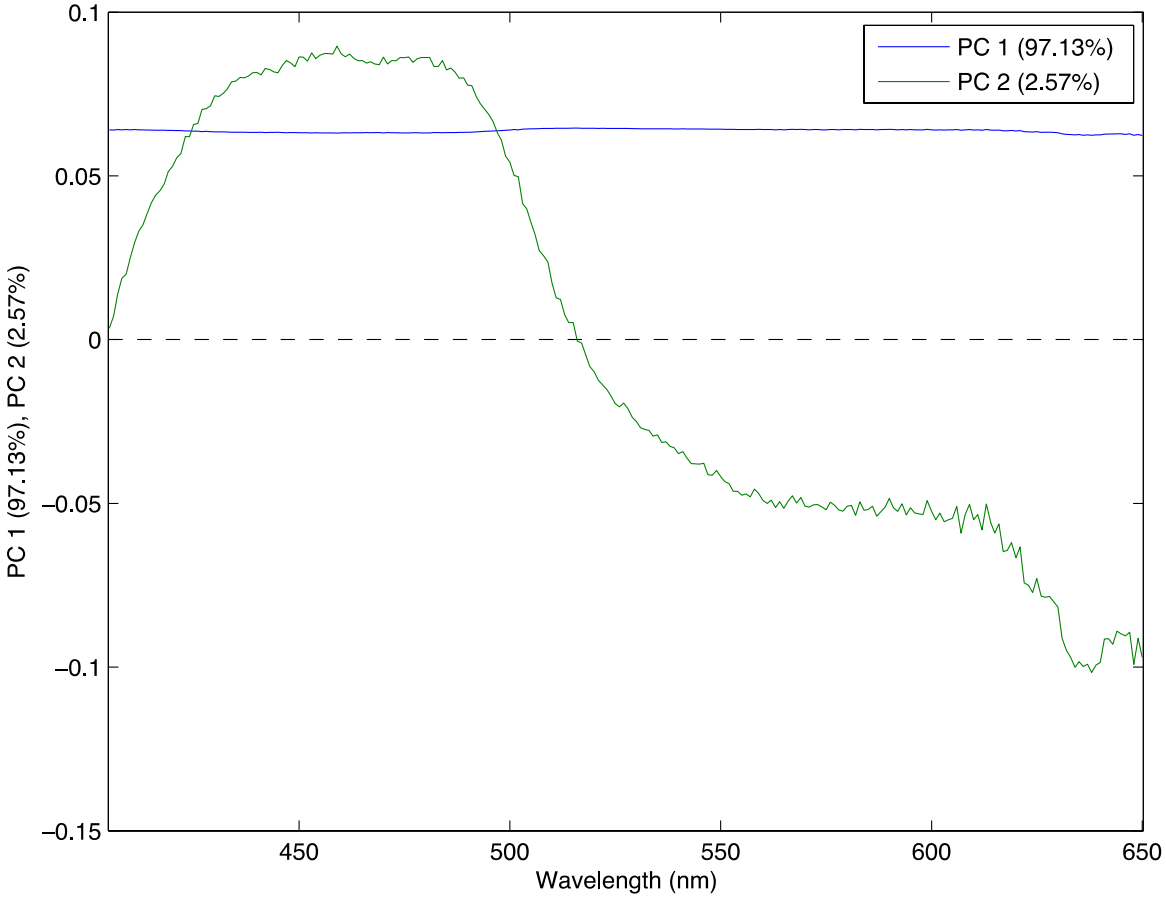

Figure 4b: Spectral pattern corresponding PCA similarity map after excitation wavelength set at 380 nm on Qishta samples during 20 days of storage.

Conclusion

From fat oxidation point of view, the physico-chemical results have shown that Qishta could be stored for 20 days without any quality deterioration. TBARS value highly increased after 20 days while peroxide value showed a slight decrease. Fluorescence spectra coupled with chemometric tools were able to detect the variation that occurred during Qishta storage. In fact, PCA showed clear discrimination between Qishta samples aged 20 days and all other samples. Tryptophan and vitamin A showed an overlapping between samples aged from 0 to 16 days, while the fluorescence spectra corresponding to riboflavin demonstrated its ability to determine the freshness level of Qishta. A high correlation was observed between the fluorescence intensity at 460 nm and peroxide values. It could be concluded that riboflavin spectra could be used as an effective tool for the evaluation of Qishta freshness.

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Conclusion

In this chapter, we demonstrated that the freshness of Qishta samples was slightly affected after being stored during 20 days at 4 °C. The peroxide values were acceptable during 16 days of storage and have decreased from 6.2 to 3.2 after 20 days of storage. This drop could be explained by the development of the secondary indicators of oxidation such as TBA. However, the quantification of the TBA values has showed that despite the increase noticed after 16 days of storage, the maximum value reached was 0.032. FA values, index reflects the quantity of fatty acids obtained from the hydrolysis of triacylglycerols, were also stable under these storage conditions. Primary and secondary lipid oxidation indicators that reflect the degree of food deterioration and the rancidity of Qishta, have revealed that Qishta samples were chemically accepted during all the experiment period.

Front face fluorescence spectroscopy, coupled with chemometric tools, was used in order to predict the freshness of Qishta samples during ageing. Fluorescence spectra of vit A, tryptophan and riboflavin were used as an efficient tool in order to distinguish between Qishta samples especially the sample aged 20 days. These results were in line with the physico-chemical analysis indicating, therefore that this technique can be used as a non-destructive and rapid method replacing the long and high cost chemical analysis.

CONCLUSION AND PERSPECTIVES

Conclusion and perspectives

Milk heat treatment is used to ensure the microbial stability of the product and therefore to increase its shelf life (Al-Saadi, Easa, & Deeth, 2013). In addition, heat treatment is used as a technological tool to create new dairy products that meet the needs of today's society. Despite the development made in the heat treatment field and the innovation of new technologies such as spray drying, there is still a lot to investigate in milk such as fouling issue that faces the majority of dairy companies today (Britz & Robinson, 2008). Cow milk, the most studied dairy product, is considered as a dynamic complex medium due to the diversity of its complements such as lactose, proteins, fat globule, calcium, phosphate and so on (da Silva & da Costa, 2019; Mohammadian, Salami, Emam-Djomeh, & Alavi, 2017). Whey proteins and caseins have different characteristics and therefore behave differently when heated or when calcium is added (Raikos, 2010). In our study we succeed to a certain point, to disassemble the black box of Qishta formation where we found that the composition and the texture of this product can be compared to those of Ricotta cheese. During milk heat treatment, whey proteins, which consists of almost 20% of the total proteins present in milk, denature and interact through disulphide bonds with the casein micelles and especially κ-casein located at the micelle surface (Hillier & Lyster, 1979; Raikos, 2010). The electrophoresis analysis has showed the presence of high molecular weight aggregates even after the addition of β-Mercaptoethanol which breaks the disulphide bridges. Then we demonstrated a relevant result about the presence of cross links in the Qishta coagulum related to the formation of lysinoalanine and lanthionine during the heat treatment and which are involved in internal proteins bound. Spectrometry analysis has confirmed the presence crosslinks in Qishta and milk samples. In addition, these analyses have allowed to identify the peptides sequences involved in the cross-link formation. A BLAST research has showed that these peptides belongs to bovine milk proteins.

Conclusion and perspectives

Our results were of high importance since, it was the first time we identify the peptide sequences involved in the crosslinks formation in protein aggregation during Qishta. Probably these crosslinks are present in any heat-treated dairy products. Electrophoresis and spectrometry analysis have showed that both whey proteins and caseins were involved in the Qishta formation. The effect of fat concentration on Qishta formation was highlighted by variating the amount of fat present in the milk. It was demonstrated that increasing fat concentration was positively correlated to the quantity of Qishta produced (yield). Replacing whole milk by a skim one did not lead to any Qishta formation. These findings showed that fat globule has a primordial role in the coagulum formation. This role was highlighted by the confocal laser scanning microscopy experiments. In fact, microscopic images showed that fat globules, colored with Nile red dyes, participate with the proteins in the network formation. Throughout the process, the size of fat globules was increasing as well as the aggregates, forming after certain time the gel of Qishta. The effect of the initial milk composition on the Qishta's yield has been investigated as well. Three different whole milk powders from different sources were used in order to produce Qishta. It was shown that increasing the proteins concentration has a positive and a significant effect on the yield of Qishta obtained without changing its texture (visually). The constraint at this step was to maintain the same process conditions while testing every milk powder separately. Something was quite uncontrollable since Qishta's yield depends on several factors such as factory temperature, employee's performance, heating intensity, milk hydration process…Despite the diversity of the factors that affect the process, it was demonstrated that increasing the percentage of proteins by 2%, has increased the yield by almost 8%.

Conclusion and perspectives

In the second part of this thesis, we investigated the effect of the storage conditions on the fat oxidation and therefore on the shelf life of Qishta particularly in its chemical stability. The chemical analysis of the primary and secondary indicators of oxidation has revealed that Qishta samples, stored at 4°C for 20 days, were not oxidized, indicating that Qishta could be stored under these conditions without any alteration on its quality for at least 20 days. The chemical analyses done on the fat extracted are time consuming and require a lot of chemical products. We investigated the use of front face fluorescence spectroscopy, as a rapid non-destructive method, in order to predict the shelf life of Qishta samples. This technique coupled with the chemometric tools was able to detect the slightly changes in the peroxide value and TBA detected after 20 days of storage. Therefore, this technique was able to discriminate the freshness of the samples according to their ages. The shelf life of a dairy product depends basically on the bacterial load that it contains (Hanson, Wendorff, & Houck, 2005). Today and according to Hallab 1881 company, Qishta has a shelf life of 4-5 days when stored at 4 °C. Increasing the shelf life of this product is of high importance since it allows to the company to export Qishta worldwide and to sell the product on the Lebanese market, where this product can only be purchased from the pastry market specialized in the oriental sweets production. In their study Kassaify et al. (2010), have showed that most of Qishta samples taken from Lebanese markets were not acceptable microbiologically. They suggest that total mesophilic bacteria and the high mold counts could be the reason behind the short shelf life of Qishta. As we demonstrated in the first chapter, during the process of Qishta making the milk temperature reaches 100 °C. Therefore, the presence of these bacteria in Qishta could be due to a post bacterial contamination as a result of the utensils used during Qishta draining or the containers used during the packaging step. In fact, a new experiment

is held in Lebanon at Hallab factory, in order to test the effect of the utensils cleaning on the microbiological results of Qishta samples. The study consists of comparing the bacterial loads before and after packaging. The results are promising so far, and confirm the hypothesis of a post contamination problem. At the end, it's of high importance to mention that the work conditions can gravely affect the shelf life since, during summer, temperature factory can reach 45 ℃. Therefore, we tried to ameliorate the process of Qishta which depends on gas as a source of energy. A promising result were obtained by using an induction system. Actually, we are trying to reproduce the technique e the results and investigate the capability of using this technique to produce Qishta at Hallab 1881 factory scale, since the first experiments were done at lab scale using a smaller plate comparing to the normal one used in Lebanon.

Finally, this thesis represents the first study held on a traditional Lebanese dairy product highly demanded and consumed called Qishta. Three years of research has allowed a better understanding of the composition of this product and the mechanisms leading to its formation. The research made on Khoa has begun long time ago and despite dozens of years of research they did not succeed to replace the artisanal process of Khoa by a new developed one capable to not change the organoleptic characteristics of this product. Ten years of research on a similar dairy product called Kajmak, in order to change its process did not succeed yet. The results obtained during this thesis can be used as a solid base in order to continue and build on. The difference between Qishta and other products as well as the difficulty of this product and its process result from the heterogeneous temperature distribution and milk addition during the process which result in a non-consistent product.

The effect of proteins and fat on Qishta must be more studied since we demonstrated that increasing both components lead to an increase in the yield of Qishta. However, we didn't studied the effect of increasing fat concentration to more than 3,6%. Also for the proteins, the increase in the yield could be related to an increase in the calcium amount in milk and this must be investigated. The addition of whey protein or casein in milk was not also investigated. Although, the effect of fat and protein has shown to have a positive effect on the yield, the impact on the texture, taste and composition was not investigated.

Concerning the microbiological shelf life of Qishta, the results obtained in this thesis has shown that the product is acceptable according to the Lebanese Norms yet, these results cannot be compared to these applied at Hallab factory. In our case, all utensils used were sterilized previously and the work environments conditions are totally different to those in Lebanon where almost 100 plates can be used at the same time leading to an increase in the temperature that can reaches almost 40 °C in summer. The effect of the microbial contamination must be studied again under Hallab 1881 conditions.

The use of the induction can be considered as an innovation in this thesis that can have an economical impact as well as a direct impact on the hygiene of the product. In fact, we have already demonstrated that replacing gas by induction has reduced three times the consumption of energy and therefore reduced the cost of Qishta production. In addition, the induction can improve the work environment conditions and this can have a positive impact on the hygiene of Qishta.

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The listed references below concern only introduction of thesis and chapters, as well as the final conclusion of the thesis

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