Cyprus University of Technology

Faculty of Geotechnical Sciences and Environmental Management

Doctoral Dissertation

# Genetic and nutritional factors affecting ovine milk production traits and milk fatty acid content in Chios sheep breed

**Simoni Symeou**

Limassol, April 2021

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Approval Form

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The approval of the dissertation by the Department of Agricultural Sciences, Biotechnology and Food Science does not imply necessarily the approval by the Department of the views of the writer.

**Acknowledgement**

"Keep Ithaka always in your mind.
Arriving there is what you are destined for.
But do not hurry the journey at all.
Better if it lasts for years,
so you are old by the time you reach the island,
wealthy with all you have gained on the way,
not expecting Ithaka to make you rich".

Constantinos P. Cavafy

What matters is not the destination, but the journey of each person. The journey lasted, passed and now it has come to an end. On this rich journey I had companions, I had teachers and friends, I had brothers and sisters, I had family.

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“Ithaka gave you the marvelous journey.
Without her you would not have set out.
She has nothing left to give you now”.

Constantinos P. Cavafy

***This Ph.D is dedicated to my parents***

**Abstract**

This thesis focuses on the nutritional and genetic factors affecting milk production traits and fatty acid (FA) content of milk in Chios sheep. Improvement of milk production traits or milk fat quality of this breed can increase the quantity and/or quality of Halloumi cheese produced in Cyprus while enriching this dairy product with beneficial for human health effects lipids.

The first part of the study aimed to evaluate feeding olive cake silage (OCS), a well-preserved by-product of olive oil production, on the FA profile of ovine milk (**Chapters 2 and 3**). In **Chapter 2**, thirty purebred Chios ewes were assigned to three diet treatments receiving 0%, 7% and 14% of OCS (on diet DM) for four weeks and the lipid content of their milk was assessed in lyophilized samples with nuclear magnetic resonance (NMR) technique. Results indicated that feeding these low and high rates of OCS reduced overall the saturated FA (SFA), increasing the unsaturated FA (UFA) content by 26% and 41% and the monounsaturated FA (MUFA) content by 30% and 46%, in milk samples, respectively. Furthermore, the content of beneficial for human health lipids, such as the conjugated linoleic acid (CLA) isomers, increased also in supplemented groups. In **Chapter 3**, the possible changes in milk of Chios sheep and related Halloumi cheese lipids with OCS diet inclusion were investigated by using gas-chromatography mass-spectrometry (GCMS) analysis. Sixty Chios ewes assigned to three diet treatments receiving 0%, 10% and 20% of OCS on DM basis and the results showed that OCS inclusion linearly increased UFA, MUFA, PUFA, CLA and oleic acid content in both milk and Halloumi cheese lipids, reducing at the same time the overall atherogenicity and thrombogenicity indices

for both dairy products. Overall, the above trials showed that OCS is an alternative feed that can be used to improve the FA profile of ovine milk and Halloumi cheese without adversely affecting the lactating performance of Chios sheep.

In the second part of the study, the objective was the identification of single nucleotide polymorphisms (SNPs) in the ovine candidate genes *ACAA2*, *DGAT1*, *SCD1*, *FASN*, and *LIPG* and assess their association with milk productions traits and the FA content of Chios sheep breed (**Chapters 4-6**). In **Chapter 4**, the aim was to investigate the potential association between a SNP of the *ACAA2* gene, a T to C substitution located in the 3' untranslated region (UTR), and important milk production traits in two extended Chios sheep populations from multiple flocks in Cyprus (742 animals in total) and Greece (632 animals in total). Databases were created containing lactation records from purebred Chios ewes in mid-lactation over 7 successive years from 20 flocks in Greece and during 8 successive years from 5 flocks in Cyprus. The mixed model association analysis revealed significant association of the investigated SNP with milk yield and milk content. In particular, the SNP was associated to milk yield with no effects on milk fat percentage in both populations, while the Cypriot population was also associated with milk protein percentage, milk protein yield and fat yield. In **Chapter 5**, molecular characterization of the UTRs of ovine *DGAT1* and *SCD1* genes in Chios breed was performed to identify polymorphisms putatively affecting milk traits due to the significant regulatory roles of those regions in gene expression. Along with those, previously identified polymporphisms of *ACAA2* and *FASN* genes were also tested in relation to their effects on milk fat content and FA profile of Chios sheep milk. For this study 429 purebred Chios ewes in mid-lactation, were used for the collection of three consecutive monthly milk samplings and mixed models were used to

analyze the association of the genotypic data identified. The 3' UTR of the *DGAT1* gene and the 5' and 3'UTRs of the *SCD1* gene appeared to be monomorphic and thus did not allow further association with milk traits. However, the *FASN* g.14777C>T SNP on exon 31 was associated with C13:0 and the *ACAA2* g.2982T>C SNP in the 3'UTR was associated with C9:0, C11:0, C12:1 *cis*-9, C13:0 and the ω6/ω3 index, while fat percentage was not affected by the identified SNPs. In **Chapter 6**, the molecular characterization of the coding regions and the partial UTRs of the *LIPG* gene of a purebred population of Chios ewes in Cyprus was undertaken. Seventeen SNPs were identified in total with three of the SNPs located in exonic regions encoded non-synonymous mutations (g134C>A: Pro→Thr, g1181G>A: Glu→Gly, g2639G>T: Val→Gly) and six of the SNPs were located in exon 10 (3' UTR), while the rest of the mutations were synonymous. Preliminary association analysis of the identified haplotypes with milk traits suggested no association with milk fat percentage, protein percentage or milk FA profile traits.

Finally, the main findings of the present work regarding both the nutritional treatments tested and the genetic polymorphisms investigated in Chios sheep breed are discussed in the final chapter of the thesis (**Chapter 7**). OCS supplementation in sheep feeding can be used as a useful tool for improvement of milk and Halloumi cheese FA content, while the identified polymorphisms in *ACAA2* and *FASN* genes could be incorporated in SNP chips used for genomic selection aiming at improving milk production traits and the milk FA content.

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# CHAPTER 1

# General Introduction

### 1.1. Small ruminant production in Cyprus

For several years now, in a rapidly growing world, there is a trend towards natural and healthy food in developed countries. Due to the rising health consciousness, part of the attention focuses on milk and dairy products driven by the scientific studies of the last decades, presenting the beneficial effects of milk on human health. According to the GlobeNewswire (2019), due to this trend, the growing population, and the income increase, it is projected that the global dairy products market is expected to increase at a compound annual growth rate of 5.2% from 2019 to reach $645.8 billion by 2025, while according to the FAOSTAT (2018), the sheep milk produced is expected to raise approximately 26%, from 10.4 Mt to 13.1 Mt by 2030.

Small ruminants comprise a significant and profitable industry in the Mediterranean region, where the production of high-quality cheese from local dairy breeds directs sheep and goat rearing (Carta et al., 2009). On the island of Cyprus and according to the most recent statistics available in 2018, there are around 400.000 sheep and goats (**Table 1.1**) (Ministry of Agriculture, Rural Development and Environment of Cyprus, 2011 and 2018). Although, the small ruminant population in Cyprus increased by 12.22% from 412.812 to 470.260 animals between 2008 and 2011, financial crisis affected the small ruminant industry. As a result, the sheep and goat population dramatically dropped by 21.3% from 2011 to 2015, decreasing from 470.260 to 370.106 animals, respectively. The following few years until 2018, however, due to the economy's rebound, the small ruminant population gradually reached 402.210 animals (7.98% increase) (**Table 1.1**). During these years, milk production mainly used for Halloumi

**Table 1.1.** The small ruminant population in Cyprus and milk production from 2008 to 2018.

| Overview year | 2008 | 2009 | 2010 | 2011 | 2012 | 2013 | 2014 | 2015 | 2016 | 2017 | 2018 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Number of animals | | | | | |
| Sheep | 190.588 | 207.129 | 234.954 | 255.540 | 249.628 | 226.067 | 213.423 | 216.381 | 219.218 | 228.834 | 229.120 |
| Goat | 222.224 | 221.336 | 217.860 | 214.720 | 195.830 | 176.302 | 159.231 | 153.725 | 161.476 | 172.674 | 173.090 |
| Total | 412.812 | 428.465 | 452.814 | 470.260 | 445.458 | 402.369 | 372.654 | 370.106 | 380.694 | 401.508 | 402.210 |
| | | | | | | Milk production (thousand Liters) | | | | | |
| Sheep | 21.340 | 22.090 | 23.035 | 26.037 | 24.477 | 21.077 | 23.553 | 25.853 | 27.477 | 31.353 | 33.150 |
| Goat | 30.382 | 28.746 | 26.867 | 27.012 | 25.856 | 20.238 | 22.218 | 22.937 | 24.146 | 29.472 | 31.472 |
| Total | 51.722 | 50.836 | 49.902 | 53.049 | 50.333 | 41.315 | 45.771 | 48.790 | 51.624 | 60.825 | 64.622 |

cheesemaking has increased by 19.96% from the year 2008 to 2018 (**Table 1.1:** 51.722 liters in 2008 to 64.622 liters in 2018) (Ministry of Agriculture, Rural Development and Environment of Cyprus, 2013 and 2018). Despite the somehow lower population of sheep and goat in 2018 compared to 2008 (**Table 1.1**), the increased milk output by a larger number of sheep compared to goats suggests that sheep production becomes more professional in Cyprus. Sheep production is expected to become more intensive and professional during the coming years due to the increased demand for Halloumi cheese. Regarding the Halloumi cheese production, from the year 2008 to 2018, there is an increasing trend in the sale of milk from farmers to the dairy industries and cheese factories, which is rarely used as drinking milk. At the same time, the milk kept from the farmers for own production of Halloumi cheese and local disposal decreases (**Table 1.2**).

### 1.2. The Chios sheep breed in Cyprus

Chios sheep is the most critical commercial breed in Cyprus it originates from the Chios island and comprises the most known breed in Greece, Cyprus and abroad due to the high potential it exhibits in milk production, prolificacy, early sexual maturity and adaptability to a vast range of climate changes (Katsaounis, 1996; Rogdakis, 2002; Chatziminaoglou, 2006; Zygoyiannis, 2006).

Chios sheep were imported in Cyprus approximately 70 years ago (Papachristoforou et al., 2000). During 2018, purebred Chios sheep accounted for 23.37% (53.535 animals) of the total sheep population and the cross-bred animals with Chios sheep consisted another 73.5% (168.394 animals) of the sheep population (**Table 1.3**) (Ministry of Agriculture, Rural

**Table 1.2.** Production and distribution of sheep milk in Cyprus

| | 2008 | 2013 | 2014 | 2015 | 2016 | 2017 | 2018 |
|---|---|---|---|---|---|---|---|
| Total milk produced (lt) | 51.721.600 | 41.315.711 | 45.771.374 | 48.789.684 | 51.623.790 | 60.824.783 | 64.622.239 |
| Sold in dairy industries and cheese factories (lt) | 41.329.073 | 34.928.888 | 40.467.994 | 43.530.041 | 47.007.542 | 55.808.038 | 60.352.389 |
| % of milk sold in dairy industries and cheese factories | 79.91% | 84.54% | 88.4% | 89.22% | 91.06% | 91.75% | 93.39% |
| Milk kept from farmers (lt) | 10.392.527 | 6.386.823 | 5.303.380 | 5.249.643 | 4.616.247 | 5.016.744 | 4.269.850 |
| % of milk kept from farmers | 20.09% | 15.46% | 11.59% | 10.76% | 8.94% | 8.25% | 6.61% |
| Milk used by farmers for Halloumi cheesemaking (lt) | 9.131.703 | 4.969.598 | 4.241.321 | 3.442.772 | 3.526.133 | 3.744.087 | 2.761.676 |
| Halloumi cheese produced by farmers (kg) | 1.074.318 | 741.731 | 633.033 | 513.847 | 526.289 | 558.819 | 412.191 |

**Table 1.3.** Total sheep number of the main sheep breeds in Cyprus (2013-2018)

| Overview year | 2013 | 2014 | 2015 | 2016 | 2017 | 2018 |
|---|---|---|---|---|---|---|
| Sheep Breeds | Sheep number | | | | | |
| Chios | 85.841 | 76.194 | 69.859 | 68.289 | 63.566 | 53.535 |
| Cross-bred with Chios | 131.261 | 130.394 | 135.588 | 144.153 | 157.497 | 168.394 |
| Cyprus Fat-tailed | 1.757 | 1.581 | 1.303 | 1.404 | 1.657 | 1.847 |
| Assaf | 1.141 | 934 | 1.337 | 2.277 | 2.796 | 2.221 |
| East Friesian | 1.577 | 224 | 236 | 313 | 2.696 | 2.392 |
| Awassi | 3.233 | 67 | 374 | 663 | 622 | 731 |

Development and Environment of Cyprus, 2014). Therefore, a total of approximately 97% of the sheep population in Cyprus are either purebred Chios sheep or crosses with Chios sheep.

Chios sheep are characterized by their burly size and the black spots on ears, nose, belly, legs, and around the eyes (**Figure 1.1**). Their conical head and the relatively long face with large

**Figure 1.1.** Chios ewe with her lambkins on left side and Chios ram on right sight

and semi-hanged ears are also characteristic of the Chios breed. Ewes can be hornless or may have small and knob-like horns, while rams have large spiral horns. Their distinctive tail classifies Chios breed as a semi-fat tailed breed with a cone-shaped tail of 9-12 cm width and 24-27 cm length (Rogdakis, 2002; Chatziminaoglou, 2006). The specific tail conformation was considered by some authors to be a disadvantage due to the difficulties it caused during mating and milking (Katsaounis, 1996; Chatziminaoglou, 2006; Zygoyiannis, 2006), although this is not confirmed by the reports from Cyprus (Mavrogenis et al., 1988; Hadjipavlou, 2011; Papachistoforou and Tzamaloukas, 2011). According to body measurements, acromial height and body weight ranges between 70-76 cm and 50-70 kg for ewes and 79-84 cm and 65-90 kg for rams, respectively (Chatziminaoglou, 2006), while thorax perimeter is approximately 95 cm

with a deep (33 cm) and narrow (17 cm) chest (Rogdakis, 2002). The weight of singletons and each twin at birth is 4,5 kg and 4 kg, respectively, and at the age of 18 weeks, they reach 18 and 16 kg, respectively (Katsaounis, 1996; Zygoyiannis, 2006). Wool quality is poor, and small teats appear on a well-developed hanging udder, which exhibits great diversity on size and shape (Katsaounis, 1996; Rogdakis, 2002; Zygoyiannis, 2006). Chios ewes appear to have early sexual maturity at the age of 7-12 months affected by the season of ewe birth (Papachristoforou, 2000; Chatziminaoglou, 2006).

According to Chatziminaoglou (2006) and Zygoyiannis (2006), in Greece, oestrus period of mature ewes, compared to the short anoestrus period (between December and March), is up to 9 months (270 ± 8 days) per year with the duration of the oestrus cycle being around 17 days. However, according to Papachristoforou et al. (2000), who studied extensively this breed in Cyprus, the reproductive cycle for multiparous Chios sheep covers most of the year, including spring and summertime, and has a long reproductive season with some animals cycling throughout the year. Based on the age and rearing system of the ewe, prolificacy has a mean of 2.03 with a prolificacy rate of 1.79, 2.09, 2.27, and 2.26 at the $1^{st}$, $2^{nd}$, $3^{rd}$ and $4^{th}$ lactation periods, respectively (Chatziminaoglou, 2006), with a quite high percentage of triplet births (Zygoyiannis, 2006; Katsaounis, 1996), and the average value of prolificacy reported in Cyprus to be 2.1 offspring per birth (Hadjipavlou, 2011).

Milk production mainly depends on the genetic background, management and rearing system followed. Genetic selection carried out at the Agricultural Research Institute of Cyprus for the last 35 years has improved the milk production of the nucleus flock (Hadjipavlou, 2011)

reporting today a total milk production for yearlings and adult ewes of 362 and 460kg per lactation lasted 214 and 263 days, respectively. In addition, milk protein content is reported to be 4.85 and 5.29%, and milk fat content 5.46 and 6.04% for yearling and adult ewes, respectively (reviewed by Agricultural Research Institute of Cyprus, 2020). According to Zygoyiannis (2006) in Greece, milk production of Chios sheep reached 275 kg in a milking period of 190 days, with an average fat content of 5.0-5.5%. In another study, Chatziminaoglou (2006) mentioned that the lactation period of the Chios sheep breed in commercial farms of Greece lasted on average for 186 days with milk output of 188 kg in the population of Chios sheep studied. In particular, during the $1^{st}$, $2^{nd}$, $3^{rd}$ and, $4^{th}$ lactation, milk production was 168, 204, 211, and 187 kg, respectively. Furthermore, at the Animal Breeding Centre of Diavata (1998-2000) in Greece, milk production reached 222 kg in 171 days.

### 1.3. Lactation anatomy and milk composition

Milk is the white fluid produced and secreted from the mammary gland after parturition because of hormonal changes, while lactation comprises a unique mammalian physiological process. Milk is capable of supporting the newborns' nutritional demands (McManaman, 2012). The udder is an exocrine epithelial gland, which in small ruminants consists of two mammary glands (de Almeida and Eckersall, 2018). The glands are composed of ductal networks with lobuloalveolar units, which terminate to the nipple. The alveoli are formed by a single layer of secretory epithelial cells, which are surrounded by myoepithelial cells, and after their contraction, they cause milk secretion into the lumen (**Figure 1.2**) (McManaman and Neville, 2003; McManaman et al., 2006; Gjorevski and Nelson, 2011; Lérias et al., 2014). Ruminants,

compared to rodents, humans, and other primates, have terminal ductal units instead of terminal end buds (Paine and Lewis, 2017). During puberty the terminal ductal units remind of multi-lobular terminal end buds due to the elongation and branching of the ducts (Paine and Lewis, 2017). The stimuli of suckling triggers the release of two hormones, prolactin, and oxytocin, with the first acting on alveolar epithelial cells causing the synthesis and excretion of milk components and the second acting on myoepithelial cells stimulating their contraction (Tsingotjidou and Papadopoulos 2008; Lérias et al., 2014; Lacasse and Ollier, 2015).

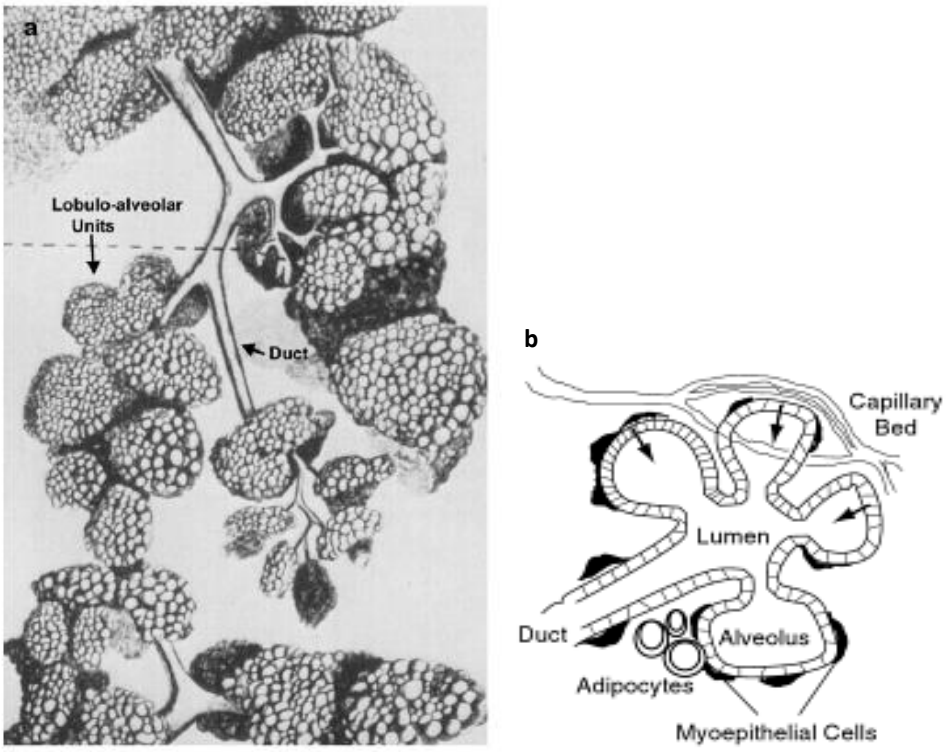


**Figure 1.2.** (a) Anatomy of a human lactating mammary gland (adopted from McManaman et al., 2006) and (b) diagram of lobuloalveolar unit and duct (adopted from McManaman and Neville, 2003).

The principal constituent of milk is water, while the other constituents are composed of major macronutrients (carbohydrates, proteins, and fat), minor nutrients (vitamins, minerals), immunoglobulins, enzymes and growth factors providing the newborn, besides energy, with all the necessary for development (Pulina and Nudda, 2004).

**Table 1.4.** Average composition of nutrients in the milk of different ruminant species and human.

| | ewe | Goat | Cow | Woman |
|---|---|---|---|---|
| Water (%) | 82.5 | 87.0 | 87.5 | 87.5 |
| Total solids (%) | 17.5 | 13.0 | 12.5 | 12.5 |
| Fat (%) | 6.5 | 3.5 | 3.5 | 4.4 |
| Fat globule (µm) | 4.0 | 3.9 | 4.4 | - |
| TN* (%) | 5.5 | 3.5 | 3.2 | 1.1 |
| Casein (%) | 4.5 | 2.8 | 2.6 | 0.4 |
| Serum protein (%) | 1.0 | 0.7 | 0.6 | 0.7 |
| Lactose (%) | 4.8 | 4.8 | 4.7 | 6.9 |
| Minerals (%) | 0.92 | 0.80 | 0.72 | 0.30 |
| Ca (mg/l) | 193 | 134 | 119 | 32 |
| Energy (kcal/l) | 1050 | 650 | 700 | 690 |
| Density | 1.037 | 1.032 | 1.032 | 1.015 |
| Acidity (*SH) | 8.5 | 8.0 | 7.1 | - |
| Ph | 6.65 | 6.60 | 6.50 | 6.85 |
| Freezing point (°C) | 0.580 | -0.570 | -0.524 | - |

*TN: total nitrogen(Nx6.38). Adapted from Pulina and Nudda (2004)

According to the reviews of Park et al. (2007), Pulina and Nudda (2004), and Wendorff and Haenlein (2017), sheep comprises the ruminant that compared to cow and goat, exhibits by far the higher composition in total solids and major nutrient components such as the fat and

protein percentage in milk (**Table 1.4**). Consequently, this makes sheep milk more desirable for cheese making since fat and protein content affect cheese yield (Pulina and Nudda, 2004; Wendorff and Haenlein, 2017).

### 1.4. Milk fat composition, dairy and health implications

Milk lipids are the essential caloric intake provided to neonates of most species. Additionally, from the dairy industries' perspective, ovine milk lipids are the most critical constituents in terms of textural and sensory characteristics of dairy products and largely affect cheese yield (Pulina and Nudda, 2004; Lamichhane et al., 2017). For that reason, a primary factor determining the milk price paid to the farmers is the concentration of fat.

Triglycerides or triacylglycerols (**TAG**) are neutral lipids and comprise the main components of milk lipids, accounting for more than 98% of the total lipids (Bernard et al., 2018; Gómez-Cortés et al., 2018). The complexity of TAG composition imputes to more than 400 fatty acids (**FAs**) esterified at the sn-1, sn-2, and sn-3 position of the glycerol backbone (Gómez-Cortés et al., 2018). In addition to TAG, milk lipids contain a small amount of 1,2-diacylglycerols (**1,2-DAG**), monoacylglycerols (**MAG**) and other complex lipids such as phospholipids, cholesterol esters, cholesterol and free fatty acids (Jensen, 2002; Park et al., 2007; Shingfield et al., 2010; Bernard et al., 2018; Moatsou and Sakkas, 2019).

In ovine milk fat, five FAs, which include C10:0, C14:0, C16:0, C18:0, and C18:1, comprise approximately 75% of milk FAs (**Table 1.5**). Short and medium-chain saturated FAs (**SFAs**: C6:0, C8:0, C10:0, and C12:0) are presented at significantly higher levels in ovine milk compared to cow milk, adding to the organoleptic characteristics of cheese (Goudjil et al., 2004; Haenlein

and Wendorff, 2006; Park et al., 2007; Shingfield et al., 2010; Moatsou and Sakkas, 2019). For instance, two of the main FAs of milk, C16:0 and C18:1, which exhibit high and low melting points, respectively, result in the production of creamy and less stable cheese when the C18:1 to C16:0 ratio is high (Lamichhane et al., 2017). The branch-chain FAs (**BCFAs**) *iso*-C14, *iso*- and *anteiso*-C15, *iso*- and *anteiso*-C17, and *iso*- C16 represent 2% of the total fat in ovine milk (Goudjil et al., 2004; Park et al., 2007; Gómez-Cortés et al., 2018). *Trans* FAs (**TFAs**) in ovine milk fat range between 2.5% to 5%, while the majority of them in ovine, caprine and bovine are the monoene TFAs (Park et al., 2007).

**Table 1.5.** Mean values of main fatty acids (% in total fatty acid methyl esters) in sheep, cow and goat milk fat.

| Fatty acid | Sheep | Cow | Goat |
|---|---|---|---|
| C4:0 | 3.5 | 3.9 | 2.2 |
| C6:0 | 2.9 | 2.5 | 2.4 |
| C8:0 | 2.6 | 1.5 | 2.7 |
| C10:0 | 7.8 | 3.2 | 10.0 |
| C12:0 | 4.4 | 3.6 | 5.0 |
| C14:0 | 10.4 | 11.1 | 9.8 |
| C15:0 | 1.0 | 1.2 | 0.7 |
| C16:0 | 25.9 | 27.9 | 28.2 |
| C17:0 | 0.6 | 0.6 | 0.7 |
| C18:0 | 9.6 | 12.2 | 8.9 |
| C18:1 cis | 18.2 | 17.2 | 19.3 |
| C18:1 trans | 2.9 | 3.9 | 2.1 |
| C18:2 n-6 | 2.3 | 1.4 | 3.2 |
| C18:3 n-3 | 0.8 | 1.0 | 0.4 |

Adapted from Gómez-Cortés et al. (2018)

During the last half of the last century and since the beginning of the 21st century, studies on milk and dairy products have been demonizing milk fat for its association with coronary heart disease (**CHD**) and the incidence of cancer (Bauman and Lock, 2012; Armas et al., 2016). Nevertheless, milk consumption contributes to blood pressure drop, and there is strong evidence that it contributes to weight loss, and reduction in the occurrence of metabolic syndrome (Parodi, 2004, 2006), while specific milk FAs exhibit anti-atherogenicity and anti-carcinogenic properties (Hamer et al., 2008; Jenkins et al., 2015; Kim et al., 2016; Gómez-Cortés et al., 2018). In addition, although epidemiological studies show the implication of fat intake from dairy products on the incidence of tumorigenesis, the results may be due to the total energy intake (Howe et al., 1997; Willett, 1997; Kolonel, 2001; Missmer et al., 2002; Parodi, 2006).

Dairy products have a high content in SFAs and are known to elevate the levels of low-density lipoprotein (**LDL**) cholesterol in plasma, when consumed in excess. As a result, the public perception is influenced since LDL is positively associated with CHD risk (Parodi, 2006; Bauman and Lock, 2012). This connection led to the reduction of SFAs intake and, consequently, of dairy products, leaving aside the fact that specific SFAs elevate the levels of high-density lipoprotein (**HDL**) cholesterol, which is negatively associated with the CHD risk (Parodi, 2006; Bauman and Lock, 2012). Various studies show that specific SFAs in isolation from milk elevate cholesterol levels to a different extent than others (Zock et al., 1994; Mensink et al., 2003; Hunter et al., 2010). Additionally, epidemiological studies did not provide evidence indicating that SFA consumption increased the levels of CHD risk factors (Parodi, 2009; Siri-Tarino et al., 2010; de Oliveira Otto et al., 2012). Furthermore, clinical trials have shown no

improvement in total, or CHD mortality when polyunsaturated oils substituted saturated fat in diets and cholesterol decreased (Ravnskov, 1998; Parodi, 2004, 2006).

Fat from dairy products constitutes the only source of specific FAs with beneficial effect on human health. For instance, butyric acid (C4:0), which belongs in the group of the short-chain SFAs (**SCSFA**), comprises the primordial energy source for intestinal epithelial cells (reviewed by Hamer et al., 2008). At the same time, butyric acid involves in the maintenance of colonic homeostasis and health. Thus, the colonic mucosa secretes mucins, which preserve its first line of defense, a barrier against pathogenic bacteria (reviewed by Gómez-Cortés et al., 2018). *In vitro* studies with human cell lines showed that butyric acid upregulates the gene expression for mucin production. It also exhibits an anti-cancer role as it inhibits the growth of various cancer cells, especially those of colon, through inhibiting histone deacetylases, reducing inflammatory processes, and inducing cell apoptosis (reviewed by Hamer et al., 2008; Gómez-Cortés et al., 2018). Likewise, the dietary medium-chain SFAs (**MCSFA**) have a positive relationship with healthy body weight management. MCFA exhibit beneficial effects on weight control and lipid metabolism and demonstrate an association with suppression of fat deposition (Nagao and Yanagita, 2010; Mumme and Stonehouse, 2015; Gómez-Cortés et al., 2018).

Concerning the dietary long-chain SFAs (**LCSFA**: C14:0 – C18:0), they are stored as body fat, depending on the carbohydrates' amount obtained from the diet. However, myristic (C14:0) and palmitic acids (C16:0) are involved in post-translational changes which control metabolic processes in the human body (reviewed by Ruiz-Núñez et al., 2016; Gómez-Cortés et al., 2018). Palmitic acid is involved in side-chain palmitoylation and facilitates protein-membrane

interactions, the intracellular movement of proteins, and the involvement in a variety of signal transduction pathways. Myristic acid is involved in N-terminal myristoylation. It includes critical components in intracellular signaling pathways, oncogenes, structural viral proteins, and common constitutive eukaryotic proteins (reviewed by Ruiz-Núñez et al., 2016; Gómez-Cortés et al., 2018). Furthermore, dairy myristic acid enhances the levels of long-chain omega-3 FA in plasma phospholipids and exerts beneficial effects by increasing HDL cholesterol and decreasing the levels of TAG, without changes in LDL cholesterol (reviewed by Gómez-Cortés et al., 2018). As far as stearic acid (C18:0) is concerned, its effects are related to oleic acid (C18:1 *cis*-9), which is an anti-atherogenic agent (reviewed by Gómez-Cortés et al., 2018).

Additionally to SCSFA, MCSFA, and LCSFA, the BCSFA, mainly detected in dairy fat, are essential bioactive components in the gut and demonstrate positive effects in chronic diseases (reviewed by Gómez-Cortés et al., 2018). BCSFA comprise 30% of the FAs in vernix covering the human fetuses, a protective waxy substance, which also suspends in the amniotic fluid and normally swallowed from them. As a result, the BCSFA end up in fetuses' gastrointestinal tract and promote the gut's colonization with specific microorganisms. Studies with neonatal rats show a reduction in the incidence of necrotizing enterocolitis when substituting dietary fat with BCSFA (Ran-Ressler et al., 2008; Gómez-Cortés et al., 2018). Moreover, in vitro studies show that BCSFA induces apoptosis in human breast cancer cells. Another SFA group which was used as a biomarker of dairy fat intake is the odd-chain SFAs (**OCSFA**) obtained from dairy fat (reviewed by Jenkins et al., 2015; Gómez-Cortés et al., 2018). The sum of the most abundant OCSFA (C15:0 and C17:0) represents 1.5% of total fat, and it was recently suggested that they are synthesized endogenously via α-oxidation. The concentration of the two most abundant

OCSFA, C15:0, and C17:0, from plasma phospholipids, has been inversely associated with CHD and a type 2 diabetes incidence (reviewed by Jenkins et al., 2015; Gómez-Cortés et al., 2018).

Apart from SFA, TFAs and CHD are positively associated as TFAs affect the levels of total, LDL and HDL-cholesterol in plasma by increasing the first two and reducing the last one, while other CHD risk factors are also affected against human health (reviewed by Park et al., 2007; Ferlay et al., 2017). However, studies conducted on TFA have shown that not all of the FAs in the specific group have the same effects (reviewed by Ruiz-Núñez et al., 2016; Gómez-Cortés et al., 2018). TFA from partially hydrogenated vegetable oils and animal fat have demonstrated that only partially hydrogenate vegetable oil was positively associated with CHD (Ascherio et al., 1994; Bolton-Smith et al., 1996; Pietinen et al., 1997; Parodi, 2006). The consumption of vegetable fats can lead to their partial hydrogenation mainly to monounsaturated *T*FAs, e.g., C18:1 *trans*-9 and C18:1 *trans*-10, compared to the main TFA in milk fat, the C18:1 *trans*-11 (**VA**; vaccenic acid), (reviewed by Parodi, 2006; Park et al., 2007; Gómez-Cortés et al., 2018). VA accounts for 45-60% of the total TFA in milk fat (in ovine, caprine and bovine) (Jensen, 2002; Goudjil et al., 2004; Park et al., 2007), and is the precursor of a conjugated linoleic acid (**CLA**), the isomer C18:2 *cis*-9, *trans*-11 (**RA**; rumenic acid). The introduction of a *cis*-double at the 9th carbon of VA by the action of the enzyme Δ9-desaturase leads to RA formation. Since elaidic acid (C18:1 *trans*-9) and C18:1 *trans*-10 only account for approximately 5% and 10%, respectively, of the total TFAs in sheep milk fat, the intake of elaidic acid and C18:1 *trans*-10 from milk fat is very little compared to its consumption from hydrogenated vegetable oils (reviewed by Park et al., 2007; Gómez-Cortés et al., 2018). In countries where there is a lack in the use of partially hydrogenated oils, VA in plasma can comprise a biomarker of ruminant fat

intake (Guillocheau et al., 2019). The review of Gómez-Cortés et al. (2018) documents the potential beneficial effects of VA found in epidemiological, clinical, and rodent studies. Although, the very high levels of VA in the diet were asserted to have a similar positive association with the risk of CVD as the non-ruminant TFAs, studies carried out on rodent models associate VA with the regulation of the expression of genes controlling cell cycle, lipid oxidation and energy balance. There is also an association with the attenuation of ectopic lipid accumulation and improved insulin secretion. Additionally, despite the importance of VA due to its role as the precursor of RA, studies suggest its independent bioactivity as a direct anti-carcinogen on human mammary adenocarcinoma and its anti-inflammatory effect on human peripheral mononuclear cells (reviewed by Gómez-Cortés et al., 2018). These shreds of evidence suggest that the enrichment of dairy products with VA has positive effects with CVD, insulin resistance, or inflammation.

Two isomers from the CLA family (RA and C18:2 *trans*-10, *cis*-12) comprise another two FA belonging in the group of the TFAs, known as conjugated *trans*. The only difference between these CLA isomers from the above mentioned monounsaturated TFAs (e.g., C18:1 *trans*-9, 18:1 *trans*-10, and C18:1 *trans*-11) is that they are polyunsaturated FAs (**PUFA**). The CLA isomers are linoleic acids with the two double bonds in conjugation at different positions and geometry (*cis-cis*, *cis-trans*, *trans-cis*, and *trans-trans*) (reviewed by Jenkins et al., 2015; Kim et al., 2016). They are almost exclusively consumed through the high-fat dairy products and ruminant meats, as they are intermediate products of the biohydrogenation of linoleic acid to stearic acid by rumen bacteria. Furthermore, RA is also produced in the mammary gland (and other tissues) by the action of Δ9-desaturase on another intermediate biohydrogenation

product, the VA reported previously. RA and C18:2 *trans*-10, *cis*-12 are the two CLA isomers with the most biological effects identified, compared to the other isomers, while RA comprises at least 75% of the total CLA in dairy fat (reviewed by Park et al., 2007; Gómez-Cortés et al., 2018). Concerning the quantity of CLA isomers in ruminant milk, studies vary as they compose a negligible percentage and are affected by various factors (e.g., genetics and nutrition). According to the review of Park et al. (2007), sheep milk exhibits a percentage of 1.08% for total CLA average content, followed by cow (1.01%) and goat (0.65%), while according to the review of Gómez-Cortés et al. (2018), cow milk is shown to exhibit a higher CLA percentage compared to sheep (**Table 1.6**). CLA was firstly known for its anti-cancer activity, and thousands of studies have been followed after that discovery. With respect to the several contradictory studies and the adverse health effects CLA has demonstrated to exhibit (reviewed by Jenkins et al., 2015), much more studies display CLA's antiobesity, antidiabetic, anti-atherosclerotic and, anti-carcinogen effects and the ability to modulate the immune system (reviewed by Park, 2014; Jenkins et al., 2015; Kim et al., 2016; Gómez-Cortés et al., 2018). More specifically, the CLA isomer C18:2 *trans*-10, *cis*-12 has been related to catabolic processes (lipolysis and fat oxidation) opposed to the main CLA isomer C18:2 *cis*-9, *trans*-11 which is related to anabolic processes and confers anti-inflammatory effects as this particular isomer is implicated in the modulation of inflammatory molecules (Gómez-Cortés et al., 2018). Clinical studies show that CLA controls the obesity epidemic, which connects with heart diseases, infertility, and insulin resistance. CLA reduces the body weight and body fat mass and improves the lean body mass, body mass index, and waist measurement. Additionally to this, CLA intake assists with the same effects to exercising individuals and has its role in fat-mass regain as it lowers body fat regain.

However, the majority of the beneficial effects were described by animal studies opposed to the fewer human studies conferring weaker results. Moreover, the extent of the effect from each individual CLA isomer is less clear as only several studies were performed with individual CLA isomers.

Regarding the atherosclerosis and CVD, the CLA isomers are associated with reduced CVD risk as they exert hypotensive effects, they lower the TAG, total cholesterol, and, very-LDL-cholesterol (Gómez-Cortés et al., 2018). However, because reports are mentioning that CLA also elevates some circulatory markers associated with heart health, more research needs to be applied. Furthermore, concerning some studies dedicated to the effects of CLA consumption with cancer and as well as immune and inflammatory responses, they show the inverse correlation between breast and colorectal incidence, the reduction of allergic symptoms and the decreases of pro-inflammatory markers, respectively (reviewed by Park, 2014; Jenkins et al., 2015; Kim et al., 2016; Gómez-Cortés et al., 2018).

The FA profile and the concentration of specific FAs in milk associated with health effects change depending on the species, breed, genotype, season, and diet (Bencini and Pulina 1997; Barillet, 2007; De La Fuente et al., 2009). Therefore, the identification of diets and specific genes modifying the levels of FAs in milk involved directly or indirectly in the prevention of cardiovascular disease and cancer risk are expected to be beneficial to human health.

**Table 1.6.** Minimum and maximum contents (percentage of total fatty acids) of individual conjugated linoleic acid isomers in cow, goat and sheep milk.

| Isomer | Cow[1] | Goat[1] | Sheep[2] |
|---|---|---|---|
| **cis-cis** | | | |
| *cis*-9 *cis*-11 | NR | 0.001–0.002 | NR |
| **cis-trans** | | | |
| *cis*-9 *trans*-11 | 0.586–1.186 | 0.685–0.828 | 0.386–0.960 |
| *cis*-12 *trans*-14 | 0.001–0.006 | 0–0.002 | 0.001–0.001 |
| **trans-cis** | | | |
| *trans*-7 *cis*-9 | 0.030–0.054 | 0.028–0.040 | 0.026–0.040 |
| *trans*-8 *cis*-10 | NR | 0.009–0.019 | 0.020–0.030 |
| *trans*-9 *cis*-11 | 0.008–0.250 | 0.002–0.026 | 0.010–0.020 |
| *trans*-10 *cis*-12 | 0.004–0.070 | 0.001–0.008 | 0.001–0.010 |
| *trans*-11 *cis*-13 | 0.012–0.120 | 0.007–0.028 | 0.010–0.020 |
| *trans*-12 *cis*-14 | NR | 0.002–0.003 | NR |
| **trans-trans** | | | |
| *trans*-7 *trans*-9 | 0.002–0.007 | 0.003–0.007 | 0.008–0.009 |
| *trans*-8 *trans*-10 | 0.002–0.004 | 0.002–0.004 | 0.010–0.011 |
| *trans*-9 *trans*-11 | 0.009–0.012 | 0.013–0.021 | 0.009–0.011 |
| *trans*-10 *trans*-12 | 0.003–0.008 | 0.003–0.003 | 0.005–0.006 |
| *trans*-11 *trans*-13 | 0.015–0.160 | 0.003–0.009 | 0.010–0.030 |
| *trans*-12 *trans*-14 | 0.007–0.017 | 0.002–0.009 | 0.010–0.024 |

NR: not reported. Adopted from Gómez-Cortés et al. (2018).

### 1.5. Milk quantity and quality are primary breeding objectives in the dairy sheep industry

The increase of productivity especially in the Mediterranean countries is driven by the increasing consumers' demands for ovine milk used to produce yoghurt and cheese. Processing ovine milk results in a higher yield of dairy products due to the higher contents in fat, protein, and total solids, compared to goat and cow milk (reviewed by Bencini and Pulina, 1997). Therefore, from the farmers' perspective, there is a need for improving milk production and

composition, as the primary factors determining milk price and affecting cheese yield are fat and protein content of milk (De Rancourt et al., 2006; Ramon et al., 2010). Apart from the need to increase the amount of milk constituents to achieve higher yields of yoghurt and cheese, milk quality affects the textural and sensory characteristics of dairy sheep products, and is associated with human health, as was analyzed in the previous section (Lamichhane et al., 2017; Gómez-Cortés et al., 2018; Mohapatra et al., 2019; Moatsou and Sakkas, 2019). The parameters affecting milk traits could be generally categorized into physiological, management and genetic factors (Bencini and Pulina, 1997; De la Fuente et al., 2009; Silanikove et al., 2016).

### 1.5.1. *Physiological factors affecting milk traits*

The physiological factors affecting ovine milk yield and quality include age and parity, weight, number of lambs, stage of lactation, and health (reviewed by Bencini and Pulina, 1997). Older ewes produce more milk compared to their yearlings (Hatziminaoglou et al., 1990; Bencini and Pulina, 1997), while during the third and fourth lactation, milk yield reaches its maximum levels and then declines (Casoli et al., 1989; Bencini and Pulina, 1997). Concerning the effects of ewe parity, milk composition, milk fat, and protein concentration become higher as the lactation number increases. The lactation stage also affects milk production where daily yield increases rapidly during the first weeks after parturition, while on the third to the fifth week, daily yields achieve a peak and then decrease, having lactose concentration following the same trend. In parallel, at the beginning and the end of lactation, when milk yield is lower, the content of fat, protein, total solids, and somatic cells is higher compared to the concentration observed at the peak of lactation (Bencini and Pulina, 1997).

Regarding dairy ewes' liveweight, usually milk production increases the heavier the ewe is (Bencini and Pulina, 1997; Ploumi and Emmanouilidis, 1999; Dhaoui et al., 2019). In Sarda breed ewes, liveweight is positively associated with fat and protein concentration during the ten first weeks of lactation (Bencini and Pulina, 1997). Furthermore, despite the small number of contradictory reports, the more lambs suckle, the more milk is generated, as in the case of rearing triplets, which displays a higher production compared to rearing twins and singletons (Bencini and Pulina, 1997; Rosales Nieto et al., 2018).

De La Fuente et al. (2009) demonstrates the variations in FA composition by examining the following factors in dairy ewes: flock, day of testing within the flock (**TD**), age, lactation stage, and season. Flock explained a 3-30% of the total variance, and TD explained 60.7% and 68.2% of the variation for specific FAs such as RA and linolenic acid (*cis*-9, *cis*-12, *cis*-15 18:3), respectively. The effects of the season, a variation factor linked to feeding was great for specific FAs (Sevi et al., 2004; De La Fuente et al., 2009). Thus, PUFA had the highest values during spring and summer, compared to winter, while the contents of CLA and linolenic acid were 44% and 30% higher during spring-summer, compared to winter. Throughout lactation, CLA and CLA/ 18:1 *trans*-11 $\Delta^9$-desaturase index increased, while ewes aging decreased the content of monounsaturated FAs (**MUFA**) and PUFA, and increased short and MCSFAs (De La Fuente et al., 2009).

### 1.5.2. *Management factors affecting milk traits*

This section summarizes the main features comprising the management factors influencing the quality and quantity of ovine milk. Milking techniques, intervals between milking, frequency of

milking, and the stripping method fall into the group of management factors that affect the quantity and quality of sheep milk (reviewed by Bencini and Pulina, 1997). Hand or machine milked ewes have no differences in the concentration of milk protein or fat (Bencini and Pulina, 1997; Sinapis, 2007), while through machine milking, ovine milk has lower microbial load, compared to hand milking (reviewed by Bencini and Pulina, 1997). Regarding the effects of the hand or machine milking on milk yield and somatic cell scores (**SCS**), the study of Sinapis (2007) has shown that higher milk yield and lower SCS was obtained from machine milked ewes, compared to the milked by hand. The daily milk output and total lactation yield increased by increasing milking frequency and the application of stripping methods removing the additional milk from the gland, also enhances milk production (reviewed by Bencini and Pulina, 1997).

Among all management factors, nutrition comprises the most crucial factor affecting milk yield and composition and therefore affecting both the cheese quantity and quality produced thereafter (Bocquier and Caja, 2001). It is well known that the energy and protein content of the ration must satisfy the animals' needs for maintenance and milk production (reviewed by Bencini and Pulina, 1997; Nudda et al., 2014), for example it has been estimated that 1.7 Mcal of metabolizable energy is necessary to cover a 7% fat content for each liter of milk produced by Sarda ewes (reviewed by Bencini and Pulina, 1997). During late pregnancy in ewes, diet supplementation results in higher milk production, while underfeeding causes a reduction in the udder development and reduces the prenatal and postnatal accumulation of colostrum (Bencini and Pulina, 1997; Charismiadou et al., 2000; Freitas-de-Melo et al., 2018). Also, early lactation underfeeding deflowers milk secretion, while the diet quality also affects milk yield (Bizelis et al., 2000). Thus, it has been shown for example that the fibre concentration has a

positive correlation with milk fat content and diets low in fibre and high in carbohydrates can cause lower milk yield and lower milk fat percentage (reviewed by Bencini and Pulina, 1997; Pulina et al., 2006; Nudda et al., 2014).

Furthermore, the addition of protected fats in the diet raises milk fat concentration and reduces milk protein, whereas protein deficient diets reduce milk protein content (reviewed by Bencini and Pulina, 1997; Pulina et al., 2006). Besides, diet comprises a critical factor controlling the FAs composition of sheep milk, as thoroughly analyzed in the forthcoming section below. Underfeeding causes body fat mobilization and consequently an increase of the long-chain FA in milk (reviewed by Bencini and Pulina, 1997; Chilliard et al., 2000a; Pulina et al., 2006), and diets supplemented with protected fat can also raise long-chain FA in milk and especially the unsaturated FAs (**UFA**) (Pulina et al., 2006).

### 1.5.3. *Effect of diet on ewe milk fat composition*

Animal nutrition comprises the most crucial determinant controlling and altering the milk FAs composition (Shingfield et al., 2013; Nudda et al., 2014; Albenzio et al., 2016). For at least the last two decades, many studies focus their attention on lipid supplementation. Review studies of diets supplemented with lipids in sheep (Pulina et al., 2006; Sanz Sampelayo et al., 2007; Nudda et al., 2014; Manso et al., 2016), cows (Jensen, 2002; Zheng et al., 2005) and goats (Chilliard et al., 2003; Sanz Sampelayo et al., 2007), demonstrate the modifications of the trials to enrich milk with beneficial to human health FAs. Specifically, in sheep during at least the last fifteen years, appreciable efforts were made with various feeding strategies (e.g., wide range of vegetable oils and agro-industrial by-products) trying to examine the modulation of FAs in ewe

milk fat (Mele et al., 2005; Pulina et al., 2006; Park et al., 2007; Sanz Sampelayo et al., 2007; Gomez-Cortes et al., 2008a, 2008b; Bodas et al., 2010; Abbeddou et al., 2011a, 2011b; Mele et al., 2011; Altenhofer et al., 2014; Abbeddou et al., 2015; Castro-Carrera et al., 2015; Gomez-Cortes et al., 2015).

An attempt of modifying the milk FA composition towards the UFA content, mainly led by nutrition studies on dairy cows, is the incorporation of high UFA supplements in diets. Fats are supplements with high energy content and enrich milk with long-chain UFA that cannot be synthesized by animal tissues. All C18 and longer FA chains, some of them very essential for various metabolic processes, are preformed and result in the tissues and milk from blood circulation. When the UFAs from a supplement reach the rumen, they undergo a biohydrogenation process partly converting them into SFA (Ferlay et al., 2017). Thus, large part of C18 UFA (linoleic acid: C18:2 *cis*-9, *cis*-12 (ω-6), linolenic acid: C18:3 *cis*-9, *cis*-12, *cis*-15 (ω-3) and C18:3 *cis*-6, *cis*-9, *cis*-12 (ω-6)) undergo a conversion into a SFA, i.e. the stearic acid (C18:0), or other biohydrogenation products (**Figure 1.3**) (Ferlay et al., 2017). The administration of vegetable lipid sources (e.g., soybean, sunflower, linseed, olive, and rapeseed) as diet supplements is either in the form of whole seeds (raw and processed) or as oils (protected or unprotected) (reviewed by Mele et al., 2005; Mele and Banni, 2010). The use of lipid sources as supplements in a free-unprotected form (e.g., vegetable oils, oilseeds) results in the loss of the UFA content and especially the loss of the essential PUFA, therefore, the use of rumen-inert fats is recommended (Palmquist, 2006; Pulina et al., 2006). Rumen-inert fats (e.g., calcium soaps) by-pass the rumen, which allows the absorption of their UFA content in higher levels of

the digestive tract and the enrichment of milk and meat products (Palmquist, 2006; Pulina et al., 2006).

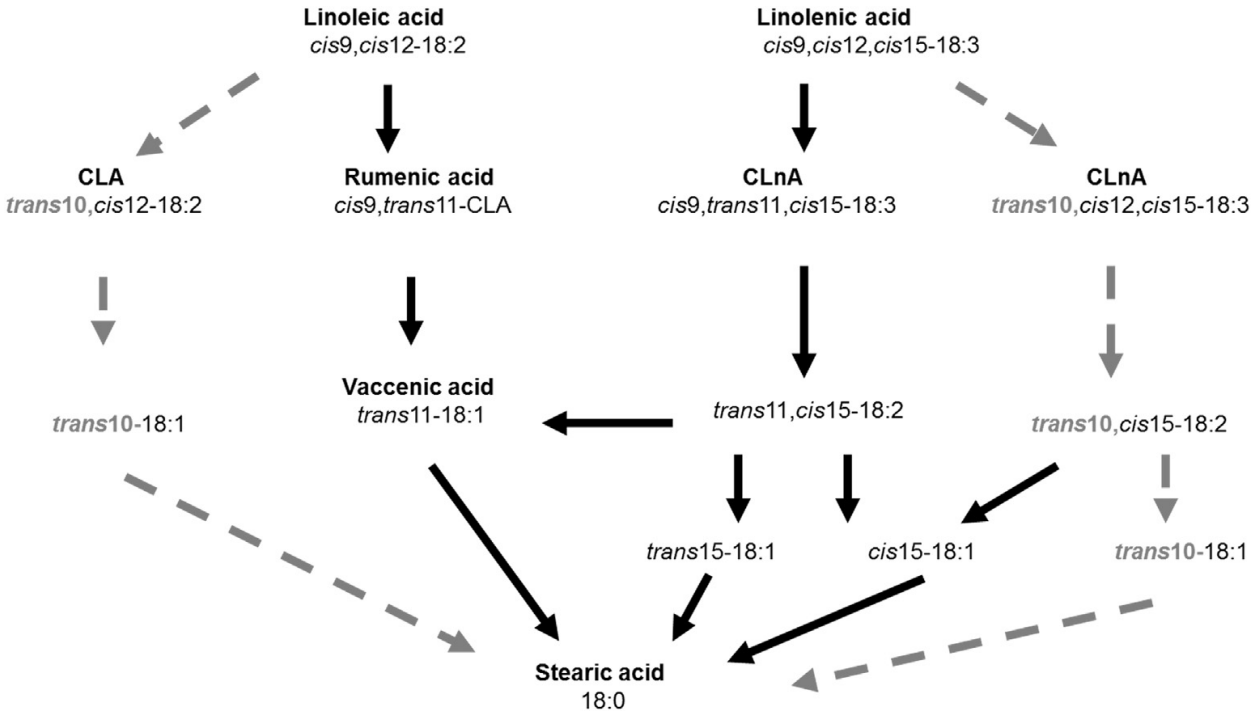


**Figure 1.3.** Schematic representation of the main ruminal biohydrogenation pathways of dietary PUFA (adopted by Ferlay et al., 2017)

Extensive nutritional studies in sheep supplementing with various substrates rich in PUFA demonstrate their effects on milk and cheese FA profile. Incorporation of sunflower seed and flax seed in diets lowers the content of milk in C16:0 and increases the content of C18:1, C18:2 and C18:3 (Zhang et al., 2006). Soybean oil, flax seed and sunflower seed supplements, enhance milk with VA and RA (Antongiovanni et al., 2004; Zhang et al., 2006). Fish oil feeds also increase the total CLA concentration in milk (Mozzon et al., 2002), while diets supplementing with protected marine oil, protected fish oil and algae enhance the milk content in eicosapentaenoic acid (**EPA**) and docosahexaenoic acid (**DHA**) (Mozzon et al., 2002; Papadopoulos et al., 2002; Kitessa et al., 2003).

Supplements rich in linoleic and linolenic acids show a positive linear relationship with the CLA content in ovine milk (**Figure 1.4**), where increasing the concentration of the supplement, the CLA content in milk increases (reviewed by Nudda et al., 2014). A few very common plant lipid supplements enhancing the levels of UFA and in particular the levels of CLA in milk are linseed (Luna et al., 2005; Gomez-Cortes et al., 2009; Bodas et al., 2010), soybeans (Gomez-Cortes et al., 2008a; Bodas et al., 2010), sunflower (Hervas et al., 2008; Luna et al., 2008; Castro et al., 2009; Gomez-Cortes et al., 2011; Maia et al., 2012; Castro-Carrera et al., 2015) and olive (Gomez-Cortes et al., 2008b; Bodas et al., 2010). All studies incorporating plant lipid supplements in sheep feeds, report the diminishment of the SFA content and increments of specific UFA and MUFA in milk fat according to the inclusion rate of the oil supplements. More specifically, **Figure 1.5** illustrates the positive effect of the administration of various fat sources as supplements in milk RA and other PUFA (Bodas et al., 2010; Manso et al., 2011; Gomez-Cortes et al., 2014; Gallardo et al., 2014). **Table 1.7** summarizes the effects of a few

comparative studies with various oil supplements in different concentrations on milk RA, VA, linoleic acid and a-linolenic acid. According to their dose, the most favorable unsaturated supplements enriching milk contents with RA, VA, C18:3 n-6 and C18:3 n-3 are linseed, soybeans, safflower and sunflower (reviewed by Nudda et al., 2014).

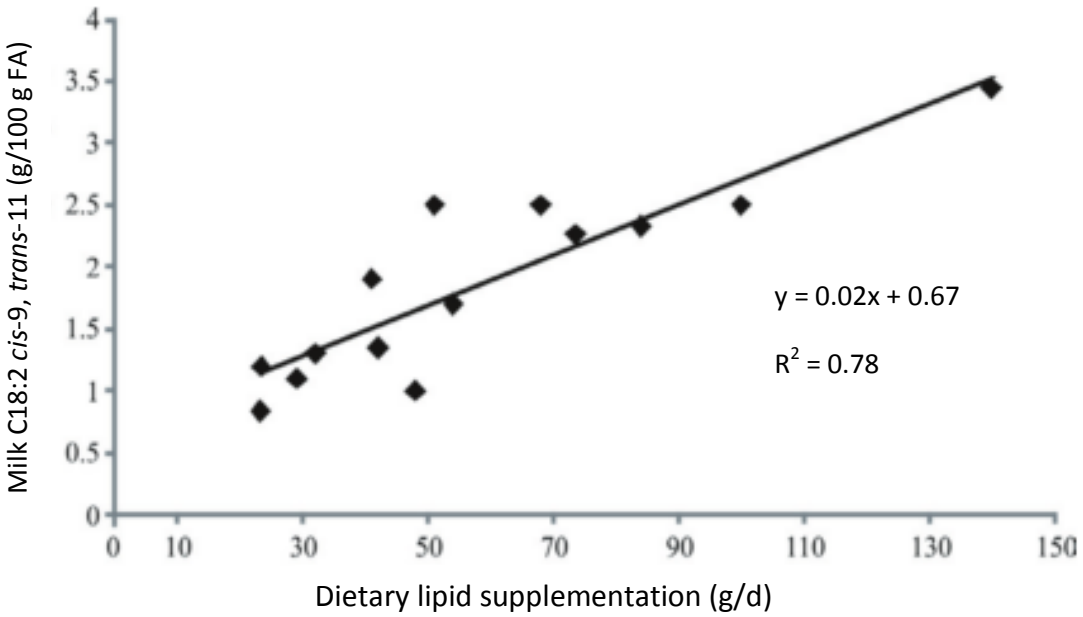


**Figure 1.4.** Relationship between fat supplementation and sheep milk CLA (C18:2 *cis*-9, *trans*-11) content according to data of different studies. Each point corresponds to a mean value of treatment (Adopted by Nudda et al., 2014).

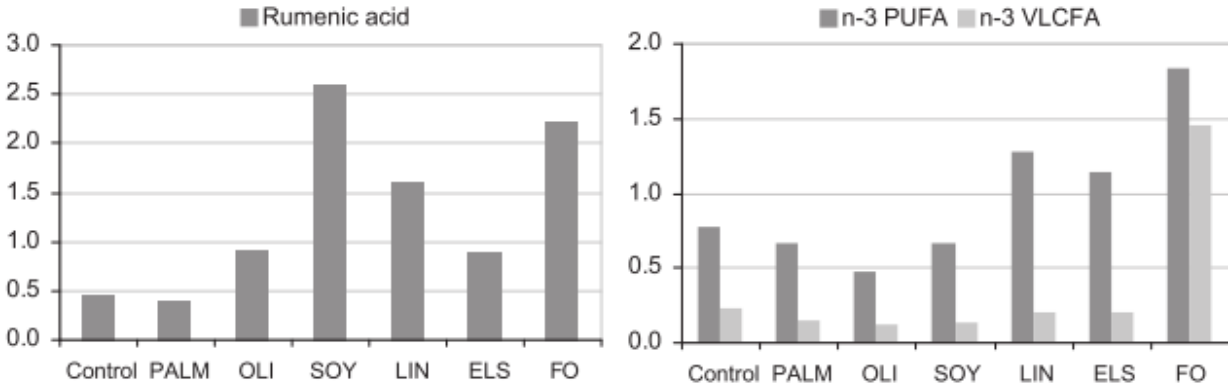


**Figure 1.5.** Comparative results on the percentage (% of total fatty acids) of rumenic acid (C18:2 *cis*-9, *trans*-11), omega-3 polyunsaturated fatty acids (n-3 PUFA) and very long chain omega-3 polyunsaturated fatty acids (n-3 VLCFA) from sheep milk under the administration of 3% of various fat sources as supplements (Control: without added fat; PALM: hydrogenated palm oil; OLI: olive oil; SOY: soybean oil; LIN: linseed oil; ELS: extruded linseed; FO: fish oil calcium soap) in ewe diets (Adopted by Manso et al., 2016).

**Table 1.7.** The effects of various lipid supplements depending the dose on C18:2 *cis*-9, *trans*-11, C18:1 *trans*-11, C18:2 *cis*-9, *cis*-12 (n-6) and C18:3 *cis*-9, *cis*-12, *cis*-15 (n-3) in sheep milk (g/100g of total fatty acids) (adapted by Nudda et al., 2014).

| Diet | Dose, g/d | c9,t11 CLA | C18:1 t11 | C18:2 n6 | C18:3 n3 | References |
|---|---|---|---|---|---|---|
| Alfalfa pellet | 0 | 0.17 | 2.04 | 3.43 | 1.05 | Reynolds et al. (2006) |
| + SO-MO | 162 | 0.27 | 6.61 | 3.85 | 0.99 | |
| Silage | 0 | 0.82 | 4.94 | 4.97 | 0.42 | |
| + SO-MO | 147 | 0.90 | 9.29 | 4.63 | 0.53 | |
| Control | 0 | 1.0 | 0.9 | 2.3 | 0.9 | Zhang et al. (2006a) |
| Linseed | 210 | 1.5 | 1.5 | 3.5 | 1.8 | |
| Sunflower seed | 182 | 2.3 | 1.5 | 4.1 | 1.4 | |
| Linseed oil | 23 | 1.2 | 3 | 2.3 | 1.6 | Zhang et al. (2006b) |
| | 32 | 1.3 | 3.8 | 2.5 | 1.7 | |
| | 41 | 1.9 | 4.2 | 2.5 | 2 | |
| Control | 0 | 0.55 | 0.88 | 2.71 | 0.32 | Castro et al. (2009) |
| Sunflower oil | 28 | 0.61 | 1.20 | 3.04 | 0.42 | |
| Palm oil | 63 | 0.39 | 0.78 | 1.99 | 0.52 | Bodas et al. (2010) |
| Olive oil | 63 | 0.91 | 2.08 | 1.51 | 0.36 | |
| Soybean oil | 63 | 2.58 | 6.52 | 3.17 | 0.53 | |
| Linseed oil | 63 | 1.59 | 4.27 | 1.76 | 1.07 | |
| Soybean oil | 0 | 0.83 | 1.88 | 2.49 | 0.38 | Gómez-Cortés et al. (2011b) |
| | 17 | 1.39 | 3.87 | 2.63 | 0.31 | |
| | 34 | 2.40 | 6.94 | 2.87 | 0.29 | |
| | 51 | 2.95 | 8.50 | 2.95 | 0.28 | |
| Control | 0 | 0.53 | 1.07 | 6.58 | 0.34 | Maia et al. (2011) |
| Canola oil | 3% | 1.29 | 3.45 | 6.91 | 0.30 | |
| Sunflower oil | 3% | 1.26 | 3.16 | 7.00 | 0.30 | |
| Castor oil | 3% | 0.54 | 1.10 | 7.68 | 0.31 | |
| Control | 70 | 0.37 | 0.85 | 2.40 | 0.31 | Gómez-Cortés et al. (2014) |
| Extruded linseed | 70 | 0.89 | 3.03 | 1.81 | 0.94 | |

SO - sunflower oil

However, attention has to be taken since the increasing concentrations of oils in diets negatively affects fibre digestion and acetic acid production and as a consequent the milk fat production (Manso et al., 2016). Therefore, a strategy to avoid the negative consequences is the inclusion of oil seeds as processed diet supplements. The administration of processed seed (extruded, heat treated, micronized or ground) instead of whole seeds has a better effect in the increase of VA and RA in milk, since the TAG fraction in the treated seeds subjects to a moderate accessibility in rumen bacteria for lipolysis and biohydrogenation and increases the duodenal flow of UFA intermediates (Doreau et al., 2009; Manso et al., 2016). Nevertheless, the utilization of processed or whole seeds defeats the obstacle of increased *trans*-10 FA levels in milk which is negatively associated with human health as they are negatively associated with CHD (Shingfield et al., 2008), compared to the administration of free oils (Manso et al., 2016).

1.5.3.1. *The effect of supplementing ewe diets with olive cake*

During the past ten years, an increase in the cost of feedstuff led farmers to the use of by-products as feed components, attracting scientists' interest for their suitability and health effects, not only to ruminants but also to humans. Crude olive cake (OC) is an agro-industrial by-product of oil extraction from olives and consists of a mixture of skins, pulp, woody endocarp, and seeds, comprising 35% of the oil extracted olives (Hadjipanayiotou 1999; Molina-Alcaide and Yáñez-Ruiz, 2008; Weinberg et al., 2008). In the Mediterranean countries, the crude OC is available in appreciable large quantities with high potential of environmental pollution and limited utilization in feedstuffs due to low digestibility, high polyphenol content, and seasonal availability. Earlier studies in Cyprus, (Hadjipanayiotou, 1999) were led to the

conclusion that ensiling crude OC comprises safe maintenance for a long-term period, with or without the use of any by-products or conventional feedstuffs for nutritional upgrading. Therefore, ensiled OC is easily utilizable from the farmers throughout the year with benefits to the environment. Weinberg et al. (2008) concluded that the addition of 3% of molasses in the ensiled OC improves the ensiling fermentation. At the same time, the ensiling procedure reduces the polyphenol content, improves its nutritional value, while the stone extraction increases digestibility by 5% (Weinberg et al., 2008). Consequently, OC, the by-product of olive oil production, represents an attractive forage replacement in ewes' diets that may improve the quantity and quality of ovine milk (reviewed by Molina-Alcaide and Yáñez-Ruiz, 2008).

However, the exploitation of OC attracted limited attention as a forage replacement. As a result, only a few studies demonstrate the effects of OC on milk yield and milk fat composition, especially as a supplement in ewe diets (reviewed by Molina-Alcaide and Yáñez-Ruiz, 2008) and more recently in dairy cows (Castellani et al., 2017; Neofytou et al., 2020) and goats (Molina-Alcaide et al., 2010). The feeding trials in dairy sheep include the use of ensiled OC alone (Hadjipanayiotou 1999; Cabiddu et al., 2004), partly destoned fresh OC (Chiofalo et al., 2004), partly destoned dried OC (Vargas-Bello-Perez et al., 2013), dried OC (Abbeddou et al., 2011a, 2011b, 2015), pelleted with extruded linseed and alfalfa hay (Mannelli et al., 2018). Also, the feeding trials include the use of ensiled OC by adding conventional feedstuffs (e.g., poultry litter and ground corn grain) (Hadjipanayiotou, 1994), poultry manure (Nefzaoui, 1991), alkali (Nefzaoui and Vanbelle, 1986) and urea (Al Jassim et al., 1997).

According to the study of Hadjipanayiotou (1999), the partial replacement of the conventional roughage with ensiled crude OC in dairy sheep caused no changes in milk yield, fat-corrected milk yield, or bodyweight, while the milk fat content was higher compared to the control. Cabiddu et al. (2004) demonstrates the loss of live-weight and drop of intake in ewes allocated in the treatment groups substituting hay grass with ensiled crude OC, however, with no differences in their performance. The study of Chiofalo and colleagues (2004), who administrated partially destoned fresh OC to lactating ewes, shows a higher milk yield, milk fat quantity (grams per day), milk MUFA and UFA/SFA ratio content, while the atherogenicity indices were reduced. The use of partly destoned dried OC in lactating ewes' diets by Vargas-Bello-Perez et al. (2013) did not affect milk yield or composition. The oleic acid, n-6/n-3 ratio, and MUFA content increased, and the SFA level and atherogenicity index decreased in milk and cheese (Vargas-Bello-Perez et al., 2013). Moreover, the studies of Abbeddou et al. (2011a, 2011b, 2015) supplementing ewe diets with dried OC also show an increase in UFA and MUFA in milk, yoghurt, and cheese. However, the levels of linoleic acid were lower than in the control milk (Abbeddou et al., 2011b, 2015), while the RA content in milk and the dairy products produced remained unchanged (Abbeddou et al., 2011a, 2011b, 2015).

The reviews of Molina-Alcaide and Yáñez-Ruiz (2008) and Vasta et al. (2008) are the latest studies reviewing the effects on milk quality by the use of olive by-products and alternative feed resources, respectively, in ruminants, including the use of OC. Until today, no studies are examining the milk FA profile derived from ewes administered diets supplemented with ensiled OC alone.

### 1.5.4. *Genetic factors affecting milk traits*

Sheep comprises a domesticated species, which during its spread around the world and through selective breeding, it was adapted to a different range of environmental conditions and met a variety of human needs regarding important economic traits (milk, meat and wool quality and quantity). Thus, due to changes in their genomes, a diversity of commercial sheep breeds emerged with distinct characteristics. The essential ingredients of the genetic factors able to affect economic traits include the breed and the genotype of sheep, with difficulties in the control of the genotype. Thus, many molecular tools are employed to reveal the source of the traits of interest; however, the financial costs are high as a result to be less prevalent in dairy sheep due to the vast diversity of the breeds.

Quantitative trait loci (**QTL**) refer to polymorphic sections on DNA (loci) explaining part of the phenotypic variations of quantitative traits (Miles et al., 2008). QTL are linked or contain genes responsible for the trait; thus, its mapping takes place by the identification of the correlated molecular markers with the trait through evaluation of the statistical association. The candidate gene approach consists of association studies focusing on significant single nucleotide polymorphisms (**SNP**) or other variations in specific genes, which according to the functional role of the gene product or based on the position of the gene within previously reported QTL, is suggested to affect complex traits of interest. In contrast, the genome wide association study (**GWAS**), a powerful observational tool widely used in farm animals the last few years, scans the entire genome for variations and associates genotypes with different

phenotypes for particular traits. Thus, GWAS identifies quantitative trait nucleotides (**QTN**) and their candidate genes for various economical traits in ruminants.

1.5.4.1. *Quantitative trait loci (QTL)*

During the last three decades, many studies investigated the genetic basis of milk production and composition. Many **QTL** studies trying to identify the genetic loci affecting milk traits in dairy cattle (Andersson and Georges 2004; Khatkar et al., 2004; Chen et al., 2013) have led the studies in dairy sheep. The detection of QTL affecting quantitative characteristics such as milk, fat, and protein yield, fat and protein percentage and SCS, could benefit the selection systems through targeting the genes responsible for the relevant characteristics; as a result increasing the rate of genetic progress due to increased accuracy of selection and reduced generation interval (Arranz and Gutiérrez-Gil, 2012).

Through the years, research accomplished mapping multiple QTL on sheep autosomal and sex chromosomes. To date, there are 3,554 QTL identified on the sheep genome and represent 270 different sheep traits compared to the 160,659 QTL identified on the cow genome representing 675 traits. In sheep, 898 out of the 3,554 QTL relate to milk traits (e.g., milk fat yield (180 days): 299 QTL, milk yield (180 days): 176 QTL, milk protein yield (180 days): 80 QTL, milk fat%: 59 QTL, milk yield: 58 QTL, milk protein%: 54 QTL) (https://www.animalgenome.org/cgi-bin/QTLdb/index; accessed at 23 December 2020).

QTL for milk yield (**Table 1.8**) were identified in different sheep breeds with the latest review study from Arranz and Gutiérrez-Gil (2012). In the Sarda x Lacaune population, QTL for milk yield on OAR3, OAR4, and OAR20 influence protein yield and fat yield, while a QTL on

OAR16 for milk yield was linked to a QTL for fat percentage (Barillet et al., 2006). In the Awassi x Merino cross population, from the QTL influencing total milk on OAR2, OAR3, OAR20 and OAR24, the genome-wise significant QTL were on OAR3 and OAR20, which also map close to other QTL for milk yield traits (Raadsma et al., 2009). In the Friesian x Dorset backcross population, two chromosome-wise significant QTL for milk yield were detected on OAR2 and OAR18. The QTL on OAR18 was also associated with effects on protein yield (Mateescu and Thonney, 2010). In the Churra sheep population, a genome-wise suggestive QTL for milk yield on OAR23 had a pleiotropic effect also influencing protein yield and fat yield (Gutiérrez-Gil et al., 2009). According to García-Gámez et al. (2013), linkage analysis in the Spanish Churra sheep population revealed three genome-wise significant QTL on OAR2 for milk, protein and fat yields. The linkage disequilibrium and linkage analysis (**LDLA**) approach revealed 34 genome-wise significant QTL, while **GWAS** revealed the most significant QTL on OAR3 for protein and fat percentage (García-Gámez et al., 2012, 2013). Arnyasi et al. (2009) detected 2 QTL for milk yield on OAR6, Li et al. (2020) reported the detection of 5 QTL for milk yield on OAR1 and a QTL on OAR2. Also, for the Sarda sheep population, Usai et al. (2019) detected 12 QTL for milk yield on OAR1-3, 5, 10-16 and 25, while for the Chios sheep, Banos et al. (2019) reported the detection of 4 distinct QTL for milk yield on OAR 2, 12, 16 and 19 (**Table 1.8**).

Regarding the studies for protein percentage and yield, Díez-Tascón et al. (2001) reported the first putative QTL for protein percentage on OAR6 in Churra sheep, while the study of Gutiérrez-Gil et al. (2009) in an extended Churra sheep population identified a suggestive QTL on OAR2 and a genome-wise significant QTL on OAR3 for protein percentage, and also a genome-wise suggestive QTL in the second half of OAR1 for protein yield. In the Sarda x

Lacaune population, a genome-wise significant QTL in the first third of OAR1 and genome-wise suggestive QTL on the second half of OAR7 were identified for protein percentage (Barillet et al., 2006). In the Lacaune-Manech population, three genome-wise suggestive QTL for protein percentage were found on OAR2, OAR5 and OAR9 (Barillet et al., 2006). In the Awassi x Merino backcross population, Raadsma et al. (2009) detected a significant QTL on the first half of OAR7 for protein percentage.

Concerning the fat percentage and yield, Barillet et al. (2006) detected a genome-wise suggestive QTL for fat percentage on OAR1, OAR9 and OAR10 in the Lacaune-Manech population. In the Sarda x Lacaune population, OAR20 was reported to have a genome-wise significant QTL for fat percentage, whereas genome-wise suggestive linkage associations were reported on OAR3, OAR7 and OAR16 (Barillet et al., 2006). In the Churra sheep population, a suggestive QTL for fat percentage was detected on OAR2, a genome-wise suggestive QTL for fat percentage was detected close to the OAR20, and suggestive associations were found to influence fat yield on OAR25 (Gutiérrez-Gil et al., 2009). In the Awassi x Merino backcross population, significant QTL for fat percentage were detected on OAR3 and OAR25, whereas the QTL on OAR3 affects several milk production traits. A suggestive QTL for fat percentage was also detected on OAR8 (Raadsma et al., 2009).

In the case of the QTL influencing the SCS which is a classical indicator of subclinical mastitis in sheep, a suggestive genome-wise significant QTL was found on OAR14 for SCS in the Lacaune-Manech population (Barillet et al., 2006). In the Sarda x Lacaune population, several traits related to mastitis resistance were recorded. For these traits, together with 11 genome-

wise suggestive QTL, two genome-wise significant QTL were found on OAR6 and OAR13 (Barillet et al., 2006). The whole-genome screening performed in Spanish Churra sheep identified a single genome-wise suggestive QTL for SCS on OAR20 (Gutiérrez-Gil et al., 2007). Moreover, in the study performed later by Gutiérrez-Gil et al. (2018), in Churra sheep the identification of chromosome-wide significant QTL on OAR5 and OAR25, and the identification of a genome-wide significant QTL on OAR20 were reported, thus confirming the later QTL firstly identified by Gutiérrez-Gil et al. (2007). In the case of the Awassi X Merino cross population, a significant QTL for SCS was recorded on OAR14, whereas two suggestive QTL for this trait were reported on OAR17 and OAR22 (Raadsma et al., 2009). Additionally, Gutiérrez-Gil et al. (2018) identified 22 significant QTL also influencing SCS through LDLA scan.

Milk FAs are another feature attracting researchers' interest due to the nutritional value they offer to dairy products and their association with human health related issues. In the Sarda x Lacaune, a chromosome-wise significant QTL for the ratio CLA/vaccenic acid was detected on OAR22 (Carta et al., 2003). Carta et al. (2008) focused their studies on milk FAs due to the nutritional value they offer to dairy products and their impact on human health. After they screened a cross-bred sheep population of Sardinian x Lacaune, the most significant QTLs mapped on OAR11, and OAR6 affected the composition of FAs such as the myristic acid and palmitic acid, and the production of MUFA, respectively (Carta et al., 2008). In the Churra sheep population, a suggesting QTL on OAR22 was detected to influence the CLA/vaccenic acid ratio (Garcia-Fernandez et al., 2010a) and on OAR11, 4 significant QTL were detected on the 5% chromosome-wise level to respectively affect the contents of C10:0, C12:0, CLA and PUFA (Garcia-Fernandez et al., 2010b).

**Table 1.8.** Summary table of various QTL for milk traits (milk, fat and protein yield, fat percentage, protein percentage, somatic cell score and fatty acids) detected on the autosomes (OAR) of several sheep breeds.

| Sheep population | Trait | QTL | Reference |
|---|---|---|---|
| Sarda X Lacaune | MY, FY, PY | OAR3, OAR4, OAR20 | Barillet et al., 2006 |
| | MY | OAR16 | |
| Awassi x Merino cross | total milk, FY, PY | OAR3, OAR20, OAR2, OAR24 | Raadsma et al., 2009 |
| Churra | MY, FY, PY | OAR23 | Gutiérrez-Gil et al., 2009 |
| Friesian X Dorset backcross | MY | OAR2 | Mateescu & Thonney, 2010 |
| | MY, PY | OAR18 | |
| Churra | MY, FY, PY | OAR2 (3QTL) | Garcia-Gamez et al., 2013 |
| | MY | OAR6 (2QTL) | Arnyasi et al., 2009 |
| | MY | OAR1 (5 QTL), OAR2 | Li et al., 2020 |
| Sarda | MY | OAR1, OAR2, OAR3, OAR5 (2QTL), OAR10, OAR11, OAR12, OAR13, OAR14, OAR16, OAR25 | Usai et al., 2019 |
| | MLACTY | OAR6 (2QTL) | Arnyasi et al., 2009 |
| Chios | MY | OAR2, OAR12, OAR16, OAR19 | Banos et al., 2019 |
| Sarda x Lacaune | PP | OAR1, OAR7 | Barillet et al., 2006 |
| Lacaune-Manech | PP | OAR2, OAR5, OAR9 | Barillet et al., 2006 |
| Churra | PP | OAR6 | Díez-Tascón et al., 2001 |
| Churra | PP | OAR2, OAR3 | Gutiérrez-Gil et al., 2009 |
| | PY | OAR1 | |
| Churra | PP | OAR3 | Garcia-Gamez et al., 2011 |
| Churra | PP | OAR3 | García-Gámez et al., 2012 |
| Awassi x Merino backcross | PP, PY | OAR3 | Singh et al., 2007 |
| Awassi x Merino backcross | PP | OAR7 | Raadsma et al., 2009 |
| | PP | OAR1 | Sutera et al., 2019 |
| | PY | OAR1 (6 QTL) | Li et al., 2020 |
| Sarda | PY | OAR1, OAR3, OAR5, OAR10, OAR11, OAR13, OAR14, OAR16, OAR19, OAR25 | Usai et al., 2019 |
| Sarda | PP | OAR1 (5QTL), OAR2 (6QTL), OAR3 (4QTL), OAR4 (3QTL), OAR5, OAR6, OAR7, OAR8, OAR9, OAR10, OAR11 (2QTL), OAR12, OAR13, OAR15, OAR16 (4QTL), OAR17, OAR19 (2QTL), OAR20, OAR22 (2QTL), OAR23, OAR24 (2QTL), OAR25 | Usai et al., 2019 |
| Sarda x Lacaune | FP | OAR20, OAR3, OAR7, OAR16 | Barillet et al., 2006 |
| Sarda x Lacaune | FY | OAR14 | Barillet et al., 2006 |

| | | | |
|---|---|---|---|
| Lacaune-Manech | FP | OAR1, OAR9, OAR10 | Barillet et al., 2006 |
| Lacaune-Manech | FY | OAR16 | |
| Churra | FP | OAR2, OAR20 | Gutiérrez-Gil et al., 2009 |
| Churra | FY | OAR25 | Gutiérrez-Gil et al., 2009 |
| Churra | FP | OAR3 | García-Gámez et al., 2012 |
| | FP | OAR1 | Sutera et al., 2019 |
| Awassi x Merino backcross | FP | OAR3, OAR8, OAR25 | Raadsma et al., 2009 |
| | FY | OAR1 (14 QTL), OAR 6, OAR13 | Li et al., 2020 |
| Sarda | FY | OAR1, OAR2, OAR3, OAR5, OAR6, OAR10, OAR11, OAR12, OAR14, OAR16, OAR25 | Usai et al., 2019 |
| Sarda | FP | OAR1 (6QTL), OAR2 (3), OAR3 (6QTL), OAR4 (3QTL), OAR5 (2QTL), OAR6 (2QTL), OAR8, OAR9 (2QTL), OAR10 (2QTL), OAR11 (2QTL), OAR12, OAR13, OAR14 (2QTL), OAR15 (2QTL), OAR16 (3QTL), OAR17, OAR18, OAR19 (2QTL), OAR20, OAR21 (2QTL), OAR22 | Usai et al., 2019 |
| Sarda x Lacaune | SCS | | Barillet et al., 2006 |
| Lacaune-Manech | SCS | OAR14 | Barillet et al., 2006 |
| Churra | SCS | OAR20 | Gutiérrez-Gil et al., 2007 |
| Churra | SCS | OAR5, OAR20, OAR25 | Gutiérrez-Gil et al., 2018 |
| Awassi x Merino cross | SCS | OAR14, OAR17, OAR22 | Raadsma et al., 2009 |
| Sarda x Lacaune backcross | CLA/VA | OAR22 | Carta et al., 2003 |
| Sarda x Lacaune backcross | CLA/VA | OAR4, OAR22 | Carta et al., 2006 |
| Sarda x Lacaune backcross | CLA | OAR4, OAR14, OAR19 | Carta et al., 2006 |
| Sarda x Lacaune backcross | MUFA<br>C14:0, C16:0 | OAR6<br>OAR11 | Carta et al., 2008 |
| Churra | CLA/VA | OAR22 | Garcia-Fernandez et al., 2010a |
| Churra | C10:0, C12:0, CLA, PUFA | OAR11 | Garcia-Fernandez et al., 2010b |

The traits analyzed are milk yield (MY), milk lactose yield (MLACTY), fat yield (FY), protein yield (PY), fat percentage (FP), protein percentage (PP), somatic cell score (SCS), conjugated linoleic acid (CLA), vaccenic acid (VA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), C10:0, C12:0, C14:0, C16:0.

1.5.4.2. *Candidate genes*

The candidate gene approach focuses on the identification of genes with DNA variants or polymorphisms, which according to their physiological role, their position on the genome within a QTL, or their expression levels, can affect or have a relationship with economically important traits. Hence, the candidate gene approach is not a random search of a gene through the genome. The association between important dairy traits and candidate genes ameliorates our comprehension on the genetic variations and leads to the development of genetic markers for the positive genetic variants, which through selection strategies these dairy traits are improved on the benefit of farmers and dairy industries.

Milk proteins are known to affect the cheese making procedure, so the genes encoding for milk proteins are the most widely studied candidate genes in ruminants since, according to studies, their variants affect the composition and properties of milk in a different extent. Moioli et al. (2007a) reviewed all the identified variants on the genes encoding milk proteins (caseins and whey proteins) in sheep. In the ovine genes encoding $\alpha_{s1}$-casein, $\alpha_{s2}$-casein, β-casein, k-casein, α-lactalbumin and β-lactalbumin, the identified variations cause the production of multiple forms of the proteins, some of which are related to the quality of the milk concerning its composition and technological properties (reviewed by Moioli et al., 2007a). The *LALBA* gene responsible for the production of α-lactalbumin was associated with protein and fat percentage in dairy sheep (Garcia-Gamez et al., 2012). Nevertheless, *LALBA* gene was not associated with any detectable signal for dairy traits in sheep by the application of selection mapping as performed from Gutierrez-Gill et al. (2014).

Furthermore, a great number of ovine genes encoding enzymes related with the FA metabolism have also been studied. According to the review of Moioli et al. (2007a), the enzyme encoded from the *Acetyl-CoA carboxylase* (***ACACA***) gene on OAR11 is found to be directly involved in the FA synthesis during lactation (Veltri, 2000) and SNP were also recorded in Promoters I and II (Moioli et al., 2005a and 2005b). No QTL were identified to map close to the ovine *ACACA* gene, and no association was detected between the gene and milk FA content (Garcia-Fernandez et al., 2010b). However, a SNP detected in Promoter III was assumed to affect the variation of fat content in milk (Moioli et al., 2013a), while in the study of Di Gerlando et al. (2017), 23 polymorphic sites were also identified.

As the *ACACA* gene, *fatty acid synthase* (***FASN***) gene encodes for a key enzyme for the FA elongation, hence it is selected as candidate gene for its potential effect on milk fat quality. The ovine *FASN* gene harbors on OAR11, where QTL for C14:0, C16:0 (Carta et al., 2008), C10:0 and the PUFA content (García-Fernández et al., 2010b) were detected. Additionally, a SNP in the ovine intron 1 was detected to affect the binding sites for Sp1 transcription factors (Sanz et al., 2015), while the g.257C>T SNP in the ovine *FASN* gene was found to affect the milk FA profile of the medium-chain FA, consistently with its function (C10:0, C10:1, C12:0, C14:0) (Crisa et al., 2010). The same SNP (g.257C>T) was detected to have opposite effects for stearic acid and CLA (Moioli et al., 2012).

The *acyl CoA-diacylglycerol-acyltransferase 1* gene (***DGAT1***) on OAR9 is considered as a positional and functional candidate gene for milk fat content due to its role intervening the last step in TAG synthesis, the QTL detected on this chromosome, which is homologous to BTA14

(Barillet et al., 2005; Moili et al., 2007a) and affecting protein percentage and content, fat percentage and content (Barillet et al., 2006; Usai et al., 2019), and due to the polymorphisms identified to be associated with milk fat content (Scata *et al.,* 2009), lactose and milk FA traits (C4:0, C16:1 *cis*-9 and n-6/n-3 ratio) in sheep (Dervishi *et al.,* 2015). However, in the study of Gutierrez-Gill et al. (2014), *DGAT1* gene was not associated with any detectable signal for dairy traits in sheep. Similarly, *diacylglycerol O-acyltransferase homolog 2* (***DGAT2***) gene is also considered as a candidate gene due to the function of the encoded enzyme intervening the last step in TAG synthesis. A haplotype for the three detected SNPs in the ovine *DGAT2* gene affected the CLA and C18:2 content in milk, the milk yield (Crisa et al., 2010), while a SNP detected in the 3' UTR of the gene revealed association with linolenic acid and CLA contents in milk (Moioli et al., 2012).

Another gene, the *stearoyl-CoA desaturase* (***SCD***) on OAR22, is a major candidate gene for milk quality due to its implication in milk technological and nutritional quality for human consumers, as the SCD enzyme, an endoplasmic reticulum-bound enzyme, has a key role in the synthesis of MUFA from SFA, and CLA in cells (reviewed by Miyazaki and Ntambi, 2003; Moili et al., 2007). According to Garcia-Fernandez et al. (2009), from the study of the coding and non-coding region of the ovine *SCD* gene, an allelic variant was detected in the promoter region and the three other in introns 2 and 3. From its intermediate frequency in the dairy breeds, the SNP in the promoter region was correlated with the degree of dairy specialization and the fat content in milk (Garcia-Fernandez et al., 2009). Two more SNP were also detected in the promoter region and a SNP in the 5'UTR (Aali et al., 2014). A SNP detected in intron 4 was associated with the CLA/VA ratio (Miari et al., 2009), while the SNP (SCD03) in intron 2 was

associated with milk fat percentage suggesting a possible effect of the gene for this trait on the specific sheep breed (Churra) where the analysis was performed (Garcia-Fernandez et al., 2010a). Furthermore, QTL influencing the CLA/VA ratio in OAR22 coinciding or adjacent with the *SCD* gene (Carta et al., 2006; Garcia-Fernandez et al., 2010a) strengthen the evidence that the *SCD* gene affects milk fat quality.

The following genes, *growth hormone receptor* (***GHR***), *growth hormone-releasing hormone receptor* (***GHRHR***) and *insulin-like growth factor 1* (***IGF-1***) on OAR16, OAR4 and OAR3, respectively, which encode enzymes of the somatotropic axis, are considered as candidate genes for their potential role in milk production. The GHR acts on carbohydrate, protein and lipid metabolism. In sheep, polymorphisms in the *GHR* gene revealed associations with milk fat content, butyric acid, stearic acid and C18:2 (Crisa et al., 2010), daily fat and protein yield, SCS, fat and lactose content and milk urea (Dettori et al., 2018). In addition, on OAR16, QTL for milk yield (García-Gámez et al., 2012; Banos et al., 2019), milk protein and fat content were also detected (García-Gámez et al., 2012), while in the Chios sheep breed, the gene was expressed in the mammary gland and the milk transcriptome, and highly expressed in liver (Banos et al., 2019). Regarding the *GHRHR* gene, which mediates the transduction of signals from its ligand growth hormone-releasing hormone (***GHRH***) gene for the transcription of *growth hormone* (***GH***) gene (Mayo, 1992; Giustina and Veldhuis, 1998), polymorphisms where detected in the ovine intronic region (Crisa et al., 2010; Dettori et al., 2018), while a SNP in the ovine exon 13 was associated with total solids and protein percentages in milk (Abousoliman et al., 2020). The ovine *IGF1* gene is chosen as a candidate gene due to its key role in the physiology of lactation. Variability in the gene revealed significant effect on milk lipid composition such as the CLA and

α-linolenic content (Crisa et al., 2010), oleic and linolenic acids (Moioli et al., 2012), while association with milk protein and casein contents were also detected (Dettori et al., 2018).

Additionally, six genes that their encoding products involved in liver metabolism and lipolysis were assumed as candidate genes affecting milk traits in sheep; *propionyl coenzyme A carboxylase, β polypeptide* (***PCCB***), *lecithin-cholesterol acyltransferase* (***LCAT***), *lipoprotein lipase* (***LPL***), *hormone-sensitive lipase* (***LIPE***), *AMP-activated protein kinase* (***PRKAA***) and *α-1-antichymotrypsin* (***SERPINA3***). According to the role of the PCCB enzyme in the gluconeogenesis in liver in cows (Murondoti et al., 2004), the haplotype [T;T;T;A] of five SNPs in the ovine gene was associated with increased levels of C18:2 (0.10%), C18:3 (0.03%) and CLA (0.12%) in milk and reduced the levels of C16:0 (0.73%) (Crisa et al., 2010). Furthermore, the association of a SNP in an ovine intronic region with C18:1 *trans*-6, C18:1 *trans*-8, C18:1 *cis*-12, C18:1 *cis*-13, C18:2 *cis*-9, *trans*-13 and linolenic acid was also recorded (Moioli et al., 2012).

The *LCAT* gene encodes its product in the liver and moves in plasma bounded with lipoproteins (Jonas, 2000). The haplotype [T;A;T] of the ovine *LCAT* gene was associated with decreased levels of C18:2 (0.06%), while the haplotype [C;G;C] showed a positive association with milk yield (40.02 g/g) (Crisa et al., 2010). Moioli et al. (2012) also studied a nonsynonymous SNP g.806G in the ovine *LCAT* gene, which was significantly associated with stearic acid and C18:1 *trans*-10.

The *LPL*, expressed in mammary gland to the enzyme hydrolyzing TAG to its precursors, is considered as a candidate gene due to its involvement in the FA metabolism (Moioli et al.,

2007a). Consistently with its function, the ovine *LPL* gene haplotype [T;T;A] had a positive influence on milk CLA content (0.09%) (Crisa et al., 2010).

In the ovine *SERPINA3* gene, haplotype [T;A] showed negative correlation with milk yield (59.11 g/d), C16:1 content (0.08%) and PUFA levels (C18:2 0.05%, C18:3 0.03%, CLA 0.13%), and positive correlation with C18:0, while haplotype [T;G] was associated with a higher milk yield (43.38 g/d) and lower C4:0 (0.15%) (Crisa et al., 2010). Due to the role of the enzyme with liver endocrine changes associated with lactogenesis, probably the haplotype is the one responsible for the variability of the UFA content in milk (Crisa et al., 2010). The second variant g.134A in the *SERPINA3* gene was also studied by Moioli et al. (2012) and was found to negatively influence VA, C18:1 *cis*-11, linoleic acid, linolenic acid and CLA content.

The genes *tyrosinase-related protein 1* (***TYRP1***) and *zona pellucida glycoprotein 2* (***ZP2***), were also chosen as candidate genes for milk traits in sheep according to their function (Crisa et al., 2010). For the ovine *TYRP1* gene, [C;C] haplotype was shown to negatively affect the short-chain milk FA C6:0 (0.05%) and C8:0 (0.04%), and [T;T] haplotype also showed a negative influence on C15:0 (Crisa et al., 2010). Milk FA contents were also affected by the ovine *ZP2* gene, where the haplotype [G;T] had a negative influence on fat (0.21%) and C16:0 (0.56%) contents, and the haplotype [G;C] had a negative effect on medium-chain FAs such as C10:0 (0.46%) and C14:0 (0.32%), and a positive effect on C18:1 (0.16%). Haplotype [A;C] showed an opposite effect compared to the first haplotype where increased fat (0.19%) and C16:0 (0.46%) contents were recorded, while CLA (0.11%) and C17:1 (0.005%) decreased (Crisa et al., 2010).

Moioli et al. (2012) studied the second SNP g.105C of the research from Crisa et al. (2010) and was found to be associated with CLA, linoleic acid, linolenic acid and other C18 isomers.

*Prolactin* (***PRL***) is the gene encoding for an essential hormone (prolactin) for milk production in mammals. In ovine it was detected that the hormone suppression inhibited lactation (Knight, 2001) and according to its role, *PRL* is considered as good functional candidate gene for milk production and composition traits. Moreover, the identification of QTL for milk, fat and protein yields (Barillet et al., 2005) and fat percentage (Gutierrez-Gill et al., 2009) on OAR20, which is in proximity with *PRL* gene, suggest it as positional candidate gene (Staiger et al., 2010). A polymorphism (intron 2) detected in the ovine gene (Vincent and Rothschild, 1997) was associated with milk yield, fat and protein in different sheep breeds (Ramos et al., 2009; Staiger et al., 2010), and a SNP in the ovine exon 3 was associated with milk yield (Miltiadou et al., 2017a; Abousoliman et al., 2020) and milk fat percentage (Miltiadou et al., 2017a).

*POU class 1 homeobox 1*(***POU1F1***) gene on OAR1, also known as *growth hormone factor 1*, can be considered as candidate gene for milk production traits as it is found to regulate the function of *GH* and *PRL* genes, and affect milk production (reviewed by Gebreselassie et al., 2020). However, association studies in the gene did not reveal association of SNPs with milk traits (Mura et al., 2012). Similarly, *signal transducer and activator of transcription 5A* (***STAT5A***) gene, activated by *prolactin receptor* (***PRLR***) gene (Jiang et al., 2012), might also be regarded as a candidate gene for milk production traits according to its function in the development of mammary gland through the transduction of signals from *PRL* and *GH* genes to milk protein

genes (Cui et al., 2004). In cow and goat, the *STAT5A* gene was found to be associated with milk FA and milk yield (Brym et al., 2004; Schennink et al., 2009; An et al., 2013), while a polymorphism in exon 11 of the ovine *STAT5A* gene was associated with lactose percentage (Abousoliman et al., 2020).

Another interesting candidate gene for the ovine milk production traits is the *ATP-binding cassette sub-family G member 2* (***ABCG2***) gene, chosen according to its function and location. The gene encodes a transporter molecule across membranes (Higgins, 1992; Klein et al., 1999) and is located on OAR6 where a microsatellite (OarAE101) was mapped and associated with milk fat percentage, protein percentage and SCS (Schibler et al., 2002), while suggestive associations were also recorded for milk yield and milk fat percentage (Garcia-Fernandez et al., 2011). Besides this, an association analysis revealed a significant effect of a SNP on the ovine gene with protein percentage and of a deletion with SCS (Arnyasi et al., 2013). According to Ruiz-Larranaga et al. (2018), the ovine gene was detected in a selected candidate region with several other genes, (*secreted phosphoprotein 1* (***SPP1***), *leucine aminopeptidase 3* (***LAP3***)), *non-SMC condensing I complex* (***NCAPG***) and *matrix extracellular phosphoglycoprotein* (***MEPE***) genes) considered to be putatively selected for ovine milk production traits, as they were previously associated with milk production traits in cattle. Additionally, the *ABCG2* gene due to its role in milk yield and composition is found to be under selection in dairy sheep (Kijas et al., 2012; Gutierrez-Gil et al., 2014; Fariello et al., 2014).

*SPP1* gene is a positional candidate gene mapped on OAR6, where variations within the ovine gene showed association with SCS (Dettori et al., 2020), in accordance to the gene's

function and position in a candidate area in the ovine genome for milk production traits and lactation regulation (Gutierrez-Gil et al., 2014; Wei et al., 2015). Similarly, the study of variations in the bovine *SPP1* gene also revealed a link between SCS and mastitis resistance (Alain et al., 2009).

Other genes chosen as candidates for milk traits according to their function are the *fatty acid-binding protein type 3* (***FABP3***), *alpha-amylase* (***AMY***), *cingulin* (***CGN***), lysophoshatidic acid phosphatase protein (***ACP6***), *annexin A9 protein* (***ANXA9***) and *alpha-amylase isoform 2* (***AMY2***) genes (reviewed by Moioli et al., 2007a). According to Calvo et al. (2006), *solute carrier family 27 (fatty acid transporter) member 3* (***SLC27A3***) gene and some of the above genes, *ANXA9*, *CGN* and *ACP6*, located on OAR1, where QTL for milk traits were detected, were selected for fine mapping and association analysis with milk traits in relation to bovine QTL on BTA3. However, the study revealed that only two of the genes, *ANXA9* and *SLC27A3*, responsible for FA transportation, are linked to QTL related to milk yield and fat content found on BTA3, a homologue of OAR1 (Calvo et al., 2006).

According to Dettori et al. (2020), variations were studied on another two positional candidate genes, the *protein O-fucosyltransferase 1* (***POFUT1***) gene on OAR13 and the *PRLR* gene on OAR16. *POFUT1* was associated with curd firmness in sheep (Dettori et al., 2020), while its enzyme has an important role for the formation of udder cells (Buono et al., 2006), and additionally, the gene is located in an area with genes responsible for the development and differentiation of the udder (Gutierrez-Gil et al., 2014). *PRLR* was associated with lactose

content, SCS, rennet coagulation time and curd firming time for ovine milk (Dettori et al., 2020), while the gene was also associated with udder changes during lactation (Szczesna et al., 2020).

*Gelsolin* (***GSN***) isoform b gene encodes for a $Ca^{2+}$ -dependent actin-regulatory protein taking part in the control of the intracellular lipid metabolism (Kusano et al., 2000). *GSN* isoform b is considered as a candidate gene for milk quality based on its function (Napolitano et al., 2014). Also the identification of three variants (not breed specific) in the consensus sequence where three known transcription factors bind, revealed their association with fat content in milk (Napolitano et al., 2014).

*Glycerol-3-phosphate acyltransferase mitochondrial* (***GPAM***), *melanocortin-4 receptor* (***MC4R***) and *perilipin* (***PLIN1***) genes are also considered as candidate genes, which according to their functions are implicated in fat quantitative traits and also the detection of polymorphic regions in the 5'UTR, all of them in regulatory binding elements for transcription factors that are implicated in lipid metabolism pathways (Sanz et al., 2015).

Other putative candidate genes for milk production traits are those for which SNPs were identified to be associated with milk traits on OAR1, OAR6, OAR7, OAR10, OAR21, OAR25 and OAR26 (Sutera et al., 2019). A SNP near the *succinate receptor 1* (***SUCNR1***) gene on OAR1 was associated with fat and protein percentage in ovine milk (Sutera et al., 2019), and previously was associated to pathways concerning cheese traits in sheep (Suarez-Vega et al., 2016). A gene with important role in the lipid metabolism in bovine is *tetratricopeptide repeat domain 7B* (***TTC7B***) gene (MacLeod et al., 2016) located on OAR7 where a SNP in an ovine intron was associated with fat and protein percentage in milk (Sutera et al., 2019). On the same

chromosome in sheep, OAR7, a SNP detected close to the *WD* repeat domain 89 (***WDR89***) gene was associated with protein yield in milk (Sutera et al., 2019). Association with protein percentage was recorded for two SNPs at 0.01 Mb of the *SH3 domain and tetratricopeptide repeats 1* (***SH3TC1***) and *klotho* (***KL***) genes on OAR6 and OAR10, respectively, while association with fat percentage in sheep milk was detected for a polymorphism on OAR14, where the *NINI* (*RPN12*) *binding protein 1 homolog* (***NOB1***) harbors. Two polymorphisms detected within the *decapping enzyme, scavenger* (***DCPS***) and *LOC101123180* genes on OAR21 and OAR25, respectively, were associated with protein yield in milk. Association with milk yield was detected for the SNP within the *DCPS* gene (Sutera et al., 2019), which is differentially expressed in the udder of sheep (Suchocki et al., 2016) and the SNP within the *teneurin transmembrane protein 3* (***TENM3***) gene on OAR26 (Sutera et al., 2019).

The ovine *leptin* (***LEP***) gene is considered as a candidate gene due to its functional role (Choudhary et al., 2005) and for its association with weaning weight, average daily gain (Abousoliman et al., 2020) and significant effects on milk traits (milk yield and fat percentage) (Mahmoud et al., 2014; Abousoliman et al., 2020). *LEP* gene is already considered as a candidate gene for body fat content (Choudhary et al., 2005) in cattle also influencing milk performance (Almeida et al., 2003). The detection of a polymorphism in the ovine *LEP* gene revealed association with milk yield (Mahmoud et al., 2014), while another SNP in exon 3 was significantly associated with milk yield and fat percentage (Abousoliman et al., 2020).

The *3-oxoacid CoA transferase 1*(***OXCT1***) gene was selected as a putative candidate gene in Chios sheep breed due to its location on OAR19, where a QTL for milk yield was identified

(Banos et al., 2019). The ovine *OXCT1* was expressed in mammary gland and immune tissues, and highly expressed in the kidney cortex, indicating a similar role to the findings in cattle where the gene was associated with milk production, mastitis resistance, and regulated the mammary gland metabolism and milk synthesis during mastitis (Banos et al., 2019). Two other genes, *lysophospholipase 1* (***LYPLA1***) and *DnaJ heat shock protein family (Hsp40) member A1* (***DNAJA1***), were also selected as putative candidate genes for milk yield due to their location in QTL affecting milk yield in Chios sheep (Banos et al., 2019).

According to the study of Saridaki et al. (2019), many positional candidate genes were identified from which only eleven were the most plausible causal genes for ovine milk traits. Association with milk yield was detected for nine genes on OAR11, with fat content for one gene on OAR16 and with protein content for one gene on OAR10. The candidate genes for milk yield are the following, where some of them possess a functional role relational to milk, while some other not: *signal recognition particle 68* (***SRP68***), *cyclin dependent kinase 3* (***CDK3***), *galanin receptor 2* (***GALR2***), *acyl-CoA oxidase 1* (***ACOX1***), *WW domain binding protein 2* (***WBP2***), *H3 histone family 3B* (***H3F3B***), *phosphoribosyl pyrophosphate synthetase associated protein 1* (***PRPSAP1***) and *integrin subunit beta 4* (***ITGB4***) (Saridaki et al., 2019). Regarding the *5-hydroxytryptamine receptor 1A* (***HTR1A***) gene, candidate for fat content (Saridaki et al., 2019), was found to regulate lactation homeostasis in the udder cell of mouse and human (Weaver and Hernandez, 2016). Also, the *glypican-5* (***GPC5***) identified as a candidate gene for protein content (Saridaki et al., 2019), involves in the growth and differentiation of cells (De Cat and David, 2001), while in dairy cows it was found to be the closest gene to a variation associated with FA in milk (Li et al., 2014).

Recently, 222 genes were illustrated within copy number variations (CNVs) which were significantly associated with health and milk production traits (Di Gerlando et al., 2019). Many of these genes were found to have a crucial role in functions related to milk traits and the identified CNVs were associated with milk and fat yield, and fat and protein percentage. Some of them are the genes *CLIP3, APLP1*, *CHST3* and *HCST* on OAR14, which were associated with fat yield in milk and the genes *ADCY7* and *PHKB*, associated with milk yield (Di Gerlando et al., 2019). Similarly, *MALRD1* gene on OAR13, was associated with both milk yield and fat yield, and *SUCLG2* on OAR19 and *ZBTB7C* gene on OAR 23 were associated with fat percentage (Di Gerlando et al., 2019).

The *palmdelfin* (***PALMD***) and *ring finger protein 145* (***RFP145***) genes on OAR1 and OAR5, respectively, were detected after comparison of the corresponding bovine genome with the ovine genome where highly divergent milk yielders had different allele frequencies at consecutive markers. *PALMD* and *RFP145* may be involved in the variation of milk yield (Moioli et al., 2013b). According to a later study of Moioli et al. (2015), 165 and 641 putative candidate genes were detected near markers for milk fat and protein content, respectively.

### 1.6. Milk Fat Synthesis and Key Enzymes Involved

Ruminants' diet contains large amounts of carbohydrates (cellulose, hemicellulose, starch, and other), which are the most important source of energy and precursors of fat and lactose in their milk (Wattiaux and Armentano, 2000). The carbohydrates degrade to glucose, a large proportion of which converts to pyruvic acid through glycolysis. Then pyruvic acid is further degraded to volatile fatty acids (**VFA**) by microorganisms in the rumen. More than 95% of the acids formed in the rumen from the carbohydrates' fermentation are VFAs (acetic, propionic, and butyric acid) (**Figure 1.6**). During the passage of VFAs through the rumen wall, before the transport to the liver, a tiny proportion of acetate and almost all of the butyrate converts to ketone bodies (β-hydroxybutyrate), used as energy sources. The significant majority of the acetate and all propionate are transfered to the liver, where the majority of propionate is converted to glucose, which is either used for the formation of lactose in the mammary gland or is converted to glycerol, the TAG backbone (Chilliard et al., 2000b; Shingfield et al., 2008, 2010). Ketone bodies and acetate are used for de novo lipogenesis in adipose tissue and mammary gland by attaching on glycerol. Diet influences the amount and percentage of each VFA produced and therefore the milk production and milk fat percentage but also affect the efficiency of converting feed into milk and the relative value of a ration for milk production as opposed to fattening (McDonald et al., 2011).

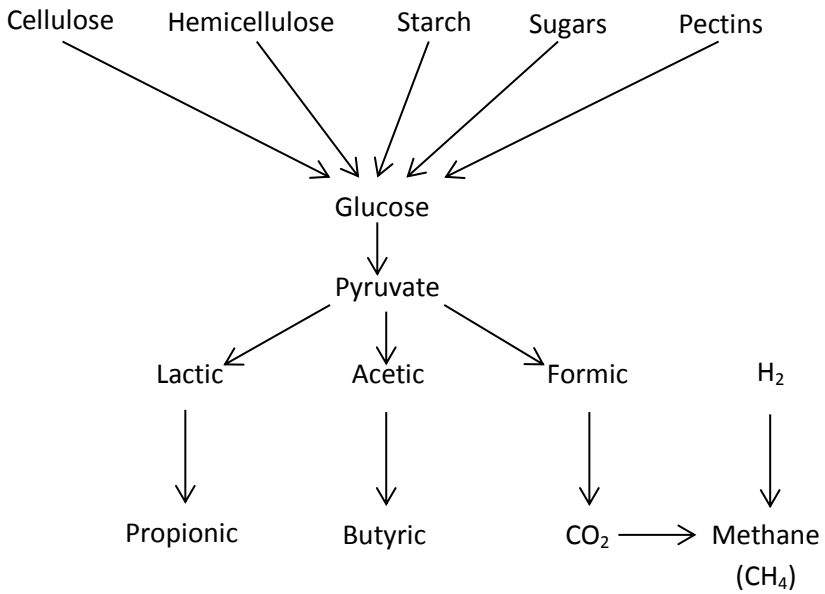


**Figure 1.6.** Rumen fermentation for the production of volatile fatty acids (VFA)

A typical diet for ruminants is high in UFA with an amount of 20 to 40g lipid/kg dry matter (**DM**) (Shingfield et al., 2010). Lipids (TAG, phospholipids, glycolipids) from diet are hydrolyzed to free FAs and glycerol by bacterial lipases in the rumen, whereas the last is fermented to VFA (Wattiaux and Grummer, 2000). As reviewed by several authors (Parodi 2004; Fox and McSweeney, 2006; Shingfield et al., 2008), free FAs in rumen follow two different directions; they either undergo biohydrogenation or are utilized as precursors for the formation of bacterial lipids. The extensive biohydrogenation by ruminal microbes converts UFA to SFA, ending up with a higher amount of SFA in meat and milk compared to those present in the ruminants' diet. Linoleic acid (**LA**; C18:2 *cis*-9, *cis*-12) and linolenic acid (**LNA**; C18:3 *cis*-9, *cis*-12, *cis*-15) are converted into conjugated FAs after the isomerization of the *cis*-12 double bond and saturation of the *cis*-15 double bond of LNA. The transient nature of conjugated products reduces to an intermediate product, the C18:1 *trans*-11 (VA), which ends up to C18:0 (stearic

acid) through a rate-limiting reduction step allowing the flow of C18:1 *trans*-11 outside the rumen. Depending on the diet, a variation in the biohydrogenation of LA (70-95%) and LNA (85-100%) indicates that C18:0 is the main product leaving the rumen. In comparison to LA and LNA, the biohydrogenation process of oleic acid (**OA**; C18:1 *cis*-9) takes place at a lower extent (58-87%). Biohydrogenation not only results in the formation of C18:0 but also in the formation of oxygenated C18:0 and C18:1 products, and C18:1-*trans* intermediates (double bonds at Δ6-Δ16) (Bauman and Lock, 2006; Shingfield et al., 2010). The majority of FAs arriving in the small intestine (85-90%) are SFAs, while bacterial lipids, mainly phospholipids, comprise the remaining 10-15%. SFAs, other free FAs, and FAs deriving from bacterial lipids digestion form a pool of FAs absorbed through the intestinal wall in the form of micelles. In the intestinal cells, TAGs reform and enter the blood system in the form of chylomicrons or very-low-density lipoproteins (**VLDL**), containing TAGs, free FAs, cholesterol and other lipids like substances coated by lipoprotein (Parodi 2004; Fox and McSweeney, 2006; Shingfield et al., 2008).

Lipids are secreted in milk from mammary epithelial cells in the form of fat globules which consist of a nonpolar core of lipids surrounded by a membrane of phospholipids, cholesterol and cholesterol esters (Spitsberg 2005; Shingfield et al., 2008, 2010). Peripheral circulation of the preformed FAs arising from diet and the de novo synthesized FAs are the two routes of FA uptake for milk TAG assembly (**Figure 1.7**). According to Palmquist (2009), an approximate 60% of FAs are taken directly from blood, while the remaining 40% are de novo synthesized. All of the C4:0 to C12:0 FAs secreted in milk, and almost 95% and 50% of the C14:0 and C16:0, respectively, are de novo synthesized, while all C18 and longer FA chains are preformed and derived from blood circulation (Chilliard et al., 2000b; Shingfield et al., 2010).

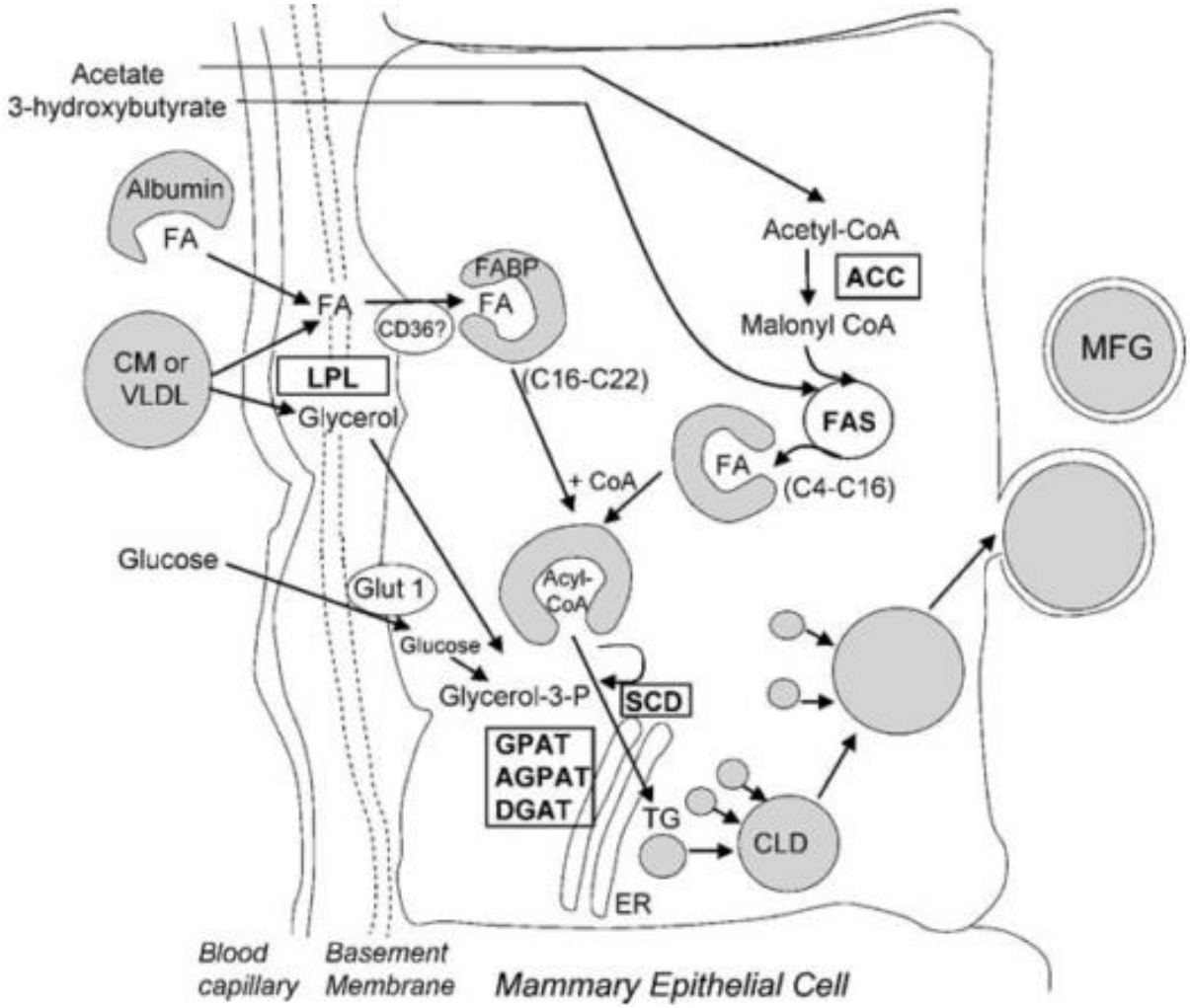


**Figure 1.7.** Representation of milk fat synthesis in the epithelial cells of the ruminant mammary gland (adopted by Bernard et al., 2008). ACC: acetyl-Coa carboxylase; AGPAT: 1-acyl glycerol 3-phosphate acyl transferase; CD36: cluster of differentiation 36; CLD: cytoplasmic lipid droplet; CoA: coenzyme A; CM: chylomicron; DGAT: diacylglycerol acyltransferase 1; ER: endoplasmic reticulum; FA: fatty acid; FABP: fatty acid binding protein; FAS: fatty acid synthase; Glut 1: glucose transporter 1; GPA: glycerol-3 phosphate acyl transferase; LPL: lipoprotein lipase; MFG: milk fat globule; SCD: stearoyl-CoA desaturase; TAG: triacylglyceride; VLDL: very low density-lipoprotein

In early lactation, the energy needs of the liver increase due to a 2-3 fold increase in gluconeogenesis for lactose production in the mammary gland, since milk yield depends on lactose synthesis. In addition, the energy needs of the mammary gland increase for TAG synthesis. The majority of the energy in need is primarily met from β-oxidation of FAs stored in adipose tissue or transferred by the blood, thus leading to an increase of acetyl-CoA (Vernon, 2005). A key component in the lipid metabolism and β-oxidation is the enzyme acetyl-CoA acyltransferase 2 (**ACAA2**). ACAA2 catalyzes the last step of FA β-oxidation (**Figure 1.8**), a pathway providing the citric acid cycle with acetyl-CoA and NADH for the production of energy, ketone bodies and of the intermediates from which glucose is formed (Eaton et al., 1996; Nelson and Cox, 2005). The *ACAA2* gene that encodes the relative enzyme is located in an area where quantitative trait loci (**QTL**) for milk, fat and protein yields map in sheep (Gutierrez-Gil et al., 2009). The detection of a SNP in the 3' untranslated region (**UTR**) of the *ACAA2* gene was associated with milk yield in the Chios sheep breed (Orford et al., 2012).

The presence of acetyl-CoA as a substrate, with two other key enzymes, acetyl-CoA carboxylase (**ACC**) (encoded from the *ACACA* gene) and fatty acid synthase (**FAS**) (encoded from the *FASN* gene), and a supply of NADH reducing equivalents, is prerequisite for *de novo* FA synthesis (Barber et al., 1997; Shingfield et al., 2008, 2010). Acetate and β-hydroxybutyrate provide the first four carbon units necessary for the FA assembly, where the first is converted into acetyl-CoA and the latter is directly incorporated into FA, after activation into butyryl-CoA (**Figure 1.7**). The conversion of acetate to acetyl-CoA takes place in the cytosol by ACAA2, where after a rate-limiting step catalyzed by the enzyme ACC, it is then converted into malonyl-

CoA, a key molecule for chain length extension during the FA synthesis (Shingfield et al., 2008, 2010).

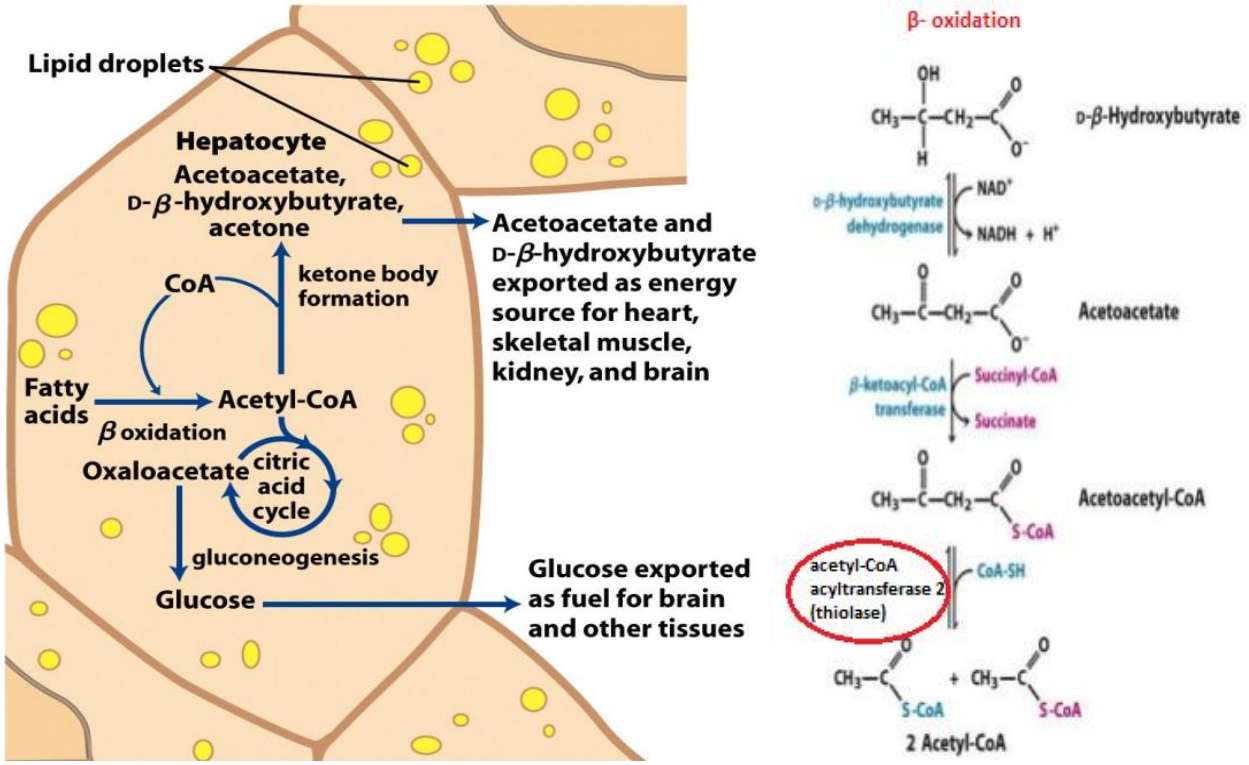


**Figure 1.8.** Combination of metabolic paths with fatty acid involvement (adapted by Nelson and Cox, 2005)

*FASN* gene encodes FAS, a large complex of a multifunctional enzyme, which catalyzes the *de novo* synthesis of small- to medium-chain FAs, through chain elongation (Bionaz and Loor, 2008). The acetyl-, butyryl-, and malonyl-CoA are condensed within the FAS enzyme, and by the additional loading of malonyl-CoA groups, FA chain elongates. Bovine mammary gland results in secreting a range of SCFA and MCFAs due to the ability to release FAs from the synthase complex (FAS) at different stages (Shingfield et al., 2008). Furthermore, in dairy cows, the *FASN* gene is found to affect the milk fat percentage and FA content (Schennink et al., 2009). In sheep, a QTL on the OAR11, where the *FASN* gene harbors, was associated with C14:0, C16:0 (Carta et al., 2008), C10:0 and the PUFA content (García-Fernández et al., 2010b) while, polymorphisms in the *FASN* gene were shown to affect the FA profile in sheep breeds (Crisa et al., 2010).

As previously mentioned, LCFA (16 and more carbon atoms) end up in mammary gland from absorption in the small intestine (from diet FA and FA synthesis in the rumen by microbes), and mobilization of body fat (Noble, 1978; Chilliard et al., 2000a). Mammary endothelial cells have the LPL enzyme on their surface (**Figure 1.7**). LPL hydrolyzes FAs transported as TAG fractions in VLDL or chylomicrons, and therefore, FAs are released from the lipoprotein core (Bionaz and Loor, 2008; Shingfield et al., 2010), via a selective cleavage at *sn*-1(3) position (Barber et al., 1997). LPL exhibits higher activity in the mammary gland compared to other tissues due to the upregulation during lactation (Bionaz and Loor 2008; Shingfield et al., 2010). Another gene, the *endothelial lipase* (***LIPG***), which belongs in the same TAG lipase gene family as *LPL* and *hepatic lipase* (***HL***), encodes the enzyme endothelial lipase (**EL**) (Hirata et al., 1999; Jaye et al., 1999). In sheep, *LIPG* gene maps on OAR23, just next to the *ACAA2* gene,

where suggestive QTL for milk, fat, and protein yields were detected (Gutiérrez-Gil et al., 2009). Although EL has similarities with the rest family enzymes, it stands out as the only lipase synthesized in endothelial cells, compared to LPL synthesized by parenchymal cells. Additionally, EL is an enzyme with much higher phospholipase activity, with significant role in HDL metabolism, compared to the other enzymes in the lipase gene family (Jaye et al., 1999; McCoy et al., 2002; Holmes et al., 2011; Yu et al., 2018).

According to knowledge from experiments with rodents and rodent cell lines, and the limited data based on ruminant studies, dietary and physiological factors (e.g., diet-derived PUFA, glucose and hormones) regulate gene expression and the amount of the produced enzyme (Palmquist et al., 2005; Shingfield et al., 2008). The same applies to the enzyme SCD also known as Δ-9 desaturase, a complex enzyme also found in mammary epithelial cells. SCD introduces a *cis* double bond at position nine, between the 9th and 10th carbon atoms, by oxidation of fatty acyl-CoA esters in a hypothesized attempt to regulate milk fluidity (Moioli et al., 2007a; Shingfield et al., 2008, 2010). SCD preferentially desaturates palmitoyl- and stearoyl-CoA into the monounsaturated palmitoleoyl- and oleoyl-CoA, respectively (Enoch et al., 1976; Bernard et al., 2008; Ntambi, 1999; Palmquist et al., 2005; Shingfield et al., 2008). Approximately 60% of the oleic acid secreted in milk originates from the desaturation of the available stearic acid (49-60%) for TAG synthesis in the mammary cells (Enjalbert et al., 1998; Shingfield et al., 2010). SCD also uses several more SFA as substrates (e.g., C10:0, C12:0, C14:0, C15:0, and C17:0) resulting in their monounsaturated products. In bovine milk fat, approximately 90% and 50-56% of the C14:1 *cis*-9 and C16:1 *cis*-9, respectively, derived from the SCD action (Shingfield et al., 2010). Similarly, 64% to 97% of C18:2 *cis*-9, *trans*-11 (RA)

secreted in bovine milk is the outcome of SCD action on C18:1 *trans*-11 (VA) (Palmquist et al., 2005; Shingfield et al., 2010). QTL studies in sheep breeds identified a QTL for the ratio of CLA/VA on OAR22. However, for the two studied sheep breed populations, Sarda x Lacaune and Churra, the QTL was found at a different position, whereas for the Sarda x Lacaune breed, the position of the QTL corresponded to that of the *SCD* gene (García-Fernández et al., 2010a). Moreover, a genome-wide association study of milk FAs in Dutch dairy cattle identified a SNP in the QTL region of the *SCD* gene on BTA26 (and in *DGAT1* gene-BTA14) known to strongly affect the medium-chain FAs and UFA (Bouwman et al., 2011).

TAG synthesis in mammary epithelial cells utilizes a pool of either de novo synthesized or dietary FAs (Shingfield et al., 2010). The FA distribution on the glycerol backbone is not a random fact in TAG assembly and highlights the importance of the responsible genes in the lipogenesis processes (Bernard et al., 2008). The esterification process of FAs on glycerol backbone is mediated via the glycerol-3-phosphate pathway. The first step in TAG synthesis is the acylation at the sn-1 position for the formation of lysophosphatidic acid (1-acyl-3-phosphoglycerol). The enzyme glycerol-3 phosphate acyltransferase (**GPAT**) catalyzes the esterification process of phosphoglycerol-3 with an acyl-CoA. GPAT uses equally long-chain saturated and unsaturated fatty acyl-CoAs as substrates for the sn-1 position (Palmquist, 2006; Bernard et al., 2008; Eenennaam and Medrano, 2008). The subsequent step is the esterification at the sn-2 position by the enzyme 1-acylglycerol 3-phosphate acyltransferase (**AGPAT**). AGPAT shows a predominance for UFA substrates in the sn-2 position; in particular, in milk MCSFA and LCSFA (C12:0-C18:0) predispose in the sn-2 position. DGAT1 is the enzyme catalyzing the last step in the esterification procedure, with a high proportion of SCSFA being esterified in the sn-3

position of milk TAG (Palmquist, 2006; Bernard et al., 2008; Shingfield et al., 2010). Jensen (2002) has reviewed the distribution of the major FAs on the glycerol backbone in the cattle. Mostly all of the C4:0 and C6:0, approximately 50% of C8:0, and around 30% and 40% of the C18:0 and C18:1, respectively, are esterified in the sn-3 position. A high proportion (44-62%) of the MCSFA and LCSFA (C8:0-C16:0) are esterified in the sn-2 position. The MCSFA C16:0 exhibits an equal distribution between sn-1 and sn-2, while 56% of the saturated C18:0 and 60% of the unsaturated C18:1 are located in the sn-1 position (Jensen, 2002).

DGAT1 comprises the only enzyme specific to TAG synthesis with a significant regulatory role. A mutation (K232A) in *DGAT1* gene, extensively studied in dairy cattle, was highly correlated with milk fat (Winter *et al.,* 2002; Grisart *et al.,* 2004; Schennink *et al.,* 2007; Vanbergue *et al.,* 2016) and milk FA content (Schennink *et al.,* 2007, 2008; Bouwman *et al.,* 2011; Houaga *et al.,* 2018). Furthermore, other polymorphisms within the bovine *DGAT1* gene in linkage disequilibrium with the K232A mutation have an association with milk FA content (Li *et al.,* 2014; Palombo *et al.,* 2018). In Dutch dairy cattle, *DGAT1* (along with the *SCD* gene) explained a large portion of the total additive genetic variation for C10:1, C12:1, C14:0, C14:1, C16:0, C16:1, C18:1 and CLA (Bouwman et al., 2011). Studies contacted in sheep, identified a QTL (OAR9) in the homologous region to the QTL in the bovine chromosome (BTA14) (Barillet et al., 2005), while the exon harbouring the K232A **SNP** is monomorphic in some breeds (Scata *et al.,* 2009; Miltiadou *et al.,* 2010). Studies suggest that two SNPs outside the coding region of the ovine *DGAT1* gene have an association with milk fat content (Scata *et al.,* 2009), while a SNP in exon 17 affect milk FA traits in Assaf sheep (Dervishi *et al.,* 2015).

### 1.7. Scope of the thesis

Many Mediterranean countries are known for the production of high quality cheese mainly made from local dairy breeds of small ruminants, thus the dairy sheep and goat industry in these countries play a pivotal role to their economies. Farmers' income mainly depends on milk yield and total solids which determine cheese yield, while the milk FA profile affects the sensory and textural characteristics of cheese and is strongly related to human health issues. Many factors are known to affect milk yield and content. Ruminant diet comprises the most crucial factor affecting milk traits and many studies supplementing ruminant feed with various substrates have highlighted the improvement of milk quality. Additionally to diet, although more difficult to control, genetics is another factor affecting milk production traits. Through the years, many studies in ruminants tried to unravel the functional role of various genes and their association with milk traits. The present work (**Figure 1.9**) aimed to evaluate the effect on milk composition caused by including ensiled olive cake in the diets of the predominant local dairy sheep breed in Cyprus, named Chios (**Chapters 2 and 3**). The study also aimed to identify variations in five candidate genes for milk traits and their association with milk production and composition traits in Chios sheep (**Chapters 4-6**). The following candidate genes, *ACAA2*, *FASN*, *DGAT1*, *SCD1* and *LIPG*, were selected according (i) to their function implicated with lipid metabolism, (ii) their position in chromosomes where QTL for dairy traits were detected to map and (iii) their expression levels or association studies between variations or SNPs with milk production traits and fatty acid content in milk.

**Chapters 2** and **3**, describe the effects on milk quality caused by the inclusion of an ensiled agro-industrial by-product, the ensiled olive cake, in Chios sheep diets. In particular,

**Chapter 2**, provides novel experimental data using nuclear magnetic resonance (NMR) fingerprinting, suggesting that the use of ensiled olive cakes in sheep rations induces desirable changes in ovine milk lipids. The present study used NMR spectroscopy as it is a powerful technique with an increasing use in dairy research and NMR methodologies have been proposed as a rapid and non-destructive technique for the identification and quantification of major FAs groups (saturated, unsaturated, monounsaturated and polyunsaturated FAs) as well as minor FAs. In this chapter, the rapid NMR technique was used as a tool for a routine analysis of milk fat, which can be used complementary to gas chromatography.

In **Chapter 3**, gas chromatography was the tool analyzing the FA content in milk and related Halloumi cheese fat. Compared to NMR spectroscopy, gas chromatography provides the full profile of the lipids from the milk and cheese produced. Thus, in this study, novel data are presented on the FA composition of these ovine dairy products when Chios sheep are fed ensiled olive cake at different rates, namely 10 and 20% on DM basis of their diets, investigating their possible beneficial effects regarding the content of health related FA.

In the next chapter, **Chapter 4**, the *ACAA2* gene was selected due to its functional role and because of a variant detected in the ovine 3'UTR, which explained 10% of the genetic variance for milk yield in Chios sheep. Based on the lack of research on the *ACAA2* gene and its functional role for energy supply, the specific gene was genotyped for the particular SNP in the 3'UTR for two independent purebred Chios sheep populations in mid-lactation; one in Cyprus consisting of 742 ewes and one in Greece consisting of 632 ewes. The lactation records for both

populations were collected during 8 and 7 successive years in Cyprus and Greece, respectively. The potential association of the gene with important sheep traits was investigated.

In **Chapter 5**, four ovine candidate genes were selected according to their functional role in lipid metabolism, position in chromosomal regions associated with milk content and research performed in ruminants, mainly in cattle. The *DGAT1*, *FASN* and *SCD* genes were selected according to studies demonstrating effects to milk fat and FA content of milk in cattle or other sheep breeds. The *ACAA2* was also selected due to the reasons mentioned in **Chapter 4**. The main objective of the current work was the detection of polymorphisms in the coding and non-coding regions of the aforementioned genes which could be associated with milk traits, particularly milk production and content and consequently cheese production, as well as milk fat composition affecting the quality of dairy products with specific FAs having potential health benefits for human. The four genes were studied in a population of 429 Chios sheep from which three consecutive milk samplings were obtained.

**Chapter 6** investigated another putative positional and functional candidate gene, the *LIPG* gene. In particular, *LIPG*, a TAG lipase gene, was chosen according to its functional role and vicinity with the *ACAA2* gene already associated with dairy traits. The main objective of the chapter was the molecular characterization of the gene with direct sequencing of all ten exons and partial 5' and 3' UTRs, for future investigation of possible association with milk traits.

The last chapter, **Chapter 7**, summarizes the main findings of the current research work and briefly discusses the main conclusions.

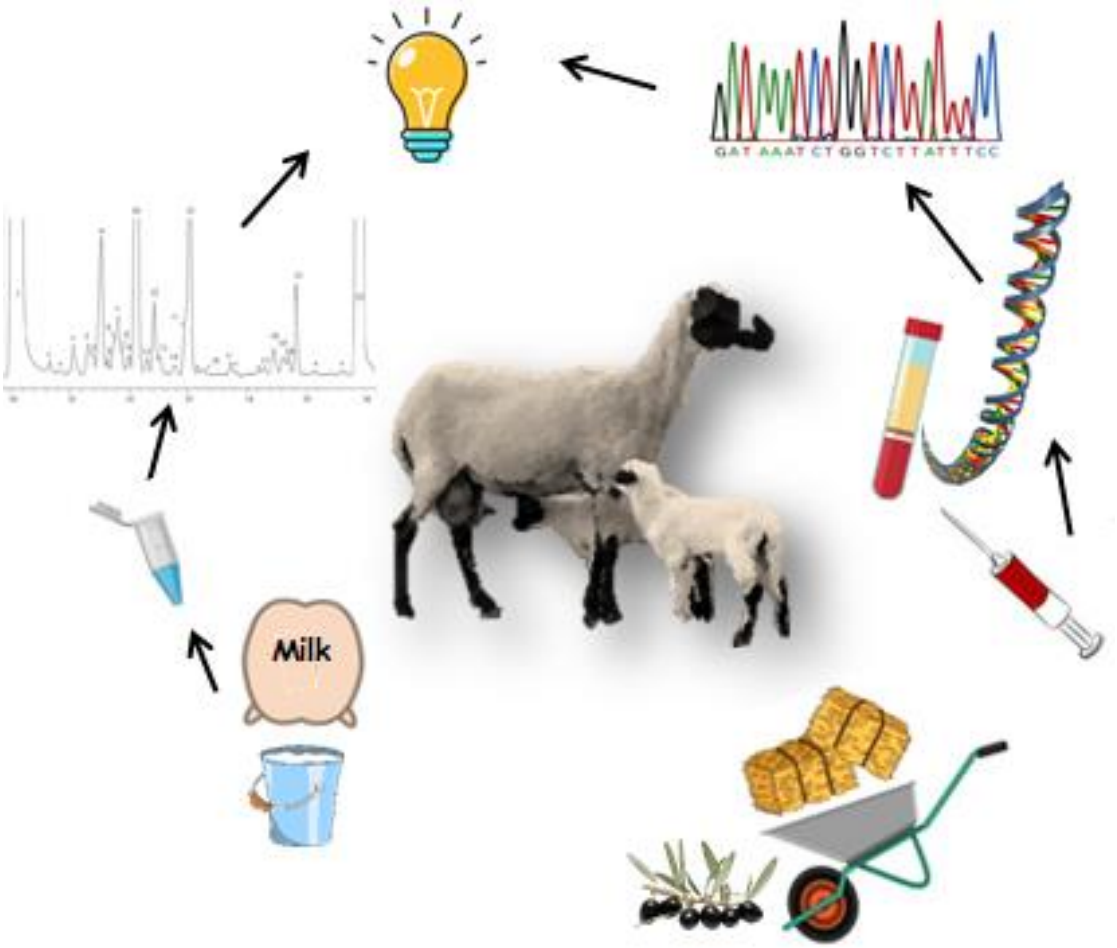


**Figure 1.9.** Schematic representation of the central idea for the present study

After including different quantities of ensiled olive cake in the diets of the predominant local dairy sheep breed in Cyprus, named Chios (**Chapters 2 and 3**), the study aimed to evaluate its effect on milk production traits and milk fatty acid content by NMR and GCMS analysis**.** The study also aimed for the molecular characterization of five candidate genes for milk traits with DNA sequencing and Taqman RT-PCR for the identification of variations and their association with milk production and composition traits in Chios sheep (**Chapters 4-6**).

# CHAPTER 2

## Nuclear magnetic resonance screening of changes in fatty acid and cholesterol content of ovine milk induced by ensiled olive cake supplementation in Chios sheep

**A slightly shorter version of this chapter has been published as:**

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**Abstract**

Lipid supplementation of dairy ewe diets has been shown to introduce changes in the fatty acid (**FA**) profile and cholesterol content of milk. The present study supplemented sheep diet with ensiled olive cake (**OC**), a conserved byproduct of olive oil production, and investigated the lipid content of lyophilized ovine milk under the employment of $^{1}$H nuclear magnetic resonance (**NMR**) technique. Thirty lactating Chios ewes were allocated into three groups of ten animals fed isonitrogenous and isoenergetic diets with different ensiled OC supplementation: G0 with 0%, G500 with 7.2% **DM** (500g on fresh weight basis) and G1000 with 14.2% DM (1000g on fresh weight basis) ensiled OC inclusion per ewe, per day. Fat percentage, FA profile and cholesterol content were determined in milk samples collected after a 4-week supplementation period. Results indicated that saturated FAs were reduced by 7.2% and 11.3% for the supplemented animals of the G500 and G1000 groups, respectively. Supplementation increased unsaturated FAs content by 26.3% for G500 and 41.4% for G1000 group and monounsaturated FAs content of milk by 30.3% for G500 and 45.6% for G1000 group. Furthermore, the inclusion of 1000 g ensiled OC significantly increased the content of potentially beneficial for human health FAs such as conjugated linoleic acids (**CLA** isomers: 9-*cis*, 11-*trans* CLA; 9-*trans*, 11-*cis* CLA and 10-*trans*, 12-*cis* CLA) by 47.2 % and linoleic acid by 14.4% compared to control group. However, any rate of ensiled OC inclusion in the diets of ewes had no effect on milk cholesterol content. Neither milk yield nor milk fat percentage of ewes were affected by the feeding treatment. Overall, the present work provides novel experimental data with NMR fingerprinting of the lyophilized milk lipid fraction, and suggests the use of ensiled OC in sheep rations since it induces desirable changes in ovine milk lipids.

## 1. Introduction

The study of milk lipid fraction has attracted the interest of research and industry alike, due to its contribution to the textural/ sensory characteristics of dairy products and due to health-relating properties. Epidemiological studies reported that fatty acid (**FA**) groups such as the monounsaturated fatty acids (**MUFA**) and polyunsaturated fatty acids (**PUFA**) are associated with human health benefits, while the saturated fatty acids (**SFA**) are related to cardiovascular diseases (Li et al., 2015). Specific FAs in milk, such as conjugated linoleic acid (**CLA**), has been linked with the prevention of chronic diseases, namely obesity, atherosclerosis, osteoporosis and cancer (Dilzer and Park, 2012), while cholesterol content in foods is strongly appreciated by consumers as a preferably avoidable risk factor in respect to cardiovascular diseases (Lewis et al., 2004).

Although genetic or management/ physiological factors affect milk lipids, animal nutrition comprises the most important determinant that can be manipulated in order to improve milk FA composition (Shingfield et al., 2013). Thus, many fat sources have been used with the scope to enrich ovine milk with beneficial for human health FA through feed supplementation with vegetable oils such as olive, soybean (Bodas et al., 2010), linseed (Mele et al., 2011; Gallardo et al., 2015) and sunflower oil (Castro-Carrera et al., 2015). However, limited attention has been given to exploit olive cake (**OC**), a by-product of olive oil production that is produced in vast quantities in Mediterranean countries, as feed supplement that may improve the lipids of milk. The few studies investigating ewe feed with olive cake by-product used previously processed material such as dried (Abbeddou et al., 2011a, 2011b and 2015), pelleted with extruded

linseed and alfalfa hay (Mannelli et al., 2018), partly destoned and dried (Vargas-Bello-Perez et al., 2013) and partly destoned fresh OC (Chiofalo et al., 2004), reported decreased SFA and increased MUFA in ewe milk fat, as well as varied results on specific FAs such as CLA or linoleic acids. Though, no data where provided regarding the cholesterol content of ovine milk under different OC forms of supplementation and this is not surprising since there is scarcity of studies on the effect of any fat supplementation of ruminant diets on milk cholesterol content (Gomez-Cortes, 2015). Reklewska et al. (2002) reported a significant decrease in cholesterol content of cow milk and an increase of unsaturated fats when linseed was supplemented. Gomez-Cortes et al. (2015) tested different oil supplementation (from palm, olive, soybean and linseed) to sheep diets on cholesterol content of cheeses and reported different effect depending on the source of oils added. This agrees with previous works of Altenhofer et al. (2014) in bovine milk who reported different results on milk cholesterol content when different sources of oils (from soybean or rapeseed) were added to cow diets.

The use of fresh OC in animal feeding is constrained by seasonal availability of olive oil extraction, rapid deterioration and rancidity formation in few days after air exposure due to lipid oxidation rendering this by-product not readily usable for animal feeding. Furthermore, drying or destoning process, proposed by other researchers (Chiofalo et al., 2004; Abbeddou et al., 2011a and 2011b; Vargas-Bello-Perez et al., 2013), add extra costs to the end by-product and time/ labor needs of its incorporation to animal feeds. An effective ensiling method overcoming the difficulties of using fresh or dried OC, are practised in Cyprus allowing the use of this byproduct in animal nutrition throughout the year (Hadjipanayiotou, 1999). However,

the effect of different quantities of this ensiled OC as supplementary feeding on the FA profile and cholesterol content of ovine milk has not been studied in any extent.

Nuclear magnetic resonance (NMR) spectroscopy is a powerful technique with an increasing use in dairy research. More specifically, NMR methodologies (Alexandri et al., 2017) have been proposed to authenticate geographical origin of dairy products (Schievano et al., 2008; Sacco et al., 2009), identify milk mixtures (Lamanna et al., 2011), investigate whole milk without any pre-treatment (Hu et al., 2004), monitor traceability and quality control, detect adulteration, or diagnose animal diseases through milk profiling (Sundekilde et al., 2013) and investigate metabolomics biomarkers of the water soluble fraction of milk (Yang et al., 2016). With regards to milk lipids, NMR methodologies have been proposed as a rapid and non-destructive technique for the identification and quantification of FAs groups (SFA, unsaturated FA (UFA), MUFA, PUFA) as well as minor FAs (Brescia et al., 2004; Tsiafoulis et al., 2014; Papaemmanouil et al., 2015). The objective of the present study was to investigate the effect of the ensiled olive oil byproduct, as a means of improving the FAs profile and cholesterol content of Chios sheep milk, using a rapid NMR protocol developed (Tsiafoulis et al., 2014; Papaemmanouil et al., 2015) as a tool for a routine analysis of milk lipids.

## 2. Materials and methods

### *2.1. Animals and experimental diets*

Thirty second parity pure bred Chios sheep in mid-lactation were used in the present study. The experiment took place at a private farm at the Limassol area (Avdimou, Cyprus) and all experimental procedures were carried out according to the regulations of the national

legislation (Animal Welfare Law, 1994) and approved by the corresponding departmental committee. All sheep weighing on average (± SEM) 48.3 (± 0.74) kg, at the start of the feeding trial, were housed indoors and randomly allocated in three balanced feeding groups of ten animals supplemented with ensiled OC or kept as control. This silage, made from crude OC, constituted from a mixture of skins, pulp, woody endocarp and seeds obtained after the extraction of olive oil from olives, which was collected by a three stage oil mill, and ensiled according to the method developed by Hadjipanayiotou et al. (1999). Immediately after olive oil extraction, crude OC was collected and accumulated on the surface of a clean and hard floor of the silos. The pile of OC was covered with a black polyethylene film without any additives or other feed material and firmly closed. The film covering the pile was stretched so the air was expelled and soil was used to cover the edges of the film. The ensiled OC was fermented in the silos for 3 to 4 months prior to use with no need of additives to improve silage fermentation. The ensiled olive cake was included in different ratios forming three isoenergetic and isonitrogenous diets as follows: (a) group G0 comprised the control, with no inclusion of ensiled OC, (b) group G500 had a daily inclusion of 500 g fresh OC per ewe, corresponding to 7.2% of DM intake and (c) group G1000 had a daily inclusion of 1000 g fresh OC per ewe corresponding to 14.2% of DM intake. The other ingredients of the diet on offer are shown in **Table 2.1**, forming similar feeding rations among treatments that covered the animal requirements for milk production in the Chios sheep breed (Economides, 1986). The chemical composition of diets, including OM, CP, crude fibre, NDF, ADF and ether extract were measured according to the standard methods of AOAC International (2005). Animals were group fed the different experimental diets and the feed consumption was measured during week four of the diets and

**Table 2.1.** Feed ingredients and chemical composition of the experimental diets G0, G500 and G1000 allocated to Chios ewes for four-week supplementation period.

| | Experimental Diets | | |
|---|---|---|---|
| Item | G0 | G500 | G1000 |
| Ingredients, g/kg of DM | | | |
| Barley hay | 105 | 104 | 106 |
| Cereal Straw | 131 | 65 | 0 |
| Barley crop silage | 262 | 261 | 264 |
| Barley grain | 219 | 218 | 215 |
| Corn | 103 | 102 | 101 |
| Soybean meal (47% CP) | 82 | 81 | 80 |
| Sunflower meal (35% CP) | 47 | 45 | 44 |
| Wheat bran | 42 | 42 | 39 |
| Olive cake silage | 0 | 72 | 142 |
| Vitamins & minerals mix | 9 | 9 | 9 |
| | | | |
| Composition g/kg of DM | | | |
| OM | 943 | 945 | 947 |
| CP | 131 | 131 | 139 |
| Crude Fibre | 250 | 257 | 280 |
| NDF | 461 | 460 | 488 |
| ADF | 317 | 329 | 374 |
| Ether extract | 20 | 26 | 32 |
| Energy, NEL Mj/kg DM | 6.61 | 6.57 | 6.54 |
| | | | |
| Fatty acid composition, % total fatty acids | | | |
| 12:0 | 0.89 | 0.60 | 0.48 |
| 14:0 | 1.16 | 0.78 | 0.50 |
| 16:0 | 19.40 | 17.67 | 15.25 |
| 16:1 | 0.00 | 0.41 | 1.23 |
| 18:0 | 3.83 | 3.57 | 4.93 |
| cis-9 18:1 | 23.22 | 35.92 | 58.96 |
| 18:2 n-6 | 38.54 | 31.24 | 13.47 |
| 18:3 n-3 | 10.36 | 6.94 | 2.37 |

NEL: net energy lactation calculated according to NRC (2001)

the feed consumption was measured during week four of the experiment. The ensiled OC supplement in both G500 and G1000 treatments was offered immediately after morning milking and ewes consumed it completely within about 15 min. The formulated feeds were then offered to the animals twice a day (morning and evening feeding). Water was provided *ad libitum*.

### *2.2. Measurements, samples and lipid isolation*

All thirty Chios ewes were machine milked twice daily (at 6.30 am and 6.30 pm) in a 2 X 12 stall – milking parlor (Fullwood Ltd., Shropshire UK). Ewe BW and individual milk yield was measured at the start and the end of the experiment (days 0 and 28). Following the morning and afternoon milking at week four of the feeding trial (day 28 of the experiment) raw milk samples of 50 mL were taken from each ewe and immediately transferred to the laboratory for the determination of the milk lipid content. Total milk fat percentage was obtained by the use of combined thermo-optical procedures (Lactostar 3510, Funke Gerber, Berlin, Germany) calibrated previously with the Gerber method 989.05 (AOAC International, 2005), while a milk subsample was stored at -80°C until lyophilized for further analyses by NMR for the determination of FAs and cholesterol content.

For the determination of the feed FAs profile, a Soxtec 2050 semi-automated solvent extractor (Foss Tecator AB, Hoganas, Sweden) was used to extract the fat according to Robinson et al., (2008) with slight modifications. Petroleum ether was used to extract the fat from homogeneously ground feed samples weighted at 3 g and placed in a cellulose extraction thimble. This was covered with a thin layer of defatted cotton to avoid splashing petroleum

ether during reflux. This thimble was attached to the metal ring and placed into the extractor. Tared aluminum cups, previously oven dried for 30 min, were placed below the thimbles to collect the extracted lipids. Initially, 90 mL of petroleum ether were added to each thimble, heat was supplied with a temperature set at 90$^{o}$ C to reflux the petroleum ether and extract the lipids from the feeds. The automated extraction programming consisted of the sample being in contact with boiling petroleum ether for 20 min, a sample rinsing stage of 40 min and a recovery stage of 10 min. After extraction, appropriate volume containing 20 mg of fat removed to a clean tube and solvent was removed by blowing a stream of nitrogen gas. Then the fat was dispersed in 1 mL of n-hexane by shaking. FA methyl esters were prepared by transesterification with methanolic potassium hydroxide according to the ISO (5509, 2000) method with slight modifications as described below. A volume of 40 µL of 11.2% KOH in methanol was added in tubes containing 1 mL of extracted lipid and shaken vigorously for 1 min. After 5 min incubation at room temperature 0.1 g of sodium bisulfate was added. Centrifugation at 350 × *g* for 3 min at room temperature followed, the particulate material was removed and aliquots of the clear organic phase were transferred to amber gas chromatography vials and stored at −70°C until analyzed (Tzamaloukas et al., 2015). Feed FAs profiles were generated by analyzing the FAME samples on a Shimadzu GCMS-QP2010 Plus Gas Chromatography mass spectrometer equipped with an HT280T auto sampler following the method of Kramer et al. (2008) and FAMEs were separated with a split ratio of 1:20 using a CP-Sil 88 fused-silica capillary column (100m x 0.25 mm i.d. x 0.2 µm film thickness). Chromatographic profiles were analysed using Shimadzu GCMS Solution software, by both comparison of their retention indices to those of a 37 FAME standard mix (Sigma Aldridge), by

using the NIST 08 and 21 mass spectral libraries and thus individual peaks were identified and quantitated by peak integration and individual FAMES expressed as a percentage of the total (**Table 1**).

For the preparation of samples for NMR measurements, the lipid fraction was extracted from lyophilized milk samples according to the Bligh and Dyer method optimized to effectively extract CLA isomers, as proposed by previous work (Tsiafoulis et al., 2014). Briefly, a quantity of 200 mg of lyophilized milk were used for extraction with the addition of methanol/ chloroform mixture (1/2, vol/vol). The chloroform fraction was collected and the solvent was evaporated under vacuum. The residue was re-dissolved using 600 μL of $CDCl_3$ (99.8%, Deutero, Kastellaun, Germany), including a concentration of 0.28 mM hexamethylcyclotrisiloxane (98%, Sigma-Aldrich, USA) which was used as an internal standard.

### *2.3. Nuclear magnetic resonance measurements*

NMR measurements of the lipid profile in lyophilized ovine milk were performed as described previously (Tsiafoulis et al., 2014). The lipid fraction of lyophilized milk was transferred in a 5mm NMR glass tube. The NMR experiments were carried out on a Bruker AV500 spectrometer equipped with a TXI cryoprobe (Bruker Biospin, Rheinstetten, Germany) which was controlled by software TopSpin 2.1 under the application of the followed experimental conditions: relaxation delay 5 s, acquisition time = 4.3 s, number of scans = 256. The line broadening factor applied for the enhancement multiplication of the spectra was 0.3 Hz, whilst, the size of the Fourier transformation was 64k. The phase and baseline were corrected for all the NMR spectra. For all samples, probe head temperature was kept at 298 K. Finally, the chemical shifts

are expressed in *δ* (parts per million) referenced to the internal standard peak, hexamethylcyclotrisiloxane at 0.173 ppm. The major and minor resonances of FA, as well as the resonance of cholesterol, which have a typical pattern in the $^{1}$H NMR spectrum of a fat sample, were assigned according to previously published data allowing for the quantitation of the composition of the SFA, UFA, MUFA, linolenic acid (C18:3 9-*cis*, 12-*cis*, 15-*cis*), linoleic acid (C18:2 9-*cis*, 12-*cis*), CLA (9-*cis*, 11-*trans* CLA; 9-*trans*, 11-*cis* CLA and 10-*trans*, 12-*cis* CLA) and cholesterol in the lipid fraction of lyophilized milk (Brescia et al., 2004; Tsiafoulis et al., 2014; Alexandri et al., 2017), based on mathematical equations described in section *2.4*. The effect of ensiled OC inclusion in the feed of ewes was studied by comparing firstly the $^{1}$H-NMR fingerprint of the lipid fraction in order to detect qualitative differences and secondly, quantitation of individual FAs and cholesterol to pinpoint quantitative differences. **Figure 2.1** illustrates a typical $^{1}$H-NMR spectrum (500 MHz) of the lipid fraction of lyophilized ovine milk samples for a control group (G0) sample. The most diagnostic resonances in the NMR spectrum, the chemical shift (δ, ppm) and multiplicity of the $^{1}$H-NMR resonances of lipid content are reported in **Table 2.2**. Results of the quality control for the NMR methodology are presented in section *2.4*.

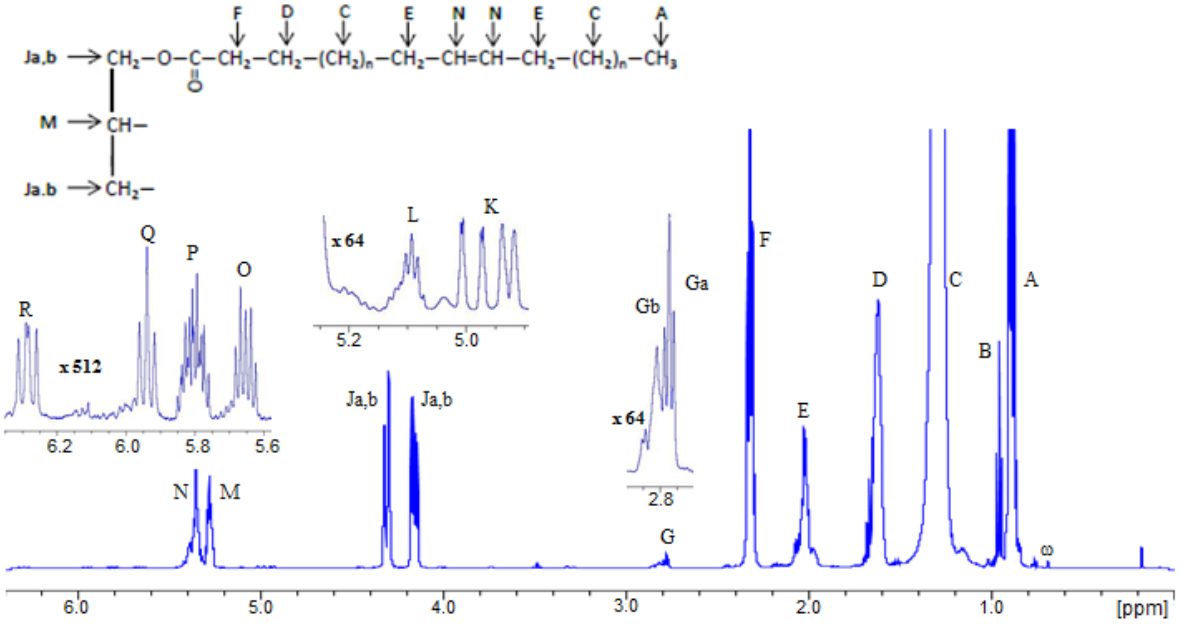


**Figure 2.1.** Typical 500 MHz $^{1}$H nuclear magnetic resonance (NMR) spectrum of the lipid fraction of an ovine milk sample in $CDCl_3$ (T, 298 K; number of scans, 256; acquisition time, 4.3 s; relaxation delay, 5 s; and total experiment time, 40 min). At the bottom, the major lipids are visible, while the insets show several minor lipids that are magnified (for the complete list of symbols see Table 2).

**Table 2.2.** Assignment of compounds of interest in the $^{1}$H-NMR spectrum of the lyophilized sheep milk lipid fraction

| Signal | δ (ppm) | Multiplicity | Compound | Protons |
|---|---|---|---|---|
| ω | 0.68 | s | cholesterol | |
| A | 0.88 | t | all FA (except butyric & linolenic acid) | methyl protons (-C<u>H</u>$_3$) |
| B | 0.95<br>0.98 | t | butyric acid<br>α-linolenic acid | methyl protons (-C<u>H</u>$_3$) |
| C | 1.32-1.27 | m | all FA | methylene protons (-C<u>H</u>$_2$-)$_n$ |
| D | 1.65 | m | all FA | methylene protons (-COCH$_2$C<u>H</u>$_2$-) |
| E | 2.02 | m | | allylic protons (-C<u>H</u>$_2$-CH=CH-) |
| F | 2.33 | t | | methylene protons (-OCO-C<u>H</u>$_2$-CH$_2$) |
| Ga | 2.77 | t | linoleic acid | bis-allylic proton H11 (-CH=CH-C<u>H</u>$_2$-CH=CH-) |
| Gb | 2.81 | m | α-linolenic acid | bis-allylic protons H11 & H14 (-CH=CH-C<u>H</u>$_2$-CH=CH-) |
| Ja,b | 4.15 | dd | *sn*-1 glycerol in TAG | (-C<u>H</u>$_2$-OOC-) |
| Ja,b | 4.28 | dd | *sn*-3 glycerol in TAG | (-C<u>H</u>$_2$-OOC-) |
| K | 4.93<br>4.97 | dd<br>dd | caproleic acid | proton H10a (=C<u>H</u>-)<br>proton H10b (=C<u>H</u>-) |
| L | 5.09 | m | glycerol in DAG | -C<u>H</u>-OOC |
| M | 5.27 | m | *sn*-2 glycerol in TAG | -C<u>H</u>-OOC |
| N | 5.34 | m | | olefinic cis and trans protons (-C<u>H</u>=C<u>H</u>-) |
| O | 5.65 | dt | 9-*cis*,11-*trans* CLA<br>9-*trans*,11-*cis* CLA | proton H12 (-C<u>H</u>=)<br>proton H9 (-C<u>H</u>=) |
| P | 5.80 | m | caproleic acid | proton H9 (-C<u>H</u>=CH$_2$) |
| Q | 5.94 | t | 9-*cis*, 11-*trans* CLA<br>9-*trans*, 11-*cis* CLA | proton H10 (-C<u>H</u>=)<br>proton H11 (-C<u>H</u>=) |
| R | 6.28 | dd | 9-*cis*, 11-*trans* CLA<br>9-*trans*, 11-*cis* CLA<br>10-*trans*, 12-*cis* CLA | proton H11 (-C<u>H</u>=)<br>proton H10 (-C<u>H</u>=)<br>proton H11(-C<u>H</u>=) |

s = singlet; t = triplet; m = multiplet; dd = doublet of doublets; dt = doublet of triplets; FA = fatty acid; DAG = diacylglycerols; TAG = triacylglycerols.

### *2.4. Critical methods for quantification*

Quantification of the linoleic and linolenic acid was based on the integrals of the signals due to bis-allylic protons at 2.79 ppm ($I_{2.79}$) and 2.81 ppm ($I_{2.81}$) using the following equations (Brescia et al., 2004):

$$\text{linolenic acid (\%)} = \frac{3\, I_{2.81}}{4(I_{0.98} + I_{0.88})} \quad (1)$$

$$\text{linoleic acid (\%)} = \frac{3\, I_{2.77}}{2(I_{0.98} + I_{0.88})}, \quad (2)$$

where $I_{0.98}$ is the total integral of the $CH_3$ groups of linolenic acid and butyric acid at 0.95-0.98 ppm and $I_{0.88}$ is the total integral of the $CH_3$ groups at 0.88 ppm.

Quantification of the total (9-*cis*, 11-*trans*) CLA and (10-*trans*, 12-*cis*) CLA was based on the integrals of the signals due to H11 and H10 protons, respectively, at 6.28 ppm, $I_{6.28}$, using the following equation:

$$[(9\text{-}cis, 11\text{-}trans) + (10\text{-}trans, 12\text{-}cis)]\ \text{CLA (\%)} = \frac{3\, I_{6.28}}{(I_{0.98} + I_{0.88})} \quad (3).$$

The percentage of MUFA can be obtained by subtracting the percentages of linoleic acid, linolenic acid and CLA from the total amount of UFA using an equation of Brescia et al. (2004) which was modified as follows:

$$\text{UFA (\%)} = \text{MUFA} + \text{linoleic acid} + \text{linolenic acid} + \text{CLA} = \frac{\frac{I_{2.02} - 2I_{4.97}}{4} + \frac{2I_{4.97}}{2}}{\frac{I_{2.33} - I_{4.97}}{2} + \frac{2I_{4.97}}{2}} = \frac{1}{2}\frac{I_{2.02} + 2I_{4.97}}{I_{2.33} + 2I_{4.97}} \quad (4)$$

where $I_{2.02}$ is the integral of the $C\underline{H}_2$ – CH = CH protons at 2.02 ppm, $I_{2.33}$ is the integral of the $C(2)H_2COOR$ protons at 2.33 ppm, and $I_{4.97}$ is the integral of the H10b of caproleic acid at 4.97 ppm. The second term in equation (4) was introduced to take into consideration that caproleic acid is the only MUFA with a terminal double bond.

SFA content was obtained by subtracting the UFA content from the total FAs using an equation of Brescia et al. (2004) which was modified as follows:

$$\text{SFA (\%)} = \frac{I_{0.88}}{I_{0.98}+I_{0.88}} + \frac{I_{0.98}}{I_{0.98}+I_{0.88}} - \frac{1}{2}\frac{I_{2.02}+2I_{4.97}}{I_{2.33}+2I_{4.97}} \quad (5)$$

Cholesterol content was obtained by using the following equation:

$$\text{Cholesterol (\%)} = \frac{I_{0.68}}{I_{0.98}+I_{0.88}} \quad (6)$$

where $I_{0.68}$ is the integral of the $CH_3$-18 group of cholesterol.

The data presented here are proportions of FAs contributing to the total milk fatty acids content rather than absolute quantities, because this corrects for potential variations in the total fat content of the samples.

### *2.4.1. Results of quality control*

#### *2.4.1.1. Effects of relaxation times*

To obtain optimum accuracy for quantification, the proton spins should fully relax between pulses, demanding recycle times [(acquisition time) + relaxation delay] of at least 5 x $T_1$, where $T_1$ is the longitudinal relaxation time of the slowest relaxing nuclei. Detailed investigation of the longitudinal relaxation time $T_1$ of various samples demonstrated that a recycle time of 10.0 sec was sufficient to achieve a complete relaxation of all nuclei.

#### *2.4.1.2. NMR analytical performance characteristics*

##### *2.4.1.2.1. Precision*

Precision can be expressed by the coefficient of variation (CV) (Danzer, 2007) which was calculated for three samples of the same origin and taking three NMR measurements per

sample. The CV was calculated to be 1.5%, thus, demonstrating the precision and effectiveness of the method.

#### *2.4.1.2.2. Limit of detection*

The limit of detection (LOD) for the 1D NMR experiments was calculated with the use of the Signal-to-Noise Ratio (SNR) method (Maniara et al. 1998) by applying the 3C / (S/N) equation in a standard sample of (9-*cis*, 11-*trans*) 18:2 CLA isomer, where C represents the concentration of the analyte as determined by $^{1}$H NMR. The LOD value was found to be 0.010 mmol $L^{-1}$ (in tube).

#### *2.4.1.2.3. Robustness – Effects of pH and temperature*

The effect of pH was investigated at three pH values: pH = 6.8 (natural pH of the milk samples), 6.5 and 7.1. The % relative deviations for (9-*cis*, 11-*trans*) 18:2 CLA isomer were found to be ≤ ±5%. Extraction experiments were performed at 25$^{o}$, 30$^{o}$ and 35$^{o}$ C to investigate the effect of temperature. The % relative deviations for (9-*cis*, 11-*trans*) 18:2 CLA isomer, were found to be ≤ ±4%. The above results demonstrate the robustness of the method in the temperature range of 25 to 35$^{o}$C and at pH = 6.8 $\pm$ 0.4.

### *2.5. Statistical analysis*

One-way ANOVA and Tukey pairwise comparisons were applied on measurements of BW, milk yield and milk fat as well as to lyophilized milk constituents, group and individual FAs and

cholesterol content, identified from calculated integrals of the specific NMR signals in order to test the effect of the feeding treatment. All statistical analyses were performed using the IBM-SPSS ver. 22 statistical software.

**3. Results**

Comparison of the $^{1}$H-NMR spectra demonstrates significant quantitative changes between the three feeding groups G0, G500 and G1000 in milk lipids as shown in **Figure 2.2** and **Table 2.3**. The total SFA contents in the milk samples were significantly affected by the supplementation showing 7.2% and 11.3% drop in the observed values from both supplemented groups (G500 and G1000, respectively), compared to the control group. The supplemented groups were also characterized by significantly ($P < 0.001$) greater content of total UFA showing 26.3% and 41.4% increase for G500 and G1000, respectively, as compared to the control group. Similarly, MUFA increased ($P < 0.001$) with OC supplementation by 30.3% for G500 and by 45.6% for G1000 groups when compared to the control. However, no differences were observed between the G500 and G1000 feeding groups for the FA groups of SFA, UFA or MUFA.

The results of the feeding treatment on specific FAs, namely CLA isomers (sum of 9-*cis*, 11-*trans* CLA; 9-*trans*, 11-*cis* CLA and 10-*trans*, 12-*cis* CLA), linoleic acid, linolenic acid and cholesterol content are also shown in **Table 2.3**. In particular, the total CLA content in sheep milk increased by 47.2% and 20.5% when animals were supplemented with 1 kg or with 0.5 kg of ensiled OC per day, respectively. However, the observed difference between the G0 and G500 group on CLA content failed to be significant. Furthermore, these differences on CLA content could also be observed qualitatively from the $^{1}$H NMR spectra obtained from the lipid

fraction and could be seen in the three lyophilized milk samples corresponding to G0, G500 and G1000 treatments shown in **Figure 2.2B**. Particularly, the illustration of the CLA resonance in the region of 6.24 to 6.34 ppm shows the increased height of the signals for the feeding groups G500 and G1000, demonstrating the increase of the total CLA isomers (**Figure 2.2C**).

**Table 2.3.** Means (± SEM) of fatty acid groups, linoleic and linolenic acids (g/100g of FA) and cholesterol content of sheep milk (g/100 g of total fat) identified by $^{1}$H nuclear magnetic resonance (NMR) spectroscopy for the three treatment groups G0, G500 and G1000 supplemented with 0, 500 and 1000 g (on fresh weight basis) of ensiled olive cake per day, respectively.

| Lipid Fraction | G0 | G500 | G1000 |
|---|---|---|---|
| SFA | $78.62^{A}$ ± 0.61 | $73.00^{B}$ ± 1.05 | $69.77^{B}$ ± 1.15 |
| UFA | $21.38^{A}$ ± 0.61 | $27.00^{B}$ ± 1.05 | $30.23^{B}$ ± 1.15 |
| MUFA | $18.21^{A}$ ± 0.58 | $23.73^{B}$ ± 0.96 | $26.51^{B}$ ± 1.09 |
| Linoleic | $2.03^{A}$ ± 0.06 | $2.08^{A}$ ± 0.08 | $2.38^{B}$ ± 0.07 |
| Linolenic | $0.57^{a}$ ± 0.02 | $0.51^{b}$ ± 0.01 | $0.52^{ab}$ ± 0.02 |
| CLA | $0.36^{a}$ ± 0.02 | $0.44^{a}$ ± 0.02 | $0.53^{b}$ ± 0.04 |
| Cholesterol | $0.24^{a}$ ± 0.02 | $0.24^{a}$ ± 0.01 | $0.27^{a}$ ± 0.02 |

SFA = saturated fatty acids; UFA = unsaturated fatty acids; MUFA = monounsaturated fatty acids; CLA = conjugated linoleic acid (9-*cis*, 11-*trans* CLA; 9-*trans*, 11-*cis* CLA and 10-*trans*, 12-*cis* CLA). Values with different superscripts within a row are significant different (a, b for $P < 0.05$; A, B for $P < 0.01$).

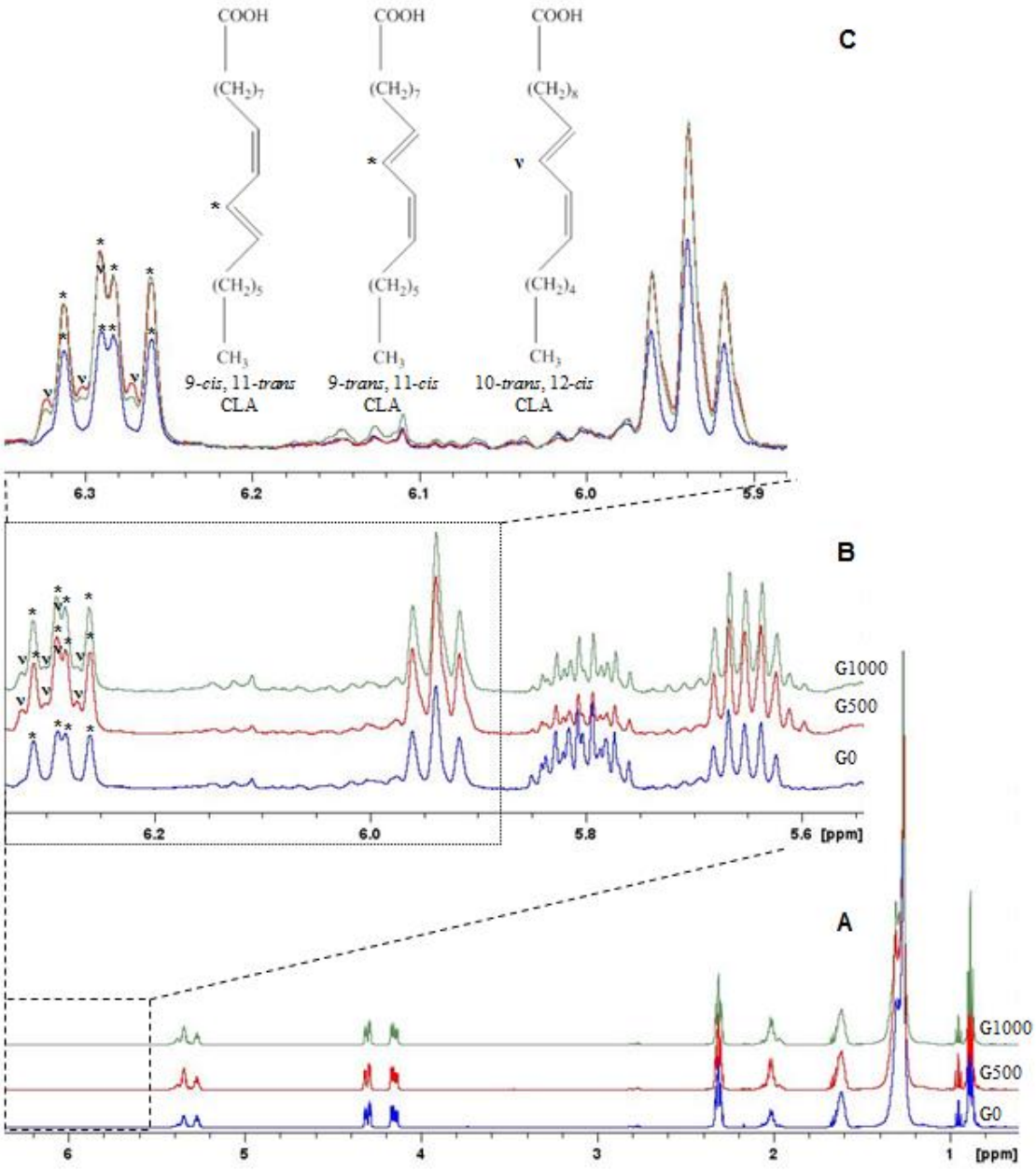


**Figure 2.2.** [1]H nuclear magnetic resonance (NMR) spectra (500 MHz) of the lipid fraction of lyophilized ovine milk lipid samples derived from the following three groups: G0, G500 and G1000 showing a typical pattern of a fat sample (T, 298 K; number of scans, 256; acquisition time, 4.3 s; relaxation delay, 5 s; and total experiment time, 40 min) (part A of the figure). In

the magnified regions qualitative differences between the lipid fractions of the three groups are shown between the areas 5.6–6.34 ppm (part B of the figure) and between 5.9-6.34 ppm (part C of the figure). The symbols indicate the following protons: **v**, **H11** proton of 10-*trans*, 12-*cis* CLA at 6.27ppm, 6.29ppm, 6.30 ppm and 6.32ppm; *****, **H11** proton of 9-*cis*, 11-*trans* CLA and **H10** proton of 9-*trans*, 11-*cis* CLA at 6.26 ppm, 6.28ppm, 6.29ppm and 6.31ppm.

Regarding specific lipids, linoleic acid had similar values in G500 samples, but significantly ($P < 0.01$) increased values in G1000 samples (in average a 14.4% increase) as compared to the control G0 samples. Supplementation with ensiled OC resulted to a reduction of linolenic acid content, in both supplemented groups, significant ($P < 0.05$) though only for the G500 samples. In contrast to the affected FAs, the results from cholesterol content of the ewe milk showed no effect of the 4-week supplementation period with OC and remained similar across all treatments (**Table 2.3**).

Milk fat percentage for the G0, G500 and G1000 groups, did not differ across different feeding regimes and had average values (± SEM) of 4.8 (± 0.17), 4.7 (± 0.11) and 4.6 (± 0.11), respectively. All diets on offer were fully consumed by sheep (with less than 5% leftovers), and we observed that the ensiled OC was very palatable for the ewes since was totally consumed shortly after feed allocation. Milk yield of ewes among the different groups was not affected and the average (± SEM) values of milk were 0.94 (± 0.08), 0.92 (± 0.07) and 1.08 (± 0.08) L per day for the G0, G500 and G1000 feeding groups, respectively. The average (± SEM) measurements for BW at the end of the 4-week feeding trial were 50.7 (± 1.4), 50.8 (± 1.5) and 51.6 (± 1.1) kg for the G0, G500 and G1000 groups, respectively, showing no significant differences between the groups.

**4. Discussion**

In this study, the effect of supplementation with ensiled OC in Chios sheep diets on milk fat was tested with $^{1}$H NMR methodology and found to affect the FA profile but not cholesterol content. More specifically, ensiled OC significantly decreased the concentration of SFA while

increased the MUFA, the total unsaturation of milk fat, and specific FAs, such as total CLA isomers and linoleic acid, which are perceived as beneficial to human health.

The lower SFA content in the OC supplemented groups agrees with previous studies where reduced SFA were reported in the milk of dairy ewes after the supplementation of feed with dried OC (Abbeddou et al., 2011a, 2011b and 2015), partly destoned fresh OC (Chiofalo et al., 2004) and partly destoned dried OC (Vargas-Bello-Perez et al., 2013). Furthermore, the decrease of SFA in the current study was followed by the increase in total UFA and MUFA for the supplemented groups, whereas, similar effects and patterns have been described in feeding studies with ewe supplemented with non-ensiled OC and vegetable oils, with high UFA content. Previous studies with non-ensiled olive cake byproducts reported an increase of total UFA and MUFA in milk ewes (Chiofalo et al., 2004; Vargas-Bello-Perez et al., 2013; Abbeddou et al., 2015), while the administration of different vegetable oils such as olive (Gomez-Cortes et al., 2008b; Bodas et al., 2010), soybean (Bodas et al., 2010), linseed (Bodas et al., 2010; Gallardo et al., 2015) and sunflower oil (Castro-Carrera et al., 2015) have also shown a reduction of SFA and an increase of MUFA and UFA levels in milk fat.

In our study the supplementation with ensiled OC in Chios ewes increased specific UFA such as the total CLA isomer (9-*cis*, 11-*trans* CLA*,* 9-*trans*, 11-*cis CLA* and 10-*trans*, 12-*cis* CLA) and linoleic acid in ovine milk fat. Results from previous studies have shown that the supplementation with dried OC, as opposed to the ensiled OC used in the present study, had no effect on the total CLA content in milk (Abbeddou et al., 2011b and 2015) or yoghurt and fresh cheese (Abbeddou et al., 2011a) of Awassi sheep. These findings may suggest that the OC

preservation method or other experimentation factors may played a role on the CLA results. The effects observed in the current study with increases of linoleic acid in ewe milk fat in OC supplemented animals agrees with previous studies, which used destoned and dried OC (Vargas-Bello-Perez et al., 2013) but not with other studies which reported a reduction (Abbeddou et al., 2011b and 2015) or no change (Chiofalo et al., 2004) in the linoleic acid content of milk fat after feeding dried or partly destoned fresh OC, respectively. Regarding linolenic acid, ensiled OC supplementation in our study resulted in a reduction of this specific FA. Similar results were reported in the study of Vargas-Bello-Perez et al. (2013) with a reduction of linolenic acid in sheep milk and cheese when supplemented with destoned dried OC. However, other studies with dried OC supplementation reported no modification in linolenic acid content of milk (Chiofalo et al., 2004; Abbeddou et al., 2011b and 2015).

The changes in the FAs profile of the sheep milk observed in the supplemented groups with ensiled OC in the present study are attributed to the lipid profile of the supplement, and its possible effects on the uptake of dietary FAs and consequently to their rumen and mammary metabolism. The OC supplement is rich in oleic acid as observed in the present study (oleic content of 36 and 59% of total FA for the G500 and G1000 diet, respectively, as opposed to 23% content of oleic acid in the control G0 diet, **Table 2.1**) and this was observed by other authors as well (Abbeddou et al., 2011b). Thus, it is likely MUFA of feed was transferred to milk FAs content through mammary uptake from the plasma dietary FAs, contributing to higher MUFA of milk observed in the present study. Furthermore, increased unsaturated fat intake leads to *trans*-MUFA formation in the rumen, which are precursors of the CLA isomers, such as 9-*cis*, 11-*trans* CLA, which produced in the udder through the mammary desaturase enzymes (Ferlay et

al., 2017). Therefore, the increase of MUFA, linoleic acid and CLA content in milk of OC supplemented ewes observed in the present study is likely to be due to (a) transfer from the feed, (b) intermediate FAs from rumen metabolism, (c) the mammary desaturase enzyme acting to 18 carbon FAs that have been transferred to the udder after absorption and rumen metabolism (Barber et al., 1997; Shingfield et al., 2013).

Additionally, our study showed no influence on the cholesterol content of ewe milk when animals were supplemented with ensiled OC. To the best of our knowledge, there is no previous study examining the effect of olive oil by-products on cholesterol content of sheep milk. However, few studies exist investigating the effect of fat supplementation on the cholesterol level of ruminant milk and their results suggest an influence of the type of fat of the diet on the milk cholesterol levels (Gomez-Cortes et al. 2015). Thus, when linseed (Reklewska et al., 2002; Gomez-Cortes et al. 2015) or soyabean oil (Altenhofer et al., 2014) were supplemented to animal diets, it resulted to a decrease in milk cholesterol content, while such reduction was not observed when olive oil or rapeseed oil (both rich in oleic acid) were incorporated to the diets (Altenhofer et al., 2014; Gomez-Cortes et al., 2015). Furthermore, when other source of fat, such as palm oil (rich in SFA) was administered to animals, an increase of cholesterol content of dairy products was observed (Gomez-Cortes et al., 2015). These results suggest the differential changes of cholesterol content of milk depending on the unsaturation degree of fat added to the diet. Ensiled OC byproduct used in the present study is also rich in oleic acid and our results agree with olive and rapeseed oil supplementation studies (both rich in oleic) which showed no effect on milk cholesterol content (Altenhofer et al., 2014; Gomez-Cortes et al., 2015) while changed the FA profile of milk when enriched sheep diets

(Gomez-Cortes et al., 2008b; Bodas et al., 2010). Nevertheless, more studies in this concern are needed to elucidate the lipid supplementation effects on cholesterol metabolism and the concomitant cholesterol content of milk and dairy products.

In the current study, BW was not affected by the different diets since all feed ingredients were balanced resulting in isonitrogenous and isoenergetic diets with acceptable fat content and according to production requirements of the ewes (Economides, 1986). These results for BW measurements are in accordance to Vargas-Bello-Perez et al. (2013) and Abbeddou et al. (2015) who reported no effect of dried OC supplementation on intake or BW gain. In contrast, Abbeddou et al. (2011b) reported a lower average daily BW gain with supplementation of dried OC, a result that the authors attributed to overestimated metabolizable energy content of OC and the lack of isoenergetic formation of the experimental diets. Furthermore, in our study, milk yield and milk fat content were unaffected for both supplemented treatments with ensiled OC (G500 and G1000 groups) compared to the control. This is in accordance with Abbeddou et al. (2011a and 2011b) and Vargas-Bello-Perez et al. (2013), while Abbeddou et al. (2015) reported a decrease in milk yield and Chiofalo et al. (2004) an increase in milk yield with different OC byproduct supplementation. These results may be attributed to the different diet formation, preservation method of OC or the protein/ energy content of the experimental diets used in different studies.

With regard to the NMR methodology, although the method cannot identify all individual FAs of milk fat, the NMR protocol applied in the present study is rapid, non-destructive and allows for the identification and quantification of major and minor FAs as well as the

cholesterol content of milk in the same run. This with respect to FAs determination provide the advantage of avoiding time-consuming derivatization steps which might lead to additional isomerization of the geometrical and positional isomers of FAs (Tsiafoulis et al., 2014) and with respect to cholesterol the need for extra methods for its determination in milk fat. Therefore, the NMR methodology could be useful as a quick screening analysis, or used complementary to gas chromatography or other techniques, and may be applied in animal experimentation requiring milk lipid identification.

## 5. Conclusions

The current work describes the effects of an ensiled agro-industrial byproduct, the ensiled OC, on FAs and cholesterol content in milk of Chios sheep by the use of the NMR methodology. From a nutritional point of view, our findings support that ensiled OC could be a valuable cost effective supplementation alternative in ruminant nutrition since it improves the FA profile of ovine milk by increasing health beneficial fats without adversely affecting milk fat percentage or milk yield. Overall, ensiled OC feeding reduced SFA and increased the UFA and MUFA contents of milk fat, while the cholesterol content was not affected. Furthermore, the supplementation of 1kg fresh ensiled OC per ewe could result in the highest contents in total unsaturated fats and particularly the CLA isomers and the essential linoleic acid. These findings support the ensiled OC byproduct use in sheep diets with the scope to improve the lipid content in ovine milk and to assist the management of olive oil wastes in Mediterranean countries. In addition, the present study demonstrates the use of NMR spectroscopy as an applicable routine analytical tool for milk lipids, including cholesterol, in nutritional studies.

**Acknowledgments**

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# CHAPTER 3

## Feeding olive cake silage increases oleic and conjugated linoleic acid content and improves atherogenicity and thrombogenicity indices in ovine milk and related Halloumi cheese

**A slightly shorter version of this chapter has been published as:**

S. Symeou, D. Miltiadou, C. Constantinou, P. Papademas, and O. Tzamaloukas (2021). Feeding olive cake silage up to 20% of DM intake in sheep improves lipid quality and health-related indices milk and ovine halloumi cheese. *Tropical Animal Health and Production*, 53:229.

**Abstract**

Feeding oil-rich products have been suggested as a strategy to enhance beneficial for human health fatty acids (**FA**) in dairy products. This study aimed to evaluate olive cake silage (**OCS**), a well-preserved by-product of olive oil production, on the FA profile of ovine milk and Halloumi cheese. Sixty second-parity purebred Chios ewes in mid-lactation were assigned to three diet treatments (2 lots of 10 animals per treatment) receiving 0%, 10% and 20% of OCS on dry matter (**DM**) basis for three weeks (treatments S0, S10 and S20 respectively). Halloumi cheese was manufactured from fresh raw milk of ewes fed the three different diets. During the trial, milk yield and milk fat, protein and total solids content were recorded weekly and were not affected by the feeding treatments. In contrast, inclusion of 20% OCS on DM basis in the diet of ewes increased the unsaturated FAs in milk by 20%, the monounsaturated FAs by 23%, polyunsaturated FAs by 11%, rumenic acid (CLA *cis*-9, *trans*-11) by 61%, oleic acid by 18%, and consequently reduced quadratically the atherogenicity and thrombogenicity milk indices by 31% and 27%, respectively, compared with the control treatment. Moreover, these differences were carried over to the lipid profile of Halloumi cheese showing, on average, more than 25% increase of unsaturated, polyunsaturated and monounsaturated FAs, with particularly enhanced oleic and rumenic acid content. These changes resulted in reduced atherogenicity by 29% and 45% and thrombogenicity by 23% and 24% of Halloumi cheese made from milk of S10 and S20 diets, respectively. Overall, the applied ensiling method of olive cake by-product produces a feed alternative that can be used as a forage substitution in lactating ewes, improving the FA profile of Halloumi cheese produced without adversely affecting the lactating performance of dairy sheep.

## 1. Introduction

Olive cake (**OC**), or olive pomace, is a by-product of olive oil production, which is a mixture of skins, pulp, woody endocarp, and seeds, and along with the wastewater consist likely environmental pollutants or unwanted by-products that have to be managed (Roig et al., 2006). One suggested way of OC utilization is the inclusion in ruminant diets (reviewed by Molina-Alcaide and Yanez-Ruiz, 2008) as an alternative to low-quality forage, a useful substitution for the Mediterranean and tropical areas with forage shortages. However, the use of fresh OC in ruminant diets is constrained by seasonal availability of olive oil extraction (2 to 3 months period at most) and rapid deterioration (lipid oxidation) occurring shortly after air exposure. Proposed processing methods such as drying (Abbeddou et al., 2011a, 2011b and 2015; Castellani et al., 2017), partly destoned and dried (Vargas-Bello-Perez et al., 2013), inclusion into multi-nutrient blocks (Molina-Alcaide et al., 2010) or pelleted with other ingredients (Castellani et al., 2017; Mannelli et al., 2018), add extra cost, time and labour and therefore possibly outweigh its usefulness as an affordable forage substitution. However, a cost-effective method of OC preservation is practiced in Cyprus through silage preparation using fresh OC, shortly after production, without any processing or additives, allowing extended storage and use throughout the year (Hadjipanayiotou, 1999; Symeou et al., 2019).

The use of OC by-products may be promoted to the farmers and subsequently to the cheese manufacturers due to likely beneficial effects on milk and cheese quality. Previous reports using dried and fresh OC in ewe and cow diets demonstrated an improvement of the fatty acid (**FA**) composition for both milk and corresponding cheese due to an increase of

monounsaturated FAs (**MUFA**) and decrease of saturated FAs (**SFA**) content (Abbeddou et al., 2011a, 2011b and 2015; Castellani et al., 2017; Chiofalo et al., 2004; Symeou et al., 2019; Vargas-Bello-Perez et al., 2018). However, these studies were not unanimous regarding the effect of supplementation on specific beneficial FAs, such as conjugated linoleic acid (**CLA** 18:2 *cis*-9, *trans*-11, rumenic acid-**RA**) content.

The objective of the present study is to investigate the likely positive effect on ovine milk and Halloumi cheese quality with regards to FAs composition when different quantities of olive cake silage (**OCS**), 10 and 20% on DM basis, are included in the diets. As shown from previous studies, these OCS quantities are not expected to affect ewe DM intake or overall performance (Hadjipanayiotou, 1999; Symeou et al., 2019). Thus, we hypothesized that the Halloumi cheese produced with milk from ewes fed OCS would be enhanced in MUFA and possibly in other minor FAs beneficial for human health (i.e. RA), on the expense of SFA content, suggesting a cost-effective method for improving quality of Halloumi cheese.

## 2. Materials and methods

### *2.1. Animals and experimental diets*

Sixty, second-parity purebred Chios ewes (bodyweight 52.4 ± 1.74 kg) in mid-lactation were distributed in 6 pens of 10 animals balanced for milk yield and live weight and allocated at random to 3 experimental feeding treatments (2 pens per treatment) for three weeks with the following inclusion of OCS in the diets: a) with no OCS, (control, S0 treatment), b) inclusion of 10% on DM basis (S10 treatment) and c) inclusion of 20% on DM basis (S20 treatment). For the needs of the current experiment, fresh OC was collected by a three-stage oil mill and ensiled

according to the method described in **Chapter 2** (Symeou et al., 2019). Briefly, olive varieties locally cultivated (Ladolia and Kalamon) where freshly harvested and immediately after olive oil extraction, crude OC was collected and accumulated on the surface of a clean and hard floor of the silos. The pile of OC was covered with a black polyethylene film (8 mil thick) and firmly closed. The film covering the pile was stretched so the air was expelled and soil was used to cover the edges of the film. The ensiled OC was fermented in the silos for 3 to 4 months prior to use with no need of additives to improve silage fermentation (**Chapter 2:** Symeou et al., 2019). The experimental rations were iso-nitrogenous and iso-energetic and formulated to meet ewe maintenance and milk production requirements at the commencement of the trial (Economides, 1986). The diet ingredients for each treatment can be seen in **Table 3.1**. Animals were group fed concentrate and forage offered twice daily in separate feed troughs, after milking, while the OCS in corresponding S10 and S20 groups was offered by hand immediately after morning milking, and ewes consumed it entirely within 15 to 20 min of allocation. Animals were housed indoors, feed residues were collected weekly, and a two-week feed adaptation period was applied. Water was provided *at libitum*. Animal weights were recorded at the beginning, and the end of the experiment. All experimental procedures were carried out according to international guidelines (Directive 2010/63/EU of for animal experimentation), national legislation (Animal Welfare Law, 1994), and approved by the corresponding departmental Ethical Committee before the commencement of the experiment. No animals were euthanized for the current study.

### *2.2. Milk yield, sample collection, and chemical analysis*

The ewes were machined milked twice daily (at 6.30 a.m. and 6.30 p.m.) in a milking parlour (Fullwood Ltd., Shropshire UK), fitted with recording and sampling equipment (Afimilk model AfiFree 155, SAE Afikim Kibbutz, Israel). For milk sample collection, following the morning and afternoon milking, raw milk samples of around 50 mL were taken from each ewe using the fitted milk sampling equipment, placed into falcon tubes in a cool box (4$^{o}$C) and immediately transferred to the laboratory for the determination of chemical content. After mixing the yield from the evening and the morning milking of each animal, milk samples were analyzed for the determination of fat, protein and total solid concentration by the use of combined thermo-optical procedures (LactoStar 3510, Funke Gerber, Berlin, Germany) previously calibrated (989.05/AOAC International 2005) in our laboratory at the Department of Agricultural Science, Biotechnology, and Food Science. For the determination of FAs profile, milk subsamples on day 21 of the trial were stored at -20$^{o}$C for subsequent analysis. For the determination of the chemical composition of OCS and other feed ingredients, representative bulk samples were collected weekly, mixed, and analyzed in duplicate following standard methods of AOAC International (2005).

### *2.3. Halloumi cheese making*

Halloumi cheese was manufactured (Papademas, 2006) on a micro-pilot scale in our laboratory at the Department of Agricultural Science, Biotechnology, and Food Science, according to the CYS94 (1985). Raw sheep milk, obtained on day 21 of the experiment, (fresh bulk milk was obtained at the end of the trial from each feeding treatment) was delivered in the laboratory at

6°C, filtered, and poured in the cheese vat. The milk was heated to 34°C, and non-animal rennet (Chymax, Chr. Hansen Denmark) was added. The milk was left to coagulate for 40 minutes, and once manually tested for desirable firmness, the curd was cut by coagulum cutting knives. The resulted cheese curd was a cube of 2cm side. The curd pieces were collected in a perforated cheese basket (10 cm diameter x 8 cm height) with a holding capacity of 400g. The cheese baskets were left to drain for approximately 60 min, with occasional (2-3 times) turning in order for the curd pieces to fuse and attain a homogeneous shape and form. The whey resulting from the curd cutting was collected back in the cheese vat and heated progressively to 90°C, where denaturation of whey proteins was initiated. The resulting solid layer floating on the surface of the cheese vat was collected by a perforated ladle and placed in cheese baskets. The drained cheese curds were transferred in the hot deproteinized whey and left to cook for about 30 min at 90°C. After cooking, curd pieces were briefly left to cool before a mixture of salt and dry mint was added and manually folding them. Whey-brine (12% w/v NaCl) was prepared, and when Halloumi pieces reached room temperature were placed in the whey-brine container and left overnight at 6°C. Next day individual Halloumi pieces (200-250g) were vacuum packed and kept at 6°C until analysis. Cheese batches were produced in duplicate per feeding treatment, and three cheese samples per batch were analyzed for FA content.

**Table 3.1.** Ingredients and chemical composition of the experimental diets S0, S10 and S20 with olive cake silage (OCS) inclusion at rates of 0, 10 and 20% of DM, respectively. Chemical composition of OCS is also shown

| | Experimental Diets | | | |
|---|---|---|---|---|
| Item | S0 | S10 | S20 | OCS |
| Ingredients, % of DM | | | | |
| Barley hay | 17.71 | 17.54 | 17.37 | |
| Lucerne hay | 8.75 | 8.66 | 8.58 | |
| Barley straw | 18.27 | 9.04 | 0.00 | |
| Olive cake silage | 0.00 | 10.02 | 19.85 | |
| Barley grain | 15.26 | 15.11 | 14.96 | |
| Corn | 11.06 | 10.95 | 10.84 | |
| Wheat bran | 4.42 | 4.38 | 4.34 | |
| Sugarbeet pulp | 6.63 | 6.57 | 6.50 | |
| Sunflower meal (35% CP) | 5.53 | 5.47 | 5.42 | |
| Distillers Dark Grain (wheat) | 4.42 | 4.38 | 4.34 | |
| Soybean meal (47% CP) | 5.97 | 5.91 | 5.85 | |
| Molasses | 0.55 | 0.55 | 0.54 | |
| Limestone | 0.99 | 0.99 | 0.98 | |
| Monocalcium Phosphate | 0.06 | 0.05 | 0.05 | |
| Salt | 0.28 | 0.27 | 0.27 | |
| Vitamins & trace mineral mix | 0.11 | 0.11 | 0.11 | |
| | | | | |
| Composition % of DM | | | | |
| CP | 15.01 | 15.08 | 15.14 | 5.82 |
| Crude Fibre | 19.86 | 19.85 | 19.84 | 56.88 |
| Ash | 6.71 | 6.40 | 6.10 | 2.51 |
| Ether extract | 2.36 | 3.19 | 4.01 | 10.84 |
| NDF | 40.70 | 40.40 | 40.20 | 78.45 |
| ADF | 25.90 | 25.01 | 24.30 | 66.21 |
| Energy, NEL Mj/kg DM | 10.44 | 10.37 | 10.30 | |

NEL: Net energy lactation calculated according to NRC (2001)

### *2.4. Fatty acid analysis*

For the lipid isolation from feeds, representative samples were collected, and a Soxtec 2050 semi-automated solvent extractor (Foss Tecator AB, Hoganas, Sweden) was used to extract the fat according to Robinson et al. (2008) as described in **Chapter 2**. The automated extraction programming consisted of the sample being in contact with boiling petroleum ether for 20 min, a sample rinsing stage of 40 min, and a recovery stage of 10 min. After extraction, an appropriate volume containing 20 mg of fat removed to a clean tube and solvent was removed by blowing a stream of nitrogen gas. Then the fat was dispersed in 1 mL of n-hexane by shaking. For the isolation of fat from milk, the rapid double centrifugation method of Feng et al. (2004) and Luna et al. (2005) was used with slight modification as described by Tzamaloukas et al. (2015), where 1.5 mL aliquots of fresh milk were first centrifuged at 17,800 × *g* for 30 min at 4°C. The resulting fat cakes were removed, placed in fresh tubes, and allowed to melt at room temperature for 20 min. The samples were then recentrifuged at 19,500 × *g* for 20 min at room temperature and 20 mg aliquots of the resulting lipid cake were removed to fresh tubes and dispersed in 1 mL of n-hexane by shaking. The fat isolation from halloumi cheese was performed according to the Folch et al. (1957) method with modifications using the mix of chloroform and methanol (2:1, v/v). In particular, 1 g of anhydrous sodium sulfate was added to a tube containing 0.5 g halloumi cheese sample. A volume of 1 ml of chloroform: methanol (2:1) was added, the tubes were vortexed and left to incubate for 10 min at room temperature. The mixture was suspended in 4 ml n-hexane and then centrifuged at 2000 x *g* for 5 min at 4 °C. A 2.5 ml aliquot containing the extracted fat was then transferred to a fresh tube.

For all isolated lipids, extracted from different matrices (feed, milk or cheese), fatty acid methyl esters (FAME) were prepared by transesterification with methanolic potassium hydroxide according to the ISO (5509, 2000) method with slight modifications described by Tzamaloukas et al. (2015) as in **Chapter 2**. FAMEs were transferred to amber gas chromatography vials and stored at −70°C until GCMS analysis which was performed on a GCMS-QP2010 Plus Gas Chromatography Mass Spectrometer (Shimadzu, Duisburgh, Germany) equipped with an HT280T autosampler (HTA, Brescia, Italy). An Agilent CP-Sil 88 fused-silica capillary column (100 m × 0.25 mm internal diameter × 0.2 µm film thickness) for milk, Halloumi, and feed samples. Aliquots of 1 µl FAME mixtures were separated with a flow rate of 1 mL/min of the carrier gas (helium) by split ratio injection 1:20 for milk and feed, and 1:50 for halloumi cheese samples.

For FAME determination from milk, halloumi, and feed, a gradient temperature program according to (Kramer et al., 2008) was used where the initial oven temperature was held at 70°C for 4 min after injection and increased 4.56°C/min to 175°C. Finally, it was raised to 215°C at 4.44°C/min, and the total run time was 86 min, and the temperature of both injector and interface was kept at 225°C. The mass spectrometer operated in electronic impact ionization mode at 70 eV, and data were collected in full scan mode, with a scan time of 0.5 s over a mass range of 35 to 350. Shimadzu GCMS Postrun Solution software was used for the identification and quantitation of all FAME. For the identification of each individual peak their retention indices were compared with commercially available authentic standards (Supelco 37-FAME standard mix, *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, *trans*-11 C18:1; Sigma-Aldrich, Gillingham, UK), using the National Institute of Standards and Technology 08 and 21 mass

spectral libraries and cross-referencing with chromatograms-spectrograms reported in the literature (Kramer et al., 2008; De la Fuente et al., 2015). For the quantitation, each peak identified was integrated and expressed as a percentage of the total FAME.

### *2.5. Statistical analysis*

The results were analyzed by the statistical program JMP PRO Ver 14.2 (SAS Intitute Inc. NC, USA) after verification of the assumptions, including normality. The analysis for the dependent variable with repeated measures including feed intake, milk yield and milk chemical composition was performed by using the following mathematical model:

$$Y_{ijkl} = \mu + \alpha_i + d_{ij} + \beta_k + (\alpha\beta)_{ik} + e_{ijkl}$$

Whilst the analysis for the dependent variables of FA percentages in total fat of feed, milk and Halloumi cheese with independent variables were evaluated only once (k=3) with the simplified mathematical model:

$$Y_{ijl} = \mu + \alpha_i + d_{ij} + e_{ijl}$$

where $Y_{ijkl}$ is the value of the response-dependent variable for the $i^{th}$ feeding treatment, in the $j^{th}$ lot (or cheese batch) during the $k^{th}$ week from the $l^{th}$ ewe (or cheese sample); $\mu$ is the overall mean response; $\alpha_i$ is the fixed main effect of treatment (level i = 0, 1, 2); $\beta_k$ is the fixed of week of lactation (week level k=1, 2, 3); $(\alpha\beta)_{ik}$ is the fixed interaction effect of treatment and week, and $d_{ij}$ is the random effect of treatment nested in lot (lot level j=1, 2) distributed $N(0,\sigma_d^2)$ and $\varepsilon_{ijlk}$ is the random residual error effect of $l^{th}$ ewe (level l =1, ..., 10) on the $i^{th}$ treatment in the $j^{th}$ lot (or batch for halloumi) during the $k^{th}$ week distributed $N(0,\sigma_e^2)$. The significance of the fixed

treatment effects was evaluated. The treatment effect was decomposed into two orthogonal polynomial contrasts (linear and quadratic) and are presented in tables when found significant (P-values less than 0.05).

## 3. Results

### *3.1. Diets composition and animal performance*

The chemical content and FA composition of the experimental diets on offer are presented in **Tables 3.1** and **3.2**, respectively. Regarding the feed FA composition, the most abundant individual FAs in OCS was oleic acid (C18:1 *cis*-9; almost 2/3 of total fat), followed to a lesser content by palmitic (C16:0) and linoleic (C18:2 *cis*-9, *cis*-12) acids. As a consequence, the inclusion of OCS resulted in a remarkable change in the linear or quadratic manner of the composition of lipids of the three diets regarding almost all individual FAs identified.

Animal performance under different diet treatments is shown in **Table 3.3**. Feed intake, milk yield, and composition or yield of fat, protein and total solids content of milk were not affected by OCS feeding.

### *3.2. Milk fatty acid composition*

**Table 3.4** shows the FA composition of individual milk samples and OCS feeding resulted in marked modifications in milk FAs compared with the control. Diet inclusion of OCS resulted in a linear increase of MUFA class in milk to the detriment of SFA content. Linear decreases occurred in most saturated fats, including odd and branch-chain FAs, and were profound in the major SFA C8:0, C10:0, C12:0, C14:0, and C16:0 ($P < 0.001$) except C18:0, which, in contrast, was

increased with the OCS inclusion in the diets. Oleic acid, other C18:1 *cis*-isomers, and C18:1 *trans*-10 were particularly enhanced ($P < 0.001$), whereas C18:1 *trans*-11 (vaccenic acid, **VA**) was not affected by olive byproduct feeding. Regarding 18-carbon dienes in milk, percentages of linoleic acid and all non-conjugated C18:2 isomers increased with the OCS participation in the diets. Notably, similar effect was observed for the major CLA, C18:2 *cis*-9, *trans*-11 which was linearly increased by 42% and 61% when the diets included 10 and 20% of OCS (DM), respectively, but no effect was observed for the other conjugated FA identified, C18:2 *trans*-10, *cis*-12. Polyunsaturated FAs (**PUFA**) such as linolenic acid (C18:3 *cis*-9, *cis*-12, *cis*-15) and C20:3 (n-6), were not affected or even decreased with OCS diets, such as the arachidonic acid (C20:4 n-6) content. Overall the total SFA percentages reduced, and particularly the group of FAs with less than 16-carbons, with a significant increase of total MUFA and the group of FAs with more than 16-carbons. Inclusion of OCS improved health-related indices of milk since the atherogenicity index reduced by 25% in the S10 and by 31% in the S20 milk, and the thrombogenicity index reduced by 22% and 27%, in S10 and S20 treatments, respectively, compared to control. The desaturation index was not affected by the treatment.

**Table 3.2.** Fatty acid composition (g/100g of total fatty acid methyl esters) of olive cake silage (OCS) and the experimental diets S0, S10 and S20 with different inclusion of OCS (0, 10 and 20% on DM, respectively)

| | | Experimental Diets | | | | Effect of treatment1 | |
|---|---|---|---|---|---|---|---|
| Item | OCS | S0 | S10 | S20 | SEM | L | Q |
| C14:0 | 0.03 | 1.11 | 0.60 | 0.30 | 0.03 | 0.005 | 0.227 |
| C16:0 | 13.57 | 19.12 | 16.79 | 15.64 | 0.17 | <0.001 | 0.124 |
| C18:0 | 3.83 | 4.15 | 3.75 | 3.55 | 0.02 | <0.001 | 0.205 |
| C18:1 cis-9 | 64.49 | 24.02 | 39.23 | 46.75 | 0.66 | <0.001 | 0.007 |
| C18:2 n-6 | 12.95 | 39.15 | 30.13 | 25.68 | 0.35 | <0.001 | 0.007 |
| C18:3 n-3 | 1.37 | 8.21 | 5.62 | 4.34 | 0.05 | <0.001 | <0.001 |
| C20:0 | 1.10 | 1.29 | 1.06 | 0.94 | 0.18 | NS | |

[1]L = Probability of linear effect, Q = Probability of quadratic effect, NS = non-significant effect.

**Table 3.3.** Feed intake, milk production and chemical composition of milk from ewes fed diets with olive cake silage inclusion at rates of 0% (control, S0), 10% (S10), and 20% (S20) of DM

| | Experimental Diets | | | | |
|---|---|---|---|---|---|
| Item | S0 | S10 | S20 | SEM | Effect of treatment |
| Intake, DM kg/d | 2.52 | 2.44 | 2.56 | 0.70 | NS |
| Yield, g/d | | | | | |
| Milk | 1933 | 1960 | 2037 | 49.30 | NS |
| Fat | 86 | 86 | 87 | 5.38 | NS |
| Protein | 84 | 87 | 88 | 4.81 | NS |
| Total solids | 311 | 318 | 322 | 17.66 | NS |
| Composition, % | | | | | |
| Fat | 4.46 | 4.37 | 4.30 | 0.14 | NS |
| Protein | 4.36 | 4.43 | 4.35 | 0.03 | NS |
| Total solids | 16.08 | 16.17 | 15.90 | 0.16 | NS |

NS = non-significant effect.

**Table 3.4.** Fatty acid profile (g/100 g of total fatty acid methyl esters) of milk fat from ewes fed diets with olive cake silage (OCS) inclusion at rates of 0% (control, S0), 10% (S10), and 20% (S20) of DM

| | Experimental Diets | | | Effect of treatment[1] | | |
|---|---|---|---|---|---|---|
| Item | S0 | S10 | S20 | SEM | *L* | *Q* |
| Saturated fatty acids | | | | | | |
| C4:0 | 1.71 | 2.10 | 2.07 | 0.14 | 0.002 | 0.023 |
| C6:0 | 2.08 | 2.03 | 1.94 | 0.09 | 0.059 | 0.821 |
| C8:0 | 2.34 | 2.07 | 2.00 | 0.12 | <0.001 | 0.174 |
| C10:0 | 5.53 | 4.53 | 4.43 | 0.29 | <0.001 | 0.029 |
| C12:0 | 4.15 | 3.31 | 3.21 | 0.17 | <0.001 | 0.003 |
| C13:0 | 0.19 | 0.16 | 0.14 | 0.02 | <0.001 | 0.209 |
| iso C14:0 | 0.28 | 0.24 | 0.20 | 0.01 | <0.001 | 0.836 |
| C14:0 | 9.25 | 7.62 | 7.44 | 0.27 | <0.001 | <0.001 |
| iso C15:0 | 0.61 | 0.54 | 0.45 | 0.03 | <0.001 | 0.606 |
| anteiso C15:0 | 0.95 | 0.87 | 0.76 | 0.04 | <0.001 | 0.625 |
| C15:0 | 1.69 | 1.45 | 1.31 | 0.05 | <0.001 | 0.117 |
| iso C16:0 | 0.73 | 0.67 | 0.58 | 0.05 | <0.001 | 0.171 |
| C16:0 | 20.37 | 18.25 | 17.82 | 0.69 | <0.001 | 0.029 |
| C17:0 | 1.11 | 0.99 | 0.92 | 0.04 | <0.001 | 0.439 |
| C18:0 | 12.78 | 14.22 | 14.32 | 0.49 | <0.001 | 0.040 |
| C20:0 | 0.79 | 0.74 | 0.71 | 0.04 | NS[9] | |
| C22:0 | 0.41 | 0.34 | 0.34 | 0.05 | NS | |
| C24:0 | 0.11 | 0.10 | 0.11 | 0.01 | NS | |
| Monousaturated fatty acids | | | | | | |
| C10:1 *cis*-9 | 0.38 | 0.33 | 0.33 | 0.03 | 0.039 | 0.326 |
| C14:1 *cis*-9 | 0.28 | 0.25 | 0.24 | 0.02 | 0.030 | 0.652 |
| C16:1 *cis*-9 | 1.18 | 1.14 | 1.05 | 0.08 | 0.033 | 0.684 |
| C17:1 *cis*-9 | 0.37 | 0.38 | 0.35 | 0.03 | NS | |
| C18:1 *trans*-10 | 0.38 | 0.78 | 0.83 | 0.15 | <0.001 | 0.102 |
| C18:1 *trans*-11 | 1.34 | 1.59 | 1.53 | 0.19 | NS | |
| C18:1 *cis*-9 | 17.72 | 20.79 | 20.97 | 0.57 | <0.001 | <0.001 |
| C18:1 *cis* other | 1.69 | 2.14 | 2.51 | 0.10 | <0.001 | 0.526 |
| Polyunsaturated fatty acids | | | | | | |
| C18:2 *trans*-8, *cis*-12/ *cis*-9, *trans*-13 | 0.19 | 0.51 | 0.63 | 0.04 | <0.001 | <0.001 |
| C18:2 *trans*-8, *cis*-13 | 0.12 | 0.20 | 0.25 | 0.03 | <0.001 | 0.283 |
| C18:2 *trans*-9, *cis*-12 | 0.06 | 0.09 | 0.13 | 0.02 | <0.001 | 0.663 |
| C18:2 *cis*-9, *cis*-12 (n-6) | 3.18 | 3.59 | 3.80 | 0.24 | <0.001 | 0.777 |
| C18:3 (n-3) | 0.47 | 0.44 | 0.44 | 0.07 | NS | |
| CLA *cis*-9, *trans*-11 | 0.75 | 1.07 | 1.21 | 0.08 | <0.001 | 0.065 |
| CLA *trans*-10, *cis*-12 | 0.30 | 0.16 | 0.16 | 0.09 | 0.078 | 0.244 |
| C20:3 (n-6) | 0.22 | 0.14 | 0.15 | 0.08 | NS | |
| C20:4 (n-6) | 0.49 | 0.40 | 0.35 | 0.05 | <0.001 | 0.598 |
| Fatty acid groups | | | | | | |
| SFA[2] | 67.33 | 62.68 | 59.86 | 1.26 | <0.001 | 0.170 |
| < C16:0 | 29.78 | 25.79 | 24.71 | 0.93 | <0.001 | 0.022 |

| | | | | | | |
|---|---|---|---|---|---|---|
| > C16:0 | 45.33 | 51.14 | 52.53 | 1.40 | <0.001 | 0.007 |
| UFA[3] | 32.24 | 36.58 | 38.99 | 1.26 | <0.001 | 0.150 |
| MUFA[4] | 25.44 | 29.44 | 31.41 | 0.93 | <0.001 | 0.055 |
| PUFA[5] | 6.80 | 7.14 | 7.58 | 0.40 | 0.007 | 0.845 |
| AI[6] | 2.07 | 1.54 | 1.41 | 0.05 | <0.001 | 0.002 |
| TI[7] | 2.74 | 2.13 | 2.00 | 0.12 | <0.001 | 0.002 |
| DI[8] | 2.92 | 3.20 | 3.13 | 0.14 | NS | |

[1]L = Probability of linear effect, Q = Probability of quadratic effect.
[2]Saturated fatty acids.
[3]Unsaturated fatty acids.
[4]Monounsaturated fatty acids.
[5]Polyunsaturated fatty acids.
[6]Atherogenicity index (C12:0 + (4 x C14:0) + C16:0)/(MUFA + PUFA(n-3:n-6)).
[7]Thrombogenicity index (C14:0 + C16:0 + C18:0)/ [(0.5 x MUFA)+[0.5 x PUFA(n-6)]+[3 x PUFA(n-3)]+(n-3/n-6)].
[8]Desaturation index (C14:1 x 100)/(C14:1+C14:0).
[9]NS = non-significant effect nor tendency (P value > 0.1).

### *3.3. Halloumi cheese fatty acid composition*

**Table 3.5** shows the FA profile of Halloumi cheese produced with raw milk from ewes fed the three treatment diets with different levels of OCS. The inclusion of OCS increased UFA in OCS treatments compared with the control cheeses, with concomitant reductions in SFA content. All individual SFA determined in cheese were linearly diminished with the addition of OCS in the ewe diets, except butyric acid (C4:0) which was not affected, and stearic acid (C18:0) which was increased in the Halloumi cheese of S20 treatment. In contrast to the SFA, total percentages of MUFA and UFA percentages were both increased by 25% in S10 and by 30% in S20 diet compared to control. With regards to monounsaturated fats, this increase is attributed to the percentages of 18-carbon monoene isomers (oleic, vaccenic and C18:1 *trans*-10 acids), since other MUFA (10:1, 14:1, 16:1, 17:1) were reduced with the inclusion of OCS. Concerning individual PUFA, OCS inclusion resulted in an increase in all 18-carbon dienes, including linoleic and RA, with the last one particularly enhanced (2 fold increment in S20 treatment). Other UFA

such as linolenic and arachidonic acid were reduced with the OCS inclusion. The inclusion of OCS in the diets of ewes quadratically reduced health-related indices in corresponding Halloumi cheese, namely atherogenicity and thrombogenicity indices which were lower by 29% and 23% in S10 and by 45% and 24% in S20 treatment, respectively, compared with the control treatment.

**Table 3.5.** Fatty acid profile (g/100 g of total fatty acid methyl esters) of Halloumi cheese made with raw milk from ewes fed diets with olive cake silage (OCS) inclusion at rates of 0% (control, S0), 10% (S10), and 20% (S20) of DM

| | Experimental Diets | | | | Effect of treatment[1] | |
|---|---|---|---|---|---|---|
| | S0 | S10 | S20 | SEM | *L* | *Q* |
| Saturated fatty acids | | | | | | |
| C4:0 | 1.56 | 1.60 | 1.74 | 0.09 | 0.061 | 0.471 |
| C6:0 | 1.78 | 1.64 | 1.55 | 0.03 | <0.001 | 0.379 |
| C8:0 | 2.02 | 1.75 | 1.55 | 0.04 | <0.001 | 0.291 |
| C10:0 | 5.60 | 4.59 | 3.71 | 0.20 | <0.001 | 0.630 |
| C12:0 | 4.59 | 3.53 | 2.90 | 0.10 | <0.001 | 0.025 |
| C13:0 | 0.13 | 0.09 | 0.07 | 0.00 | <0.001 | 0.017 |
| iso C14:0 | 0.21 | 0.14 | 0.11 | 0.01 | <0.001 | 0.001 |
| C14:0 | 10.80 | 9.17 | 7.96 | 0.21 | <0.001 | 0.209 |
| iso C15:0 | 0.49 | 0.30 | 0.25 | 0.01 | <0.001 | <0.001 |
| anteiso C15:0 | 0.90 | 0.59 | 0.50 | 0.02 | <0.001 | <0.001 |
| C15:0 | 1.42 | 1.06 | 0.88 | 0.03 | <0.001 | 0.006 |
| Iso C16:0 | 0.46 | 0.32 | 0.30 | 0.02 | <0.001 | 0.004 |
| C16:0 | 24.14 | 23.32 | 20.79 | 0.58 | <0.001 | 0.082 |
| C17:0 | 0.73 | 0.52 | 0.51 | 0.04 | <0.001 | 0.015 |
| C18:0 | 13.59 | 13.16 | 17.30 | 0.24 | <0.001 | <0.001 |
| C20:0 | 0.45 | 0.37 | 0.49 | 0.02 | NS[8] | |
| C22:0 | 0.16 | 0.12 | 0.17 | 0.01 | NS | |
| C24:0 | 0.04 | 0.09 | 0.05 | 0.01 | NS | |
| Monounsaturated fatty acids | | | | | | |
| C10:1 | 0.26 | 0.19 | 0.17 | 0.01 | <0.001 | 0.006 |
| C14:1 *cis*-9 | 0.22 | 0.15 | 0.13 | 0.01 | <0.001 | 0.005 |
| C16:1 *cis*-9 | 1.02 | 0.83 | 0.67 | 0.02 | <0.001 | 0.498 |
| C16:1 *cis*-9 | 0.17 | 0.10 | 0.07 | 0.01 | <0.001 | 0.002 |
| C17:1 *cis*-9 | 0.25 | 0.17 | 0.14 | 0.01 | <0.001 | 0.002 |
| C18:1 *cis*-9 | 21.39 | 27.83 | 29.83 | 0.48 | <0.001 | <0.001 |
| C18:1 *trans*-10 | 0.32 | 0.64 | 0.63 | 0.10 | 0.01 | 0.067 |
| C18:1 *trans*-11 | 0.30 | 0.56 | 0.63 | 0.05 | <0.001 | 0.035 |
| Polyunsaturated fatty acids | | | | | | |
| C18:2 *trans*-9, *trans*-12 | 0.09 | 0.23 | 0.25 | 0.06 | 0.018 | 0.195 |
| C18:2 *cis*-9, *cis*-12 | 2.80 | 3.48 | 3.23 | 0.15 | 0.014 | 0.005 |
| C18:2 *cis*-9, *trans*-11 CLA | 0.41 | 0.74 | 0.86 | 0.03 | <0.001 | 0.004 |
| C18:3 (n-3) | 0.28 | 0.24 | 0.20 | 0.02 | 0.001 | 0.899 |
| C20:4 (n-6) | 0.22 | 0.19 | 0.16 | 0.01 | <0.001 | 0.345 |
| Fatty acid groups | | | | | | |
| SFA[2] | 70.87 | 63.52 | 61.95 | 0.96 | <0.001 | 0.005 |
| < C16:0 | 30.47 | 25.02 | 21.69 | 0.51 | <0.001 | 0.262 |
| > C16:0 | 43.3 | 49.9 | 55.9 | 0.97 | <0.001 | 0.636 |
| UFA[3] | 29.13 | 36.48 | 38.05 | 0.96 | <0.001 | 0.005 |
| MUFA[4] | 25.17 | 31.54 | 33.08 | 0.70 | <0.001 | 0.003 |
| PUFA[5] | 3.96 | 4.94 | 4.71 | 0.27 | 0.0153 | 0.020 |
| AI[6] | 2.51 | 1.78 | 1.43 | 0.08 | <0.001 | 0.019 |
| TI[7] | 3.21 | 2.47 | 2.42 | 0.11 | <0.001 | 0.004 |

[1] L = Probability of linear effect, Q = Probability of quadratic effect.
[2]Saturated fatty acids.
[3]Unsaturated fatty acids.
[4]Monounsaturated fatty acids.
[5]Polyunsaturated fatty acids.
[6]Atherogenicity index (C12:0 + (4 x C14:0) + C16:0)/(MUFA + PUFA(n-3:n-6)).
[7]Thrombogenicity index (C14:0 + C16:0 + C18:0)/ [(0.5 x MUFA)+[0.5 x PUFA(n-6)]+[3 x PUFA(n-3)]+(n-3/n-6)].
[8]NS = non-significant effect nor tendency (P value > 0.1).

## 4. Discussion

The current study investigated the effects of OCS inclusion in the dairy Chios sheep diet, focusing on the FA profile of milk and corresponding Halloumi cheese. The OCS treatments were found to have a positive influence on the FA content of milk and Halloumi cheese with regards to beneficial FAs for human health. More specifically, OCS significantly decreased the SFA content and increased the total UFA and MUFA content of milk and Halloumi cheese. These results agree with the findings of other researchers on milk, cheese, and yoghurt by the utilization of fresh or dried forms of OC (Chiofalo et al., 2004; Abbeddou et al., 2011a, 2011b and 2015; Vargas-Bello-Perez et al., 2013) or the addition of vegetable oils (olive: Gomez-Cortes et al., 2008b; Bodas et al., 2010; soybean and linseed: Bodas et al., 2010; sunflower: Castro-Carrera et al., 2015) in ewe diets. In the present study, the significant effect of the OCS treatments on the unsaturation of milk and Halloumi cheese fat contributed to the decrease in the atherogenicity and thrombogenicity indices in both products. This outcome is similar to those reported in ovine milk by Chiofalo et al. (2004) and Vargas-Bello-Perez et al. (2013) after the inclusion of partly destoned fresh or dried OC, respectively, and in bovine milk after inclusion of dried OC (Castellani et al., 2017). The improved health indices in the present and

the previous studies support the use of this by-product as a forage substitution for the improvement of lipid quality in dairy products.

The milk and Halloumi cheese obtained from the OCS groups in the present study had increased long-chain FA (**LCFA**, > C16:0 carbon atoms) and decreased short and medium-chain FA (4 to 14 carbon atoms) compared to control. This outcome is similar to studies testing high in LCFA diets (reviewed by Dorea and Armentano, 2017) and is attributed to either a higher secretion of LCFA from the blood, derived from the feed and/or a lower de novo FA synthesis in the mammary gland. The lower synthesis is being provoked by bioactive FAs, such as CLAs and *trans* monoenes, known for their inhibitory action on de novo FA synthesis and reduced overall milk fat content (Chilliard et al., 2000b; Shingfield et al., 2013). However, in the current study the milk fat percentage remained unaffected and the most well studied inhibitory FA, CLA *trans*-10, *cis*-12, had a tendency of lower content in milk of OCS treatments compared to control ($P < 0.01$). Therefore the milk fat depression mechanisms seem not to affect the present results and the observed milk FA profile and consequently the lipids profile of corresponding Halloumi cheese.

In the present study, the content of stearic and oleic acid, both in milk and Halloumi cheese, was higher for the OCS groups compared to the control. Previous studies with the use of different OC processed forms (Chiofalo et al., 2004; Abbeddou et al., 2011a, 2011b and 2015; Vargas-Bello-Perez et al., 2013) and vegetable oils (Gomez-Cortes et al., 2008b; Bodas et al., 2010; Castro-Carrera et al., 2015), also reported higher contents of stearic and oleic acids in milk and cheese. Except for the stearic acid content of the feed that can be transferred in milk,

this FA is also produced in the rumen as the final hydrogenation product of the C18 MUFA and PUFA and is then incorporated in milk fat through the blood circulation (Doreau et al., 2016). Since OCS treatments in the present study had lower stearic content and significantly higher oleic content compared to control diet, it is suggested that the second mechanism of bio-hydrogenation has been involved in increased stearic content in milk and consequently in Halloumi cheese fat. In the case of oleic acid, it has been reported that may well originate from the mammary uptake of dietary oleic acid but also derived by the desaturation of stearic acid from Δ-9 desaturase in the rumen or mammary gland (Doreau et al., 2016). In our study, the desaturation index was not affected by the treatment suggesting that the transfer from the rich in oleic diets may have been involved.

Among the UFA of our study, the OCS inclusion in ewe diets has positively influenced the linoleic acid in milk and Halloumi cheese. Increased contents of linoleic acid in milk (**Chapter 2:** Symeou et al., 2019) and cheese (Vargas-Bello-Perez et al., 2013) were also reported in previous studies feeding ewes with OCS and destoned dried OC, respectively, while lower contents were reported with dried OC (Abbeddou et al., 2011b and 2015) or no effects when partly destoned fresh OC was included in the ewe diets (Chiofalo et al., 2004). As far as linolenic acid (n-3) content in milk is concerned, there was no response to the OCS treatments in the present study, while the linolenic acid (n-3) content in Halloumi cheese, made of the S10 and S20 milk groups, was lower compared to the control. Other researchers supplementing diets with dried OC (Abbeddou et al., 2011b and 2015) and partly destoned fresh OC (Chiofalo et al., 2004) reported no influence to the content of linolenic acid (n-3) in milk, while the inclusion of OCS (**Chapter 2:** Symeou et al., 2019), destoned dried OC (Vargas-Bello-Perez et al., 2013) and

olive oil (Gomez-Cortes et al., 2008b; Bodas et al., 2010) in the diets led to the reduction of the linolenic acid content in milk and cheese fat. In the present study, linolenic acid in Halloumi cheese is shown to be altered compared to the milk content, suggesting that this particular FA is affected during the Halloumi cheese-making procedure. Previous studies have reported that the cheese FA composition can be affected by the cheese type (Domagala et al., 2010) and through various stages of the cheese processing such as the heating procedure, the microbial cultures and the ripening time (Collomb et al., 2006; Santillo et al., 2016).

The OCS inclusion at 10 and 20% of the diet in the current study positively affected the levels of RA in milk and, consequently, in Halloumi cheese. Milk and dairy products comprise the primary source of CLA, which are known for their beneficial effect on human health (Dilzer and Park, 2012; Gómez-Cortés et al., 2018). Previous studies supplementing with dried OC reported no changes in the RA content neither for ovine milk (Abbeddou et al., 2011b and 2015) nor ovine yoghurt or cheese (Abbeddou et al., 2011a). However, researchers assessing OCS feeding in ovine (**Chapter 2:** Symeou et al., 2019), and dried OC in bovine (Castellani et al., 2017) and caprine (Molina-Alcaide et al., 2010) milk reported increased CLA contents. Moreover, the olive oil inclusion in ewes diets resulted in contradictory results showing high (Bodas et al., 2010) and low (Gomez-Cortes et al., 2008b) contents of RA in the ovine milk. The main source of RA is the ruminal bio-hydrogenation of linoleic acid (18:2, *cis*-9, *trans*-12), which produces, as a first intermediate, the most predominant CLA isomer, the RA, and as a second intermediate the vaccenic acid (reviewed by Palmquist, 2006). These intermediates are secreted in milk since they end up to the mammary gland through the blood circulation. Part of vaccenic acid reaching the mammary gland is desaturated to RA by Δ-9 desaturase (Doreau et

al., 2016). However, since the desaturation index was not affected by the OCS diet inclusion in the present study, it remains unclear the contribution of each of the previously referred mechanisms in the increments of RA content in milk.

In the current study, the three week OCS feeding treatment did not affect feed intake or milk yield. This result agrees with previous studies where dairy ewes were allocated to treatments with comparable inclusion rates of OCS (Hadjipanayiotou, 1999; Symeou et al., 2019), dried OC (Abbeddou et al., 2011a and 2011b) or partly destoned dried OC (Vargas-Bello-Perez et al., 2013). Similarly, Castellani et al. (2017) included dried olive pomace in cow feeds at rates of 10% on a DM basis with no effect on the milk yield. In contrast to those studies, Abbeddou et al. (2015) reported a decrease in milk yield and Chiofalo et al. (2004) an increase in milk yield with OC by-product inclusion in ewe diets. These differences in milk production may be attributed to the OC processing, the inclusion rates used or the different protein and energy content of experimental diets as has been suggested previously (Abbeddou et al., 2015).

**5. Conclusions**

Overall, the present study suggests that the ensiling of OC can provide an applicable forage replacement feed in sheep diets at rates up to 20% DM improving the fat quality of milk, and without adversely affecting milk yield, milk composition or the feed intake of ewes. Moreover, this substitution affects positively the lipid quality of Halloumi cheese produced with raw milk from ewes fed OCS with decreased saturation, atherogenicity and thrombogenicity and concomitant increased MUFA and CLA content.

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# CHAPTER 4

## Association of a SNP in the 3'untranslated region of the ovine *acetyl-coenzyme A acyltransferase 2* gene with milk traits

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**Abstract**

The *acetyl-CoA acyltransferase 2* (***ACAA2***) gene encodes an enzyme of the thiolase family that is involved in mitochondrial fatty acid elongation and degradation by catalyzing the last step of the respective β-oxidation pathway. The increased energy needs for gluconeogenesis and triglyceride synthesis during lactation are primarily met by the increased fatty acid oxidation. Therefore, the ACAA2 enzyme plays an important role in the supply of energy and carbon substrates for lactation and may thus affect milk production traits. The aim of this study was to investigate the potential association between the *ACAA2* gene and important sheep traits in two independent sheep populations of the Chios dairy sheep in Greece and Cyprus. A database was created containing lactation records from a total of 632 and 742 purebred Chios ewes in mid-lactation collected during 7 successive years from 20 flocks in Greece and during 8 successive years from 5 flocks in Cyprus, respectively. A single nucleotide substitution, a T to C transition located in the 3' untranslated region of the *ACAA2* gene, was used in mixed model association analysis with milk yield for the sheep population in Greece and with milk yield, milk protein yield and percentage, milk fat yield and percentage for the sheep population in Cyprus. The single nucleotide polymorphism was significantly associated with total lactation production in both sheep population, and furthermore, it was associated with milk protein percentage and yield and milk fat yield in the populations in Cyprus. Homozygous TT and heterozygous CT animals in Cyprus exhibited higher milk yield compared with homozygous CC animals, whereas the latter exhibited increased milk protein percentage. On the contrary, the genotypic classes TT and CT of the sheep in Greece were shown to have lower milk yield than the CC sheep.

## 1. Introduction

In Mediterranean countries, most of the ovine milk produced is used for the production of cheese commercialized as protected designation of origin (PDO) and other quality labels (Arranz and Guttierez Gil, 2012). Milk yield represents more than two thirds of the total income of the dairy sheep industry (Carta et al., 2009) and therefore the improvement of milk production continues to be the most profitable breeding objective. Farmers' income is additionally determined by total solids (Othmane, 2002) that affect cheese yield (Bencini and Pulina, 1997). Therefore, increased milk fat and protein content is also highly desirable from a financial perspective (Ramon et al., 2010). Although traditional breeding programs have achieved appreciable genetic gains mainly for milk yield, application of selection schemes assisted by molecular information could expedite improvement and facilitate selection of organizationally demanding and costly to measure selection goals, such as milk composition (Carta et al., 2009).

In a whole genome quantitative trait loci (**QTL**) study performed in Churra ewes, Gutiérrez-Gil et al. (2009) detected suggestive QTL for milk, fat and protein yield, mapped in a region of the ovine chromosome 23 harbouring the *acetyl-CoA acyltransferase 2* (***ACAA2***) gene. The *ACAA2* gene encodes the Acetyl-CoA acyltransferase 2 enzyme, also known as 3-oxoacyl-CoA thiolase or mitochondrial 3-ketoacyl-CoA thiolase. ACAA2 catalyzes the last step in mitochondrial fatty acid (**FA**) β-oxidation, thus playing a central role in the supply of energy for the animal (Bartlett and Eaton, 2004). Therefore, due to the chromosomal location of the

*ACAA2* gene and its functional role in lipid metabolism, it was regarded as a putative functional and positional candidate gene that may affect milk yield and composition.

The entire coding region and the untranslated regions (**UTRs**) of the *ACAA2* gene are monomorphic in Chios sheep, with the exception of a SNP located in the 3' UTR of the gene (HM537015:g.2982T>C). The SNP was significantly associated with milk yield in Chios sheep from a research flock at the Agricultural Research Institute of Cyprus (Orford et al., 2012). Animals with the g.2982TT or g.2982CT genotype had significantly higher milk yield than those with the g.2982CC genotype and the g.2982T>C SNP explained 10% of the additive genetic variance for milk yield (Orford et al., 2012).

The objective of the present study was to provide additional genetic support about the influence of the *ACAA2* gene in dairy traits by performing association analysis of the g.2982T>C SNP with milk yield, fat percentage and yield and protein content and yield in two extended populations of Chios sheep from available commercial farms keeping production records in Cyprus and in Greece, in an effort to investigate whether the associations found in the two populations could be confirmed.

## 2. Materials and methods

### *2.1. Animals, DNA extraction and SNP genotyping*

Genomic DNA was extracted from whole blood samples obtained from each individual 632 Chios ewes farmed in 20 flocks in Greece using standard methods. Genotyping of the g.2982C>T SNP of the *ACAA2* gene was performed by developing a Taqman RT-PCR assay.

Primer sequences and Taqman probes were designed to flank and detect, respectively, the g.2982C>T polymorphism located in exon 10, in the 3'-UTR of the *ACAA2* gene, based on the *ACAA2* sequence we have previously published with accession number HM537015 (Orford et al., 2012). Primers and probes were designed and synthesized by VBC-Biotech Service, Vienna. TaqMan RT-PCR reactions were performed in 10-µl reaction volumes comprised of 1× KAPA probe fast qPCR Master Mix (Kapa Biosystems, USA), 200nM each of the PCR primers: ACAA2_SNP_Forward (AACCAGGTGACCTTCAGAGC) and ACAA2_SNP_Reverse (AATGGGTCTTTGCTTCACCA) (4 µM each), and 200nM each of TaqMan probes: SNP_2982_C (5'FAM/3'BHQ1) CACGGAGAGTGACCAGGTTTGGGTAAGG (5 µM), Taqman probe SNP_2982_T (5'-JOE/3'BHQ1) CACGGAGAGTGACTAGGTTTGGGTAAGG (5µM), 1x ROX low and 25 ng of genomic DNA. A 7500 Real Time PCR system (Applied Biosystems, USA) was used with a cycling profile that included a 3 min denaturation step at 95°C and 40 cycles of denaturing for 3 sec at 95°C, primer annealing for 10 sec at 66°C and extension for 30 sec at 72°C. Genotyping of 50 genomic DNA samples was confirmed by sequencing (**Figure 4.1**), and ensured that there was no other detected polymorphism. In particular, PCR reactions were set up in 25-µl volumes containing 25 ng of genomic DNA, 0.5U of Taq DNA polymerase (Qiagen Inc., Valencia, CA), 0.5 µM each primer, and 2.5 mM $MgCl_2$. After an initial 5 min denaturation step, the PCR reactions were subjected to 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, followed by a final elongation step for 5 min at 72 °C (Orford et al., 2012). The primers amplifying the $10^{th}$ exon of the *ACAA2* gene were those previously designed Orford et al. (2012) (Forward primer: TTGATTATCTAATTCCGTACATTCTTT, Reverse primer: TTTAATGCCACTGAAGTGTATGC). Subsequently, a cost-effective direct DNA sequencing protocol was performed according to

Miltiadou et al. (2017a), where the obtained PCR products generated 96-well plates, which were precipitated by the addition of 5μL of 3M sodium acetate, pH 5.3, with 25μL of isopropanol. After incubation at -70C for 20 min and centrifugation at 4°C for 45 min (2,720 x g), the supernatant removal took place by inverting the plates on paper towels and briefly spinning the plates upside down for 2 s at 500 x g. A washing step followed by the addition of 100μL of 70% ethanol without mixing with the precipitated products. Then, another centrifugation carried on for 10 min at 2,720 x g at 4°C, where the supernatants were removed as before. The final step was a 5 min drying at 50°C completed with the addition of 40 μL of deionized water in each well to re-dissolve the purified DNA (Miltiadou et al., 2017a). For the sequencing cycle 10-μL volume reaction were set up with the use of 2 μL of the purified templates, 0.5 μL of Big Dye Terminator Reagent v.3.1 (Applied Biosystems), 0.5 μL of the reverse primer (5 μM), 1.75 μL of 5 x sequencing buffer, and 5.25 μL of distilled water. After an initial 2-min denaturation step at 96°C, the sequencing reactions were subjected to 30 cycles at 96°C for 15 s, 50°C for 15 s, and 60°C for 4 min. When the reaction ended, 10 μL of water were added, followed by 5 μL of 125 mM EDTA and 60 μL of 100% ethanol to precipitate the termination products. First, the plates were incubated at room temperature for 15 min in dark, centrifuged at 2,720 × g at 4°C for 45 min, and the supernatants were removed as before. Following a wash with 250 μL of 70% ethanol, the plates were dried for 5 min at 50°C and the termination products were dissolved in 10 μL of formamide before reading on an ABI 3130 genetic analyzer (Applied Biosystems) (Miltiadou et al., 2017a).

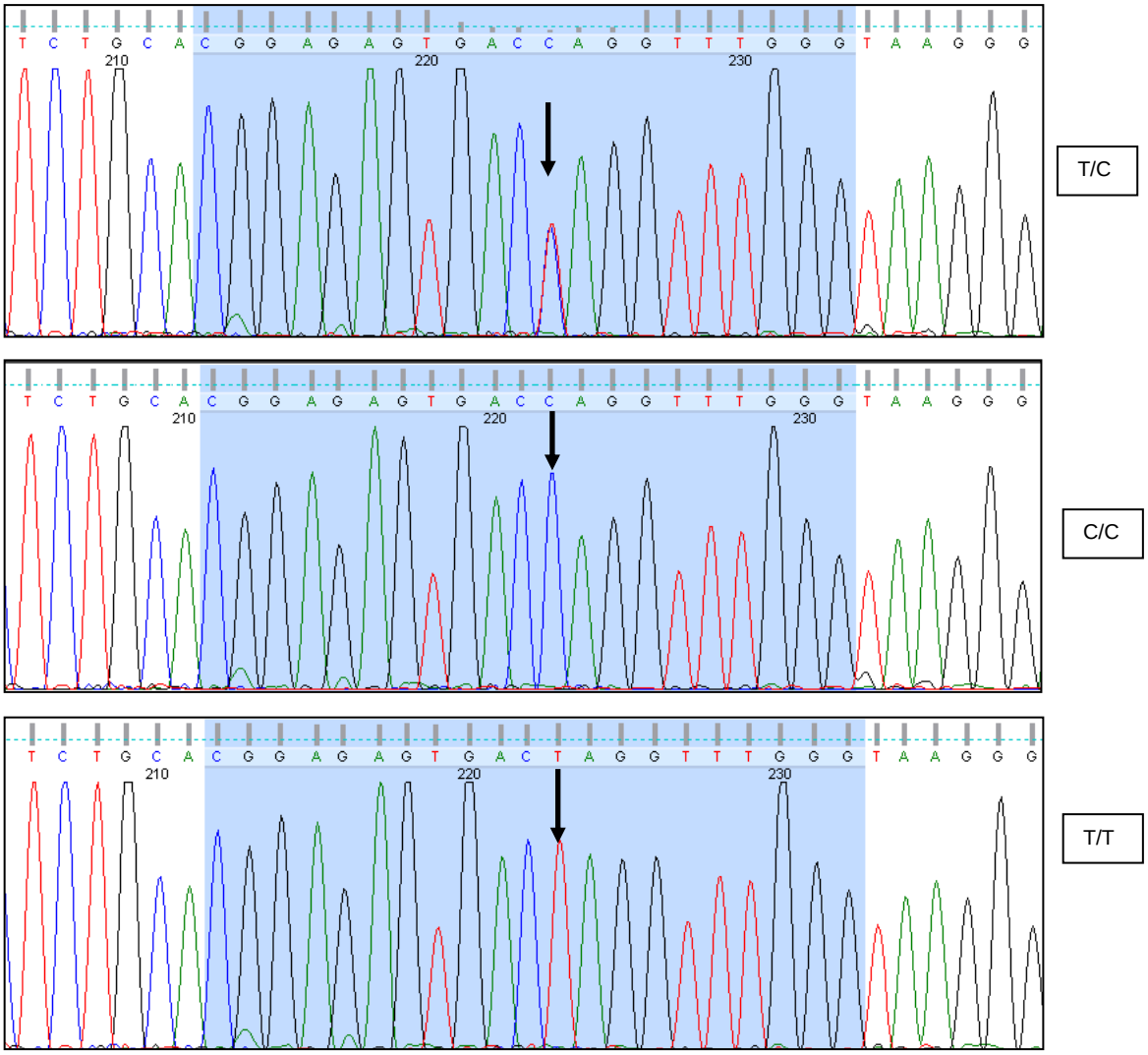


**Figure 4.1.** Sequencing of the HM537015:g.2982T>C polymorphism within the 3' untranslated region (UTR) of the *ACAA2* gene. The position of a T/C SNP is indicated by a black arrow.

Genomic DNA was also isolated from whole blood samples collected from each 742 ewes of five purebred Chios ewes' farms in Cyprus using the Genomic DNA Blood kit (Macherey-Nagel), according to the manufacturer's instructions. DNA quality and quantity were estimated by UV absorption at 260 and 280nm, while genotyping of the g.2982T>C SNP of the *ACAA2* gene (**Figure 4.1**) was performed using a cost effective direct DNA sequencing protocol as previously described for sequencing the DNA samples obtained from the Greek Chios sheep population according to Orford et al. (2012) and Miltiadou et al. (2017a).

### *2.2. Phenotypic data*

Data were collected from all 632 purebred Chios ewes from 20 commercial farms in North Greece, keeping phenotypic records according to the regulations of the International Committee for Animal Recording (**ICAR**). For all animals, standard records were collected, including month of lambing, year of lambing, lactation number and age of lambing. Phenotypic data were obtained for lambing years between 2003 and 2009 and included 1,325 individual records for total lactation milk yield for six lactations, with 1,275 milk observations from the first three lactations.

Phenotypic data from Cyprus were collected from 742 purebred Chios ewes from one governmental and four commercial farms. Those were the only commercial farms in Cyprus keeping phenotypic records according to the regulations of the ICAR. Inbreeding in the flocks was controlled by the use of at least two sires from other flocks and by avoiding mating of close relatives. For the sheep population in Cyprus, standard records were collected, including month of lambing, year of lambing, lactation number and age of lambing. Phenotypic data were

obtained for lambing years between 2009 and 2016 and included 1,514 individual records of total milk yield and 1,203 and 615 measurements of fat and protein percentage, respectively, for the first up to the seventh lactation with 1,272 milk records from the first three lactations.

For both sheep populations, total lactation yield was calculated for each animal with the Fleischmann method with monthly tests on actual yields (sum of a.m. and p.m. records, ICAR 2014). Milk samples were obtained for fat and protein analysis using combined thermo-optical procedures (LactoStar 3510, Funke Gerber, Berlin, Germany), previously calibrated for protein with the Lowry protein assay and for fat with the method 989.05 (AOAC International, 2005).

### *2.3. Mixed model association analysis*

The impact of each identified genotype in the g.2982T>C SNP locus on the studied traits obtained from the Chios ewes in Greece and Cyprus was determined with the following mixed linear model; each trait was analyzed separately:

$$Y_{jklmn} = \mu + F_i + YS_j + L_k + b_1 age + b_2 dur + G_l + A_m + ei_{jklmn}, \quad [1]$$

where, Y refers to the phenotypes of lactation milk yield record *n* of animal *m* in the Greek Chios population while in the Cypriot Chios population Y refers to the phenotypes of lactation milk yield, fat yield/ percentage or protein yield/ percentage record *n* of animal *m*; μ = overall population mean for the trait; F = fixed effect of flock *i* (Greece: 1-20 and Cyprus: 1-5); YS = fixed effect of year (Greece: 2003-2009 and Cyprus: 2009-2016) by season (1-2) of lambing interaction *j*; L = fixed effect of number of lactation *k* (Greece: 1-6 and Cyprus: 1-7); $b_1$ = linear regression on age at lambing (age); $b_2$ = linear regression on lactation duration (dur); yield traits

only; G = fixed effect of genotype *l* in the g.2982T>C locus (1-3; CC, CT, TT); A = random effect of animal *m*; e = random residual effect.

Mixed model association analysis of the g.2982T>C SNP was performed in the Chios ewes population in Greece for total milk yield at all lactations, first lactation, across first to third lactation and across second to sixth lactations, while, for the Chios ewes population in Cyprus the mixed model association analysis was performed for the lactation milk yield, fat yield/ percentage and protein yield/ percentage at all lactations, first lactation, across first to third lactation and across second to seventh lactations. Predicted trait values for each SNP genotype class and standard errors were obtained by utilizing the predict directive in ASREML (Gilmour *et al.*, 2009). Briefly, the predicted trait values are derived from the best linear unbiased estimate of the significant fixed and random effects in the model. The predicted trait values were used to estimate additive and dominance SNP effects on traits, and the proportion of phenotypic and animal variances for each trait explained by the SNP locus. The equations used were:

Additive effect, a=(TT-CC)/2,

Dominance effect, d=CT-[(TT+CC)/2],

Percentage of phenotypic variance ($V_P$) due to SNP =100 * $[2pq\ (a+d\ (q-p))^2]/V_P$,

Percentage of animal variance ($V_A$) due to SNP=100 * $[2pq\ (a+d\ (q-p))^2]/V_A$,

where TT, CC and CT are the predicted trait values for each genotype class; and p, q are the allele frequencies at the SNP locus. Variance components were estimated with the same model after excluding the genotype effect.

## 3. Results

### *Genotyping and association with production traits*

The g.2982C/T polymorphism of the *ACAA2* gene was genotyped from 632 Chios ewes from North Greece. Allelic frequencies were 0.60 for the g. 2982T allele and 0.40 for the g.2982C allele. Genotypic frequencies were 0.370, 0.454 and 0.176 for the genotypes g.2982TT, g.2982CT and g.2982CC, respectively and were found to be in Hardy-Weinberg equilibrium.

Mixed model association analysis of the g.2982T>C SNP for the sheep population in Greece was performed for total milk yield at all lactations, first lactation, across first to third lactation and across second to sixth lactations. Predicted trait mean values and standard errors for the three genotype classes for the traits are presented in **Table 4.1**.

For the sheep population in Greece, a significant negative dominance effect of -12.63 ± 3.63 ($p<0.001$) and of -12.71 ± 3.60 ($p<0.001$) was detected for total lactation milk yield across first to third lactation and for all lactations, respectively. Based on the estimated allelic effects and the allele frequencies observed in the sample, it was estimated that the g.2982T>C SNP explained 0.62% of the phenotypic variance of milk yield for all lactations and 2.34% of the animal variance (**Table 4.2**).

**Table 4.1.** Predicted genotype means and SE[1] of the genotypic classes CC, CT and TT at the g.2982T>C *ACAA2* locus and significance of genotype contrasts for each trait for the Chios ewes farmed in Greece.

| Trait | All lactations | 1st lactation | 1st-3rd lactations | 2nd to 6th lactations |
|---|---|---|---|---|
| | Milk yield (kg) | | | |
| CC | 249.74±4.82[c] | 231.99±5.08[a,c] | 245.44±4.86[c] | 235.48±6.71 |
| CT | 232.31±3.01[d] | 222.66±3.26[b] | 227.84±3.04[d] | 214.41±4.16 |
| TT | 240.30±3.23[e] | 227.05±3.56[d] | 235.51±3.26[d] | 225.67±4.27 |

[1]Marginal ggenotyped means (±standard error) predicted from the mixed model association analyses, after adjusting for all significant fixed and random effects.

[a-e]Means within a column with two different superscripts differ as: [a,b]P<0.001; [c,d]P<0.01; [c,e]P<0.025

**Table 4.2.** The SNP allelic effects and percentage of phenotypic and animal variance explained by the g.2982T>C SNP of the *ACAA2* gene for the Chios ewes farmed in Greece.

| Trait | All lactations | 1st lactation | 1st-3rd lactations | 2nd to 6th lactations |
|---|---|---|---|---|
| | Milk yield (kg) | | | |
| a[1]±SE | -4.72±2.90 | -2.47±3.10 | -4.96±2.93 | -4.90±3.98 |
| P (sed)[2] | NS | NS | NS | NS |
| d[3]±SE | -12.71±3.60 | -6.86±3.86 | -12.63±3.63 | -16.16±4.95 |
| P[4] | 0.0004 | NS | 0.0005 | 0.0011 |
| % $V_P$ due to SNP[5] | 0.62% | 2.26% | 0.66% | 0.68% |
| % $V_A$ due to SNP[5] | 2.34% | | 2.50% | 1.91% |

NS: not significant

[1] a= additive effect; positive additive genetic effect (a>0) indicates T allele increased the trait

[2] p value for assessing the additive effect on the trait

[3] d=dominance effect

[4] p value for assessing the dominance effect on the trait

[5] Estimated using allele frequencies observed in sample (p=0.40 for allele C and q=0.60 for T)

Concerning the Chios sheep population in Cyprus, the allelic frequencies in the g.2982C/T SNP locus were 0.54 for the T allele and 0.46 for the C allele; genotypic frequencies were 0.27, 0.54 and 0.19 for g.2982TT, g.2982CT and g.2982CC, respectively. Genotypic frequencies were found to deviate from the Hardy-Weinberg equilibrium (p=0.019).

Mixed model association analysis of the g.2982T>C SNP of the sheep population in Cyprus was performed for total lactation milk yield, fat percentage and yield, protein percentage and yield at all lactations, first lactation, across first to third lactation and across second to seventh lactations. Marginal predicted means for the three genotype classes for each individual trait are presented in **Table 4.3**.

The SNP was significantly associated with milk yield at first lactation ($p<0.01$), across first to third lactation ($p<0.01$) and at all lactations ($p<0.025$) with respective additive effects of 10.61 ± 3.56 kg, 7.62 ± 3.06 kg and 6.81 ± 2.95 and respective positive dominance effects of 13.02 ± 4.26 kg, 9.49 ± 3.67 kg and 8.67 ± 3.53 kg (**Table 4.4**). Significant differences were found between the CC and CT genotype pairs and between the CC and TT classes at the g.2982T>C SNP locus for milk yield at all lactations, at first lactation and across first to third lactation, but not between TT and CT (**Table 4.3**). These results suggest a complete dominance effect at the locus, since heterozygous CT animals exhibit similar predicted mean values compared to homozygous TT animals. Based on the estimated allelic effects and the allele frequencies observed in the sample, it was estimated that the g.2982T>C SNP explained 2.25%, 0.80% and 0.62% of the phenotypic variance of milk yield for first, across first to third and for all lactations, respectively (**Table 4.4**). Overall, these results suggest a stronger association in first-lactation

milk yield, whereas the effect was reduced in subsequent lactations. None of the analyses showed a significant association of the SNP genotype with fat percentage.

After applying Holm-Bonferroni correction on the results obtained for sheep population in Cyprus the SNP was also associated with protein percentage at all lactations ($p<0.01$) and from second to seventh lactation ($p<0.0001$) (**Table 4.3**) with respective significant additive effects of -0.05±0.02 and -0.07±0.02 (**Table 4.4**). Pairwise contrasts between the predicted protein percentage values showed significant differences between the genotype classes CC and CT and between CC and TT at all lactations and across second to seventh lactation, with CC animals exhibiting higher protein percentage, whereas differences between CT and TT were not significant (**Table 4.3**). It was estimated that the SNP explained 1.68% and 3.59% of the phenotypic variance of protein percentage for all lactations and across second to seventh lactation, respectively (**Table 4.3**). Additionally, we noted a dominance effect of 0.46 ± 0.21 kg ($p=0.027$) and 0.41 ± 0.21 kg ($P = 0.048$) of the SNP on fat and protein yields, respectively. Homozygous TT and heterozygous CT animals exhibited significantly higher milk fat yield compared with homozygous CC ewes ($P < 0.025$ and $P < 0.001$, respectively).

**Table 4.3.** Predicted genotype means and standard errors[1] of the genotypic classes CC, CT and TT at the g.2982T>C *ACAA2* locus and significance of genotype contrasts for each trait for the Chios ewes farmed in Cyprus.

| Trait | All lactations | 1st lactation | 1st-3rd lactations | 2nd to 7th lactations |
|---|---|---|---|---|
| | | | **Milk yield (kg)** | |
| CC | 245.23 ± 4.44[c] | 170.21 ± 5.28[a,c] | 247.93 ± 4.60[c] | 238.72 ± 5.80 |
| CT | 260.71 ± 2.76[d] | 193.85 ± 3.33[b] | 265.03 ± 2.87[d] | 249.47 ± 3.64 |
| TT | 258.85 ± 3.88[e] | 191.44 ± 4.77[d] | 263.17 ± 4.04[d] | 246.78 ± 5.08 |
| | | | **Milk protein (%)** | |
| CC | 5.29 ± 0.02[c] | 4.49 ± 0.04 | 5.22 ± 0.02 | 5.20 ± 0.03 |
| CT | 5.23 ± 0.01[d] | 4.53 ± 0.02 | 5.21 ± 0.01 | 5.10 ± 0.02 |
| TT | 5.20 ± 0.02[d] | 4.55 ± 0.04 | 5.16 ± 0.02 | 5.06 ± 0.03 |
| | | | **Milk protein yield (kg)** | |
| CC | 8.78 ± 0.25 | | | |
| CT | 9.42 ± 0.16 | | | |
| TT | 9.23 ± 0.25 | | | |
| | | | **Milk fat (%)** | |
| CC | 5.19 ± 0.05 | 5.48 ± 0.08 | 5.03 ± 0.05 | 4.71 ± 0.05 |
| CT | 5.20 ± 0.03 | 5.49 ± 0.05 | 5.05 ± 0.03 | 4.71 ± 0.04 |
| TT | 5.14 ± 0.04 | 5.48 ± 0.06 | 4.97 ± 0.04 | 4.62 ± 0.05 |
| | | | **Milk fat yield (kg)** | |
| CC | 12.87 ± 0.26[a,c] | | | |
| CT | 13.62 ± 0.17[b] | | | |
| TT | 13.44 ± 0.23[e] | | | |

[1]Marginal ggenotyped means (±standard error) predicted from the mixed model association analyses, after adjusting for all significant fixed and random effects.

[a-e]Means within a column with two different superscripts differ as: [a,b]P<0.001; [c,d]P<0.01; [c,e]P<0.025

**Table 4.4.** SNP allelic effects and percentage of phenotypic and animal variance explained by the g.2982T>C SNP for the Chios ewes farmed in Cyprus.

| Trait | All lactations | 1st lactation | 1st-3rd lactations | 2nd to 7th lactations |
|---|---|---|---|---|
| | | **Milk yield (kg)** | | |
| a[1]±SE | 6.81±2.95 | 10.61±3.56 | 7.62±3.06 | 4.03±3.86 |
| P (sed)[2] | 0.021* | 0.003* | 0.013* | NS |
| d[3]±SE | 8.67±3.53 | 13.02±4.26 | 9.49±3.67 | 6.72±4.64 |
| P[4] | 0.014* | 0.002* | 0.010* | NS |
| % $V_P$ due to SNP[5] | 0.62% | 2.25% | 0.80% | 0.20% |
| % $V_A$ due to SNP[5] | 2.53% | | 3.08% | 0.59% |
| | | **Milk protein (%)** | | |
| a[1]±SE | -0.05±0.02 | 0.03±0.03 | -0.03±0.02 | -0.07±0.02 |
| P (sed)[2] | 0.003* | NS | NS | 0.000* |
| d[3]±SE | -0.02±0.02 | 0.01±0.03 | 0.01±0.02 | -0.03±0.02 |
| P[4] | 0.41 | NS | NS | NS |
| % $V_P$ due to SNP[5] | 1.68% | 0.72% | 0.66% | 3.59% |
| | | **Milk protein yield (kg)** | | |
| a[1]±SE | 0.23 ± 0.18 | | | |
| P (sed)[2] | 0.20 | | | |
| d[3]±SE | 0.41 ± 0.21 | | | |
| P[4] | 0.048* | | | |
| % $V_P$ due to SNP[5] | 0.44 | | | |

| | Milk fat (%) | | | |
|---|---|---|---|---|
| a[1]±SE | -0.03 ± 0.03 | 0.00 ± 0.05 | -0.03 ± 0.03 | -0.05 ± 0.04 |
| P (sed)[2] | 0.37 | 0.98 | 0.40 | 0.19 |
| d[3]±SE | 0.04 ± 0.04 | 0.00 ± 0.06 | 0.05 ± 0.04 | 0.04 ± 0.04 |
| P[4] | 0.32 | 0.95 | 0.18 | 0.37 |
| % $V_P$ due to SNP[5] | 0.07 | 0.00 | 0.06 | 0.24 |
| % $V_A$ due to SNP[5] | 0.33 | | 0.28 | 0.80 |
| | **Milk fat yield (kg)** | | | |
| a[1]±SE | 0.28 ± 0.17 | | | |
| P (sed)[2] | 0.10 | | | |
| d[3]±SE | 0.46 ± 0.21 | | | |
| P[4] | 0.027* | | | |
| % $V_P$ due to SNP[5] | 0.41 | | | |

NS: not significant

[1] a= additive effect; positive additive genetic effect (a>0) indicates T allele increased the trait

[2] p value for assessing the additive effect on the trait; *significant post-Holm-Bonferroni adjustment for 3 independent tests

[3] d=dominance effect

[4] p value for assessing the dominance effect on the trait; *significant post-Holm-Bonferroni adjustment for 3 independent tests

[5] Estimated using allele frequencies observed in sample (p=0.46 for allele C and q=0.54 for T)

## 4. Discussion

The g.2982C/T polymorphism of the *ACAA2* gene was genotyped from 632 Chios ewes, farmed in Greece and from 742 Chios ewes, farmed in Cyprus. Sequencing of the 10th exon including the UTR did not reveal any additional polymorphism to the g.2982C/T previously detected (Orford et al., 2012). Allele frequencies for both sheep population were similar to the previously observed (T:0.56; C:0.44; Orford et al., 2012). Our study confirmed the previously observed association of the HM537015:g.2982T>C *ACAA2* SNP with milk yield in an extended population of Chios sheep from multiple flocks in Cyprus and an independent Chios sheep population from multiple flocks in Greece. The g.2982T>C SNP of the *ACAA2* gene showed association with milk protein percentage, milk protein yield and milk fat yield in the Cypriot population having data for those traits. The allele frequencies for the sheep population in Greece were found to be in Hardy-Weinberg equilibrium consistently with Orford et al. (2012), while the frequencies for the sheep population in Cyprus showed deviation from the Hardy-Weinberg equilibrium in contrast to the previously observed frequencies resulted from a single experimental flock (Orford et al., 2012). The deviation from the equilibrium could be possibly explained by the fact that farmers select animals mainly based on milk yield, due to low meat prices during the last decade, whereas the selection indices used at the experimental flock (Orford et al., 2012) combine the individual capacity of young stock for growth and milk production (Mavrogenis and Constantinou, 1991).

Consistent with our previous study (Orford et al., 2012), the g.2982T>C SNP, was significantly associated with milk yield, with the T allele of the sheep population in Cyprus

exhibiting positive additive and dominance effects, mainly attributed to first lactation production data. Based on predicted g.2982T>C SNP values from mixed model association analyses, Chios ewes with a CC homozygous genotype were found to produce approximately 12% and 11% significantly less milk from the CT ($P < 0.001$) and TT ($P < 0.01$) animals, respectively. The current results shed more light in the confirmation of a previously detected association as the use of a bigger and different data set strengthens the evidence for the observed association (Sasaki et al., 2013). However, the T allele of the sheep population in Greece had negative dominance effects on milk yield, while the predicted g.2982T>C SNP values from the mixed model association analyses have shown that Chios sheep in Greece with a CC homozygous genotype produce significantly more milk rather than the heterozygous CT and homozygous TT animals, with the first producing 6.98% and 3.78% more milk from the CT ($P < 0.01$) and TT ($P < 0.025$) animals, respectively.

According to the study of Orford et al. (2012), the heterozygous genotype in the g.2982T>C SNP locus was nearer the homozygous TT genotype based on its phenotype, while the T allele was suggested to have a putative partial dominant effect on milk yield. In the present study, the use of two extended and independent sheep populations has shown over-dominance of the CT genotype on milk yield as its phenotype lies outside the phenotypical range of both homozygous genotypes. In the case of the sheep population in Cyprus and Greece the CT genotype was found to be the superior and inferior genotype, respectively. The overall effects estimated in the present study from the sheep in Cyprus were lower compared with Orford et al. (2012), possibly due to the increased variation introduced from the use of multiple flocks managed in different ways, as a substantial fraction of the environmental

variance for production traits is attributed to farm (Carta et al., 2009; Sasaki et al., 2013). In the current analysis, we adjusted for the systematic effect of the flock and, therefore, the estimates are more representative and indicative of the true effect of the gene in the population. The reasons why the effects are more important in the first lactation than in the rest of lactations are not clear at this stage; therefore, further research is needed. It is therefore likely that the SNP is not the causative mutation but is in linkage or linkage disequilibrium with the causative SNP that differs in both populations possibly due to founder effects. Indeed, the Cypriot sheep population has been established from a small number of less than 30 animals (Mavrogenis A., personal communication), while the Greek population was endangered in the past (Rogdakis, 2002). In addition, the Cypriot population has suffered from a bottleneck effect due to selection for scrapie resistance (Brown et al., 2014).

In the present study, additional evidence about the correlation of the *ACAA2* gene with important sheep dairy traits is provided from the available data of the sheep in Cyprus, as the g.2982T>C SNP was found to be significantly associated with milk protein percentage. Homozygous CC animals exhibited superior values for protein percentage compared with both homozygous TT and heterozygous CT animals, in contrast to their inferior values estimated for total milk yield (**Table 4.3**). This is consistent with the negative genetic correlation between those 2 traits (Bencini and Pulina, 1997; Fuertes et al., 1998). As genotypic classes for protein yield differ marginally, the decrease in protein content could be attributed to a dilution effect due to the increase of milk yield (Emery, 1988).

Similarly, milk yield is known to be negatively correlated with fat content (Fuertes et al., 1998). However, although the marginal predicted mean for the fat content from CC ewes in Cyprus was higher compared with that of the other genotypes in the present study, the differences were not significant (**Tables 4.3**) and the investigated SNP was not significantly associated with milk fat percentage (**Tables 4.4**), consistent with the results of Orford et al. (2012). In agreement with other studies where fat is the most variable component of ovine milk (Othmane et al., 2002; Pulina et al., 2005), in the current study standard errors for fat content were twice as much as those for protein content. Therefore, low precision of data for fat content may be a reason for not detecting the association of the studied SNP with fat percentage; however, we found a significant association of the SNP with fat yield (**Table 4.4**). In addition, homozygous CC animals exhibited significantly lower fat yields compared with heterozygous CT and homozygous TT animals (**Table 4.3**), which could be attributed to decreased milk yield with similar fat content.

In mammals, excess energy is stored primarily as triglycerides, which are mobilized when energy demands arise such as during periods of underfeeding or in early lactation (Drackley et al., 1999). FAs coming from triglycerides are taken up by the liver where they are either used as energy source or converted to ketone bodies that may be released into the blood and can be used as energy or substrates for de novo FA synthesis in the mammary gland. In addition, it is estimated that gluconeogenesis is usually increased two- to three-fold during early lactation to meet the demands of the mammary gland for lactose and triglyceride synthesis (Drackley et al., 2000; Vernon, 2005). The increased energy needs for gluconeogenesis and triglyceride synthesis are met primarily by increased FA oxidation. The acetyl-CoA acyltransferase 2

enzyme, also known as 3-oxoacyl-CoA thiolase or mitochondrial 3-ketoacyl-CoA thiolase is encoded by the *ACAA2* gene and although is predominantly expressed in liver, *ACAA2* has been reported to be expressed in many other tissues, including the mammary gland (http://www.genecards.org/cgi-bin/carddisp.pl?gene=ACAA2; Shi et al., 2015). The energy produced by the fatty acid oxidation, in which the ACAA2 enzyme is involved by catalysing the last step in mitochondrial FA β-oxidation, can induce non-thermodynamically favourable reactions related to milk yield and content through a mechanism which needs to be elucidated.

## 5. Conclusions

In the current study, the results obtained from the use of two extended and independent Chios ewes populations in Greece and Cyprus confirmed that g.2982T>C SNP in the 3'UTR of the *ACAA2* gene is associated with milk yield. In addition, the current study, from available data obtained from the sheep population in Cyprus demonstrated the association of the *ACAA2* gene with important dairy sheep traits such as the milk protein percentage, protein and fat yields. Future studies should examine the association effects of the g.2982T>C SNP with milk traits in other dairy sheep breeds and the putative functional involvement of the gene in dairy traits. The molecular information could be exploited in appropriate breeding schemes for the improvement of milk production and content of dairy sheep.

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# CHAPTER 5

## *ACAA2* and *FASN* polymorphisms affect fatty acid profile of Chios sheep milk

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**Abstract**

The objective of the present research paper was the identification and association of single nucleotide polymorphisms (**SNP**) in the ovine *acetyl-CoA acyltransferase 2* (***ACAA2***), *acyl-CoA:diacylglycerol acyltransferase 1* (***DGAT1***), *fatty acid synthase* (***FASN***) and *steroyl-CoA desaturase 1* (***SCD1***) genes with milk fat percentage and fatty acid content. Three consecutive milk samplings took place from a total of 429 purebred Chios ewes from four farms during mid-lactation. Milk fat percentage was recorded and the fatty acid profile was determined using gas chromatography-mass spectrometry. The exon 10 (3'UTR) of the *ACAA2* gene, the 3' untranslated region (UTR) of the *DGAT1* gene, the exon 31 of the *FASN* gene and the 5'UTR and 3'UTR of the *SCD1* gene were sequenced and genotyped for all ewes. Genotypic data were jointly analyzed with 1,184 fat content records and 37,718 fatty acid percentage records using mixed models. *DGAT1* and *SCD1* genes appeared to be monomorphic, while SNP g.14777C>T identified on exon 31 of the *FASN* gene was found to be associated with C13:0. SNP g.2982T>C on the 3'UTR of the *ACAA2* gene was shown to be associated with C9:0, C11:0, C12:1 *cis*-9, C13:0 and the ω6/ω3 index. Fat percentage was not shown to be affected by the identified SNPs. The results could be useful information for breeding programs aiming to improve the quality and nutritional value of ovine milk.

## 1. Introduction

Ovine milk production is a significant industry in the Mediterranean region, mainly directed to the production of high quality cheese, due to the higher solid content of sheep milk compared to other ruminants (reviewed by Carta et al., 2009). Milk fat and protein content, as well as the milk fatty acid (**FA**) profile, determine the quality and nutritional value of ovine milk since they affect cheese quality and organoleptic characteristics (reviewed by Lamichhane et al., 2017), while specific FAs have been reported to be related to non-communicable disease by several studies (reviewed by Gómez-Cortés et al., 2018). Although variation of fat and FA content in sheep milk is mainly affected by nutrition (reviewed by Nudda et al., 2014) and management practices (Sitzia et al., 2015), genetics play an important role (Barillet 2007; Carta et al., 2009).

Acyl-CoA:diacylglycerol acyltransferase 1 (**DGAT1**) is an enzyme essential for the last step of triacylglycerol (**TAG**) synthesis (Coleman et al., 2000). In dairy cattle, a mutation (K232A) of the *DGAT1* gene was highly correlated with milk fat (Winter et al., 2002; Grisart et al., 2004; Schennink et al., 2007; Vanbergue et al., 2016) and milk FA content (Schennink et al., 2007, 2008; Bouwman et al., 2011; Houaga et al., 2018). Furthermore, other polymorphisms within the bovine *DGAT1* gene in linkage disequilibrium with the K232A mutation were associated with the milk FA content (Li et al., 2014; Palombo et al., 2018). In sheep, although the exon harboring the K232A single nucleotide polymorphism (**SNP**) was found to be monomorphic in some breeds (Scata et al., 2009; Miltiadou et al., 2010), two SNP outside the coding region of the ovine *DGAT1* gene were suggested to be associated with milk fat content (Scata et al.,

2009), while a SNP in exon 17 was shown to affect milk FA traits in Assaf sheep (Dervishi et al., 2015).

The acetyl-CoA acyltransferase 2 (**ACAA2**) enzyme catalyzes the last step in FA β-oxidation, thus serving as a key component in the lipid metabolism for energy production and supply of substrates for FA synthesis (reviewed by Eaton et al., 1996). In addition, quantitative trait loci (**QTL**) for milk, fat and protein yields have been mapped in the area of the *Ovis aries* chromosome (**OAR**) 23 where the *ACAA2* gene is located (Gutierrez-Gil et al., 2009). A SNP in the 3' untranslated region (**UTR**) of the gene explained 10% of the additive genetic variance for 1$^{st}$ lactation milk yield (Orford et al., 2012), while more recently, the same SNP was also associated with total milk yield, milk protein percentage and milk fat yield in dairy Chios sheep (**Chapter 4**) and was shown to exhibit differential allelic expression (Miltiadou et al., 2017b).

The *fatty acid synthase* (***FASN***) gene encodes a large complex of a multifunctional enzyme which catalyzes the *de novo* synthesis of small to medium chain FAs (Bionaz and Loor, 2008). In dairy cows, the gene has been found to affect the milk fat percentage and FA content (Schennink et al., 2009). In sheep, Carta et al. (2008) detected a QTL on the OAR11, where the *FASN* gene is harbored, associated with C14:0 and C16:0, whereas García-Fernández et al. (2010b) reported that this gene was near a QTL region affecting C10:0 and the polyunsaturated FAs (**PUFA**) content of milk in Spanish Churra breed. Moreover, polymorphisms in the *FASN* gene have been shown to affect the FA profile in Altamourana and Gentile di Puglia sheep breeds (Crisa et al., 2010) and in goats (Haile et al., 2016).

Another candidate and well investigated gene shown to affect the milk FA content is the *steroyl-CoA desaturase 1* (***SCD1***). The key enzyme encoded, also known as Δ-9 desaturase, synthesizes monounsaturated FAs (**MUFA**) (Enoch et al., 1976) and produces the 9-*cis*, 11-*trans* conjugated linoleic acid (**CLA)** from vaccenic acid (**VA**) (Bauman et al., 1999). Various studies in dairy cow breeds have reported the association of a polymorphism (A293V) on exon 5 with milk FA and FA index (Mele et al., 2007; Moioli et al., 2007b; Schennink, 2008; Conte et al., 2010; Bouwman et al., 2011; Buitenhuis et al., 2014; Palombo et al., 2018). In sheep, *SCD1* is located on OAR22 which harbors a QTL affecting the CLA/VA ratio in a Sardinian X Lacaune sheep population (Carta et al., 2006), while, a SNP detected in intron 4 of the ovine *SCD1* gene has been associated with the CLA/VA ratio in the same sheep population (Miari et al., 2009). Moreover, studies in goats reported a deletion in the untranslated region of the *SCD1* gene associated with the FA profile of milk (Zidi et al., 2010).

In the present study, we hypothesized that, since DGAT1, ACAA2, FASN and SCD1 play fundamental functional roles in lipid metabolism and/or located in chromosomal regions associated with milk content, polymorphisms within those genes may partially explain the variation of fat content and/or FA composition in sheep milk. Therefore, our objectives were to (i) characterize at molecular level polymorphisms in the *DGAT1*, *ACAA2*, *FASN* and *SCD1* genes and (ii) examine the association of identified variations with milk fat percentage and FA profile in the Chios sheep breed, a highly productive and well adapted breed of the Mediterranean region.

## 2. Materials and methods

### *2.1. Animals, phenotypic data and sampling*

A total of 429 purebred Chios dairy ewes in mid-lactation from four farms in Cyprus were used for the present study. All ewes were machine milked twice daily. Representative milk samples of 50 mL were collected during morning milking from each ewe and placed in 50mL falcon tubes in a cool box (4 °C) which was immediately transferred to the laboratory. Sampling took place three times in three consecutive months during one lactation period (2012-2013) from each individual ewe. Standard records for the date of lambing, lactation number, ewe age at lambing and date of sampling were also collected. All experimental procedures that involved animal handling were carried out according to the regulations of the national legislation (Animal Welfare Law, 1994) and international guidelines (Directive 2010/63/EU for animal experimentation) and approved by the corresponding departmental committee.

### *2.2. DNA extraction, SNP identification and genotyping*

Blood samples were collected from all 429 ewes for DNA extraction. The Genomic DNA Blood kit (Macherey-Nagel, Duren, Germany) was used for genomic DNA isolation, according to manufacturer's instructions. UV absorption at 260 and 280 nm was used for the estimation of DNA quality and quantity. A cost effective direct DNA sequencing protocol was performed for SNP identification and genotyping as described in **Chapter 4**. Exon 10 of the *ACAA2* gene, the 3'-UTR of the *DGAT1* gene, exon 31 of the *FASN* gene and the 5'-UTR and 3'-UTR of the *SCD1* gene were sequenced from 30 randomly selected animals using an outer set of primers amplifying the genomic regions presented in **Table 5.1** (section SNP identification). Subsequently, an inner

set amplifying a shorter area was used for SNP genotyping of all animals. Outer primers ACAA2_3'UTR_F1 (TTGATTATCTAATTCCGTACATTCTTT)/ ACAA2_3'UTR_R1 (TTTAATGCCACTGAAGTGTATGC) and DGAT1_3'UTR_F1 (AGGAACTCGGAGTCCATCAC)/ DGAT1_3'UTR_R1 (CTCCTCAGGGGCAGAAAAG), used for amplification of the *ACAA2* and *DGAT1* gene regions, were obtained from Orford et al. (2012) and Scatà et al. (2009), respectively. All other primers presented in **Table 5.1** were designed for this study using primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/). For this purpose, publically available sequences of the gene area of interest from multiple genus species of the bovidae family were aligned together aiming to design outer primers in highly conserved areas (Genbank accession numbers for each gene region: *FASN exon 31*: *Ovis aries* JN570752.2, GQ150557.1 and XM_015098375.1, *Capra hircus* DQ915966.3, DQ223929.1 and NM001285629.1, *Bos taurus* AF285607.2, NM00112669.1, XM005220997.7 and AY343889.1; *SCD1 5'-UTR* and *3'-UTR*: *Ovis aries* GQ904712.2, FJ513370.1, JX944473.1 and JX9444751, *Capra hircus* AH011188.2, *Bos taurus* AY241932.1 and AH011561.2). Inner primers for all gene regions were designed according to the amplicons obtained with the outer primer sets after SNP identification. PCR reactions for *DGAT1*, *FASN* and *SCD1* were set up in 25-µL volumes containing 25 ng of genomic DNA, 0.5 U of Taq DNA polymerase (KAPABIOSYSTEMS, Boston, Massachusetts, US), 0.5 µM of each primer, 0.2 mM dNTPs and 1.5 to 2.5 mM $MgCl_2$ for each primer pair (**Table 5.1**). After an initial 5-min desaturation step at 94°C, the reaction mix was subjected to 30 cycles at 94°C for 30 sec, 55 to 60°C annealing temperature for 30 sec (**Table 5.1**) and 72°C for 30 sec with a final elongation step at 72°C for 5-min. PCR reactions for the *ACAA2* gene were performed as described in Orford et al. (2012).

**Table 5.1.** Primers and reaction conditions for PCR used to amplify the area of interest of the *ACAA2*, *DGAT1*, *FASN* and *SCD* genes

| Primer | Sequence 5'→ 3' | SNP[1] | OAR[2] | Annealing T (°C)[3] | MgCl (mM) | Amplicon size (bp) | Covered region[4] | GenBank accession no. |
|---|---|---|---|---|---|---|---|---|
| **For SNP identification** | | | | | | | | |
| ACAA2_3'UTR_F1<br>ACAA2_3'UTR_R1 | TTGATTATCTAATTCCGTACATTCTTT<br>TTTAATGCCACTGAAGTGTATGC | g.2982T>C | 23 | 55 | 2.5 | 598 | exon 10 - 3' UTR | HM537015.2 |
| DGAT1_3'UTR_F1<br>DGAT1_3'UTR_R1 | AGGAACTCGGAGTCCATCAC<br>CTCCTCAGGGGCAGAAAAG | | 9 | 58 | 1.5 | 739 | exon 14 - 3' UTR | EU178818.1 |
| FASN_exon31_F1<br>FASN_exon31_R1 | TGTGGGCTATGTGGTGAGG<br>CATCTGGGTGGGTTTGGTC | g.14777C>T | 11 | 59 | 2.5 | 400 | partial intron 30 - partial exon 32 | JN570752.2 |
| SCD_5'UTR_F1<br>SCD_5'UTR_R1 | AAATTCCCTTCGGCCAATGAC<br>TCTCACCTCCTCTTGCAGCAA | | 22 | 58 | 1.5 | 527 | partial promoter - exon 1 | GQ904712.2 |
| SCD_3'UTR_F<br>SCD_3'UTR_R | GAAAGTATCCAAGGCTGCCG<br>TGCTTCTTCTTTATCCTCAGCC | | 22 | 59 | 2 | 842 | partial exon 6 - 3' UTR | GQ904712.2 |
| **For Genotyping** | | | | | | | | |
| ACAA2_3'UTR_F2<br>ACAA2_3'UTR_R2 | ATTGA**C**AACACAGCCTGAGGAGAGC<br>TGGATTTCAGTTCAGAAATGCTGCAT | | 23 | 60 | 2.5 | 247 | partial exon 10 - 3' UTR | |
| DGAT1_3'UTR_F2<br>DGAT1_3'UTR_R2 | ATGATGGCRCAGGTGAGCA<br>AGGAGGCAGCTTYCACCAG | | 9 | 59 | 2 | 364 | intron 16 - 3' UTR | |
| FASN_exon31_F2<br>FASN_exon31_R2 | ACCATGTGGCAGGAAGTGTC<br>AATGACCACTTTGCCGATGT | | 11 | 59 | 2.5 | 150 | partial exon 31 | |
| SCD_5'UTR_F2<br>SCD_5'UTR_R2 | ATCCCCAGCACAGCAGGT<br>CATTACTCGGAAGCTCTCACC | | 22 | 59 | 2.5 | 249 | 5' UTR - partial intron 1 | |

[1] Single nucleotide polymorphism; [2] *Ovis aries* chromosome; [3] Annealing temperature; [4] UTR: untranslated region.

### *2.3. Milk analysis*

From each milk sample, one subsample was kept at 4°C for fat content determination through thermo-optical procedures (LactoStar 3510, Funke Gerber, Berlin, Germany) calibrated previously with the Gerber method 989.05 (AOAC International, 2005). Another subsample of 1.5 mL milk was kept at -20°C for FA analyses. For the isolation of fat from milk, the rapid double centrifugation method of Feng et al. (2004) was used with slight modifications (Tzamaloukas et al., 2015), as described previously in **Chapter 3**. A transesterification reaction was used for the formation of fatty acid methylesters (**FAME**) according to the ISO method (2002) as previously described in **Chapter 3**. FAME were transferred to amber gas chromatography vials and stored at −80°C until they were analyzed on a GCMS-QP2010 Plus Gas Chromatography Mass Spectrometer (Shimadzu, Duisburgh, Germany) equipped with an HT280T auto sampler (HTA, Brescia, Italy) by split ratio injection (1:20) of 1 µl FAME aliquots onto an Agilent CP-Sil 88 fused-silica capillary column (50 m × 0.25 mm internal diameter × 0.2 µm film thickness). A gradient temperature program was used, where the initial oven temperature was held at 80°C for 1 min after injection and increased at 20°C/min to 120°C. Temperature was then raised to 193°C at 1°C/min, and finally increased to 220°C at 5°C/min. The carrier gas (helium) had a flow rate of 1 mL/min and the temperature of both injector and interface were kept at 225°C. Shimadzu GCMS Postrun Solution software was used for the identification and quantitation of FAME. For the identification of each individual peak, retention indices were compared to those of commercially available authentic standards (Supelco 37-FAME standard mix, *cis*- 9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, *trans*-11 C18:1; Sigma-Aldrich, Gillingham, UK), using the National Institute of Standards and Technology 08 and 21 mass

spectral libraries and cross referencing with chromatograms-spectrograms reported in the literature (Kramer et al., 2008; Tsiafoulis et al., 2014). For the quantitation, each individual peak identified was integrated and expressed as a percentage of the total, as this quantification method is widely used and allows sound comparisons of FA profiles in milk samples (Butler et al., 2008). At the end of the process, 1,184 fat content records and 37,718 FA percentage records were available for statistical analysis.

### *2.4. Statistical analysis*

The impact of each identified genotype in each locus on the studied traits was determined with the following mixed linear model; each trait was analyzed separately:

$$Y_{jklmn} = \mu + F_i + YS_j + L_k + b_{1age} + G_l + A_m + e_{ijklmn} \quad [1]$$

where, Y refers to the phenotypes of fat percentage and 43 individual milk FAs, 9 FA (saturated FAs (SFA), unsaturated FAs (UFA), MUFA, PUFA, short chain FAs (SCFA), mammary origin FAs, medium chain FAs (MCFA), long chain FAs (LCFA), Odd chain FAs) and 3 constructed indices (ω6/ω3, atherogenicity index (AI), elongation index (EI)); μ = overall population mean for the trait; F = fixed effect of flock *i* (1–4); YS = fixed effect of the year (2012-2013) by season (1–2) of lambing interaction *j*; L = fixed effect of lactation number *k* (1–7); $b_1$ = linear regression on age at lambing (age); G = fixed effect of genotype *l* (1–3; CC, CT, TT); A = random effect of animal *m*; e = random residual effect.

The predicted values for all traits and their respective standard errors were derived for each SNP genotype in each locus and reflected the marginal genotypic effect on the trait adjusted for all other effects fitted in the model. Equations [2] and [3] were applied on the predicted phenotypic values for the estimation of the additive (a) and dominance (d) SNP

effects on traits. The proportion of phenotypic variance ($V_P$) for each trait accounted for by the SNP locus was estimated with the equation [4].

$a = (TT - CC)/2,$ [2]

$d = CT - [(TT + CC)/2],$ [3]

$V_P$ % due to SNP= $100 \times \{2pq[a + d(q - p)]2\}/V_P$ [4]

where TT, CC, and CT are the predicted trait values for each genotype class; p and q are the respective allele frequencies at the SNP locus. The statistical model [1] was used to estimate the variance components after excluding the genotype effect. For all statistical analyses the ASReml3 software was used (Gilmour et al., 2009). Pairwise t-test between the genotype classes (TT, CT and CC) was performed on the corresponding predicted trait values.

**3. Results and Discussion**

The descriptive statistics obtained from Chios sheep milk regarding the mean (± SD) of fat percentage and each individual FA, the calculated FA groups and the FA indices of this study are presented in **Table 5.2**. Mean fat percentage was 5.3 (± 0.9) which, was shown to be in consistency with the fat percentages recorded in previous studies for the Chios sheep breed in Cyprus (Orford et al., 2012; Tzamaloukas et al., 2015; Miltiadou et al., 2017b). In the present study, the vast majority of milk fat fraction expressed in wt/wt included approximately 73% SFA, of which the highest proportion pertained to palmitic acid (C16:0; 24.96%) followed by myristic acid (C14:0; 11.19%), capric acid (C10:0; 9.03%), stearic acid (C18:0; 8.70%), lauric acid (C12:0; 5.47%), caprylic acid (C8:0; 3.32%), butanoic acid (C4:0; 3.27%), caproic acid (C6:0; 3.18%) and other SFA with percentages less than 1%. Milk fat contained 26.26% wt/wt UFA

**Table 5.2.** Descriptive statistics for milk fat content (%) and milk fatty acid (FA) composition (%, wt/wt fat)

| | | Range | | | |
|---|---|---|---|---|---|
| Trait | Mean | Minimum | Maximum | Std. Deviation | CV (%) |
| Milk fat % | 5.27 | 2.69 | 8.02 | 0.91 | 17.28 |
| Saturated Fatty Acids | | | | | |
| C4:0 | 3.27 | 1.18 | 11.32 | 1.56 | 47.63 |
| C5:0 | 0.04 | 0.01 | 0.12 | 0.02 | 51.28 |
| C6:0 | 3.18 | 0.93 | 10.73 | 1.56 | 48.98 |
| C7:0 | 0.07 | 0.01 | 0.32 | 0.04 | 65.15 |
| C8:0 | 3.32 | 0.86 | 10.47 | 1.52 | 45.69 |
| C9:0 | 0.11 | 0.01 | 0.31 | 0.05 | 48.56 |
| C10:0 | 9.03 | 1.69 | 27.85 | 4.27 | 47.27 |
| C11:0 | 0.13 | 0.02 | 0.47 | 0.07 | 52.79 |
| C12:0 | 5.47 | 1.90 | 11.60 | 1.49 | 27.25 |
| C13:0 | 0.12 | 0.02 | 0.58 | 0.07 | 62.28 |
| *iso* C14:0 | 0.10 | 0.02 | 0.29 | 0.05 | 45.93 |
| C14:0 | 11.20 | 5.95 | 15.33 | 1.56 | 13.95 |
| *iso* C15:0 | 0.23 | 0.04 | 0.59 | 0.10 | 43.66 |
| *anteiso* C15:0 | 0.46 | 0.04 | 1.44 | 0.19 | 40.68 |
| C15:0 | 1.03 | 0.16 | 2.47 | 0.35 | 34.25 |
| *iso* C16:0 | 0.28 | 0.02 | 2.90 | 0.24 | 86.42 |
| C16:0 | 24.96 | 11.04 | 35.18 | 3.74 | 14.99 |
| *iso* C17:0 | 0.35 | 0.03 | 0.76 | 0.15 | 42.38 |
| *anteiso* C17:0 | 0.49 | 0.03 | 1.55 | 0.32 | 64.93 |
| C17:0 | 0.57 | 0.04 | 1.43 | 0.23 | 40.97 |
| C18:0 | 8.70 | 1.63 | 21.64 | 3.25 | 37.32 |
| C20:0 | 0.30 | 0.03 | 0.98 | 0.22 | 73.95 |
| C22:0 | 0.14 | 0.03 | 0.31 | 0.07 | 49.05 |
| Monounsaturated Fatty Acids | | | | | |
| C10:1 | 0.39 | 0.06 | 0.93 | 0.14 | 36.27 |
| C12:1 *cis*-9 | 0.12 | 0.01 | 0.37 | 0.06 | 50.00 |
| C14:1 *cis*-9 | 0.24 | 0.003 | 0.88 | 0.14 | 58.42 |
| C16:1 *cis*-9 | 0.92 | 0.06 | 3.38 | 0.55 | 59.23 |
| C16:1 *cis*-7 | 0.44 | 0.02 | 1.23 | 0.29 | 64.61 |
| C17:1 *cis*-9 | 0.21 | 0.02 | 0.66 | 0.11 | 53.09 |
| C18:1 *cis*-9 | 18.71 | 3.67 | 35.96 | 5.23 | 27.97 |
| C18:1 *trans*-11 | 0.96 | 0.03 | 3.00 | 0.61 | 63.47 |
| C18:1 *cis*-12 or *cis*-13 | 0.45 | 0.06 | 1.33 | 0.28 | 63.38 |
| C20:1 *cis*-9 | 0.16 | 0.02 | 0.58 | 0.12 | 73.87 |
| Polyunsaturated Fatty Acids | | | | | |
| C18:2 other | 0.15 | 0.02 | 0.52 | 0.09 | 62.49 |
| C18:2 *cis*-9, *cis*-12 | 2.72 | 0.29 | 6.67 | 1.10 | 40.38 |
| C18:2 *trans*-10, *cis*-12 CLA | 0.17 | 0.01 | 0.51 | 0.11 | 65.30 |
| C18:2 *cis*-9, *trans*-11 CLA | 0.56 | 0.05 | 1.61 | 0.31 | 55.54 |
| C18:3 *cis*-6, *cis*-9, *cis*-12 | 0.08 | 0.01 | 0.25 | 0.05 | 53.90 |
| C18:3 *cis*-9, *cis*-12, *cis*-15 | 0.27 | 0.03 | 0.73 | 0.13 | 46.03 |
| C20:4 *cis*-5, *cis*-8, *cis*-11, *cis*-14 | 0.21 | 0.03 | 0.52 | 0.10 | 46.02 |
| Fatty acid groups | | | | | |
| SFA[1] | 72.92 | 51.89 | 93.81 | 7.21 | 9.88 |
| UFA[2] | 26.28 | 6.16 | 47.32 | 6.88 | 26.20 |
| MUFA[3] | 22.43 | 5.87 | 39.83 | 5.70 | 25.41 |
| PUFA[4] | 3.85 | 0.29 | 9.03 | 1.54 | 39.88 |
| SCFA[5] | 19.35 | 5.28 | 58.60 | 8.76 | 45.26 |
| Mammary origin FA[6] | 38.43 | 15.42 | 80.03 | 10.53 | 27.40 |
| MCFA[7] | 17.20 | 7.25 | 25.6 | 2.87 | 16.70 |
| LCFA[8] | 60.77 | 19.97 | 82.6 | 10.32 | 17.98 |
| Odd chain FA[9] | 3.32 | 0.43 | 7.01 | 1.16 | 34.98 |
| ω6/ω3[10] | 12.03 | 0.28 | 64.37 | 6.68 | 55.54 |
| AI[11] | 3.19 | 1.10 | 13.09 | 1.39 | 43.40 |
| EI[12] | 0.52 | 0.26 | 0.75 | 0.08 | 15.07 |

[1]Saturated fatty acids
[2]Unsaturated fatty acids
[3]Monounsaturated fatty acids
[4]Polyunsaturated fatty acids
[5]Short chain fatty acids (SFA and UFA from C4 to C10)
[6]Mammary origin fatty acids (SFA and UFA from C4 to C15)
[7]Medium chain fatty acids (SFA and UFA from C12 to C14)
[8]Long chain fatty acids (SFA and UFA from C16 to C24)
[9]Odd chain fatty acids (C11:0 + C13:0 + C15:0 + C15:1 + C17:0 + C17:1)
[10]ω6/ω3 (C18:2 *cis*-9, *cis*-12 + C18:3 *cis*-6, *cis*-9, *cis*-12 + C20:4 *cis*-5, *cis*-8, *cis*-11, *cis*-14)/ C18:3 *cis*-9, *cis*-12, *cis*-15
[11]Atherogenicity index (C12:0 + (4 x C14:0) + C16:0)/(MUFA + PUFA)
[12]Elongation index (C18:0 + C18:1)/(C16:0 + C16:1 + C18:0 + C18:1

from which 22.43% wt/wt were MUFA and 3.85% wt/wt were PUFA. The highest percentage of MUFA was measured for oleic acid (C18:1 *cis*-9; 18.71% wt/wt). For PUFA the highest percentage (expressed in wt/wt) was measured for the following three individual FAs with decreasing order; linoleic acid (C18:2 *cis*-9, cis-12; 1.10%), CLA (C18:2 *cis*-9, *trans*-11; 0.56%) and linolenic acid (C18:3 *cis*-9, *cis*-12, *cis*-15; 0.27%). Similar results have been previously obtained for most of the FAs from different sheep breeds (Carta et al., 2008; De La Fuente et al., 2009) and those reported for Chios sheep breed in Cyprus under similar farming practices (Tzamaloukas et al., 2015).

In the present work, informative polymorphisms were only observed in the *ACAA2* and *FASN* genes. One SNP located in the 3'UTR (g.2982T>C) of *ACAA2* gene and one SNP detected in the coding region of exon 31 (g.14777C>T) of the *FASN* gene, were identified. Across all 429 animals examined, allelic frequencies in the *ACAA2* SNP locus were 0.47 for the C allele and 0.53 for the T allele and the genotypic frequencies were 0.20, 0.55 and 0.25 for CC, CT and TT animals, respectively. This outcome is consistent with the allele frequencies (0.44 for C allele and 0.56 for T allele) obtained by Orford et al. (2012) and (0.46 for C allele and 0.54 for T allele) **Chapter 4** from Chios sheep, while genotypic frequencies were found to deviate from Hardy-

Weinberg equilibrium, consistently with our latest study where the frequency of the genotypes carrying the T allele was higher probably due to directional animal selection for increased milk yield (**Chapter 4:** Miltiadou et al., 2017b). In the *FASN* SNP locus, the respective frequencies for the C and T alleles were 0.67 and 0.33, while the genotypic frequencies were 0.46, 0.44 and 0.10 for CC, CT and TT, respectively, and also deviated from Hardy-Weinberg equilibrium. The gene frequencies observed for the *FASN* locus in Chios sheep are intermediate, whereas the frequency for the C allele that has been reported for the Altamurana and Gentile di Puglia breeds was 0.93, while Sarda breed was monomorphic for the same allele (Crisa et al., 2010), suggesting important inter-breed differences.

In the present study, no polymorphisms were detected in the UTRs of *DGAT1* and *SCD1* genes. Since the coding regions of *DGAT1* and *SCD1* genes have been previously reported to be either monomorphic or exhibit limited polymorphism with low frequencies for the minor allele in other sheep breeds studied (*DGAT1*: Scata et al., 2009; Dervishi et al., 2015; *SCD1*: García-Fernández et al., 2009; Miari et al., 2009), the UTRs of those genes were investigated in the present study, because of the well-established roles of those regions in regulating gene expression (Xie et al., 2005; Sugimoto et al., 2015). In the ovine 5'UTR of the *DGAT1* gene a SNP has been detected and found in low frequency in breeds exhibiting high milk fat content such as the Altamurana and Gentile di Puglia breeds (Scata et al., 2009). Regarding the *SCD1* gene, mutations have been detected in the promoter region across several other sheep breeds (García-Fernández et al., 2009; Aali et al., 2014), while a SNP located within the 5'UTR was detected in meat reared sheep breeds and was associated with the FA profile in meat (Aali et al., 2016). The lack of polymorphisms in the UTRs of the *DGAT1* and *SCD1* genes in the present

study could be possibly attributed to a founder effect due to low number of animals initially used to establish the Chios sheep population in Cyprus (**Chapter 4:** Miltiadou et al., 2017b).

Concerning the *ACAA2* gene, our interest focused on SNP HM537015:g.2982T>C we firstly identified in previous studies where it was found to be associated with milk yield (Orford et al., 2012; **Chapter 4:** Miltiadou et al., 2017b), milk protein percentage and milk fat yield in Chios sheep (**Chapter 4:** Miltiadou et al., 2017b). The present study aimed to elucidate the possible association of *ACAA2* with ovine milk fat percentage and FA profile. The *ACAA2* gene was found to be significantly associated with the FAs composition in ewe milk. In particular, the g.2982T>C SNP exhibited negative dominance effects on C9:0 ($P$ = 0.032), C11:0 ($P$ = 0.020), C12:1 *cis*-9 ($P$ = 0.004) and C13:0 ($P$ = 0.038), while a negative additive effect for the FA index ω6/ω3 (-0.0371 ± 0.0179, $P$ = 0.04) was also found. This SNP explained 0.76% of the total phenotypic variance of that index (**Table 5.3**). Although the heterozygous CT Chios ewes were found to have a comparatively lower content of C9:0, C11:0, C12:1 *cis*-9 and C13:0 in milk fat compared to the other two genotypes (**Table 5.4**), pairwise contrasts showed significant differences only for C13:0 (**Table 5.4**). In the case of ω6/ω3 FA index, homozygous TT animals tended to have a lower ω6/ω3 content compared to CC and CT animals (P<0.1). Therefore, the SNP previously associated with milk yield (Orford et al., 2012; **Chapter 4:** Miltiadou et al., 2017b) is also correlated with ω6/ω3 content, an index associated with anti-inflammatory effects which prevent chronic diseases such as coronary heart disease, diabetes, cancer and osteoporosis (reviewed by Simopoulos, 2008). Similarly to our previous work (**Chapter 4**), fat percentage was not affected by the identified *ACAA2* polymorphism in the current study.

**Table 5.3.** Single Nucleotide Polymorphism (SNP) allelic effects in the *ACAA2* and *FASN* gene loci and corresponding percentage of total phenotypic variance explained

| Trait | a[1] ± SE | *P*-value Pa[2] | d[3] ± SE | *P*-value Pd[4] | %Vp due to SNP[5] |
|---|---|---|---|---|---|
| | | | *ACAA2* | | |
| C9:0 | 0.008 ± 0.023 | 0.723 | -0.059 ± 0.028 | 0.032 | 0.01% |
| C11:0 | 0.010 ± 0.025 | 0.698 | -0.068 ± 0.029 | 0.020 | 0.01% |
| C12:1 *cis*-9 | 0.020 ± 0.028 | 0.479 | -0.096 ± 0.033 | 0.004 | 0.07% |
| C13:0 | 0.020 ± 0.020 | 0.318 | -0.050 ± 0.024 | 0.038 | 0.13% |
| ω6/ω3 | -0.037 ± 0.018 | 0.038 | 0.015 ± 0.021 | 0.469 | 0.76% |
| | | | *FASN* | | |
| C13:0 | 0.010 ± 0.024 | 0.694 | -0.058 ± 0.028 | 0.042 | 0.32% |

[1]a = additive genetic effect; negative additive effect (a>0) indicated T allele decreased the trait.

[2]*P*-value for assessing the additive effect on the trait.

[3]d = dominant genetic effect.

[4]*P*-value for assessing the dominant effect on the trait.

[5]$V_p$ = percentage of phenotypic variance; based on the allele frequencies observed in sample (*ACAA2:* p = 0.47 for C and q = 0.53 for T; *FASN:* p = 0.67 for C and q = 0.33 for T).

**Table 5.4.** Genotypic means for milk fatty acids (FA) and milk FA indices associated with the g.2982T/C SNP in the *ACAA2* gene locus and the g.14777C/T SNP in the *FASN* gene locus

| | Genotypic means ± SE[1] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | g.2982T/C (*ACAA2*) | | | | | | g.14777C/T (*FASN*) | | | | | |
| Trait | CC[2] | N | CT[2] | N | TT[2] | n | CC[2] | n | CT[2] | n | TT[2] | n |
| C9:0 | 0.113[c] ± 0.004 | 191 | 0.109[c] ± 0.002 | 526 | 0.115[d] ± 0.004 | 238 | | | | | | |
| C11:0 | 0.136 ± 0.005 | 192 | 0.128[c] ± 0.003 | 530 | 0.137[d] ± 0.005 | 229 | | | | | | |
| C12:1 *cis*-9 | 0.152[d] ± 0.005 | 190 | 0.143[c] ± 0.003 | 540 | 0.151[c] ± 0.005 | 239 | | | | | | |
| C13:0 | 0.089 ± 0.003 | 193 | 0.086[b] ± 0.002 | 539 | 0.094[a] ± 0.003 | 245 | 0.088[c] ± 0.002 | 405 | 0.083[d] ± 0.002 | 423 | 0.090[c] ± 0.005 | 91 |
| ω6/ω3 | 13.67[c] ± 0.29 | 178 | 13.58[c] ± 0.18 | 508 | 12.95[d] ± 0.29 | 221 | | | | | | |

[a-d]Means within a row with 2 different superscripts differ as follows: [a,b]$P < 0.05$, [c,d] $P < 0.1$

[1]Predicted from the mixed model association analyses, after adjusting for all significant fixed and random effects.

[2]Significance of pairwise genotype contrast assessed using a 2-sample *t*-test.

The association detected between the *ACAA2* gene and the FA contents (odd chain FA and ω6/ω3 ratio) could be attributed to either a functional role directly affecting the studied traits or linkage and/or linkage disequilibrium with the causal locus or loci. LCFA reach the udder through blood circulation. Similarly, the ω6/ω3 ratio depends on the availability of ω6 and ω3 from diet, while C18:2 ω6 and C18:3 ω3 FA are metabolized to longer chain ω6 and ω3 FAs (Palmquist, 2009). Therefore, acetyl-CoA produced in mitochondria by the ACAA2 enzyme, could be used in the formation of malonyl-CoA, which is utilized for the FAs chain elongation (reviewed by Eaton et al., 1996). According to the function of the ACAA2 enzyme, we can deduce that the g.2982T>C SNP may be indirectly associated with the ω6/ω3 ratio by producing the substrates for elongation of the C18:2 ω6 and C18:3 ω3 precursors. On the same basis, even-chain and odd-chain FAs are elongated with the aid of acetyl-CoA. Furthermore, β-oxidation provides energy for such anabolic reactions.

In the current study, we confirm the presence of the g.14777C>T SNP (Crisa et al., 2010) in the *FASN* gene of the Chios sheep breed and provide evidence of a significant association with the FA composition in ewe milk. In particular, the SNP identified in the *FASN* gene was associated with C13:0 with a negative dominant effect of -0.06 ± 0.03 ($P$ = 0.04). The proportion of total phenotypic variance explained by the SNP was 0.32%. According to previous studies, a QTL on the OAR11, where the *FASN* gene is located, may affect the C14:0 and C16:0 content of ewe milk (Carta et al., 2008), while in another study the identification of a QTL near the *FASN* gene was reported to affect the C10:0 and PUFA contents of ewe milk (García-Fernández et al., 2010b). Crisa et al. (2010) described that the allele T of the *FASN* SNP in exon 31 had a positive substitution effect on the MCFA of milk (C10:0, C10:1, C12:0 and C14:0) in two sheep breeds

(Altamurana and Gentile di Puglia), which may justify the absence of the polymorphism from Sarda sheep, a breed which according to Signorelli et al. (2008) is known for a lower MCFA content compared to the other studied breeds. In the present study, we identified an association of *FASN* with an odd-chain FA in milk. Odd-chain FAs are in low percentage in milk compared to the corresponding FAs (C10:0, C12:0, C14:0 and C16:0) with even number of carbon atoms that have been previously associated with the *FASN* gene (Crisa et al., 2010; García-Fernández et al., 2010b). Although the majority of odd-chain FAs derives from ruminal bacteria, another small proportion is *de novo* synthesized (C5:0 to C13:0) from propionate in the mammary gland of ruminants (Massart-Leen et al., 1983; reviewed by Vlaemink et al., 2006). Heck et al. (2012) also suggested that odd-chain FA are *de novo* synthesized as a much larger proportion of their variance is explained by genetic rather than herd parameters, suggesting a possible involvement of *FASN* in odd chain FA synthesis.

**4. Conclusions**

Studies in cows have yielded important findings for consistent and well established gene effects on the milk FA profile. This kind of knowledge would be useful for the small ruminant industry as it could possibly facilitate the improvement of the milk and cheese FA composition. In the case of dairy sheep, a large number of locally adapted breeds are commonly used in different countries and, therefore, breed specific associations have been detected. In the present study, we report the association between polymorphisms within the *ACAA2* and *FASN* genes and milk FA profile in ewe milk. The effect of *ACAA2* SNP on ω6/ω3 content, an index with well-established effects in human health, could be possibly incorporated in a selection scheme

including other SNPs affecting the ω6/ω3 index towards improvement of milk FA quality. Further studies are needed to investigate if the association of the *ACAA2* gene with the FA profile is breed specific or could be also observed in other ovine breeds.

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# CHAPTER 6

## Molecular characterization of the ovine *endothelial lipase* (*LIPG*) gene in Cypriot Chios sheep

**S. Symeou, M. Orford, O. Tzamaloukas, and D. Miltiadou**

**Abstract**

The *LIPG* gene encodes for the endothelial lipase (**EL**) and is part of the triglyceride (**TAG**) lipase gene family. EL as the only lipase synthesized by vascular endothelial cells has the higher phospholipase activity from the rest TAG lipase members and a major role in determining the levels of high-density lipoprotein (**HDL**) cholesterol in the body. The *LIPG* gene maps to *Ovis aries* chromosome ((OAR)) 23, just before the *acetyl-CoA acyltransferase 2* (***ACAA2***) gene already associated with dairy traits. The objective of the current work was the molecular characterization of the complete coding region of the ovine *LIPG* gene of a purebred population of Chios ewes in Cyprus. Characterization was accomplished by direct sequencing of all ten exons, including partial 5' and 3' untranslated regions (**UTRs**). Seventeen single nucleotide polymorphisms (**SNPs**) were identified. Three SNPs located in exons 1, 4, and 8 (KC76441: g134C>A, g1181G>A and g2639G>T, respectively) encoded non-synonymous mutations. Six of the identified SNP were located in exon 10 (3' UTR) (KC76441: g3140C>T, g3210G>A, g3285G>A, g3292C>T, g3356G>A, g3373C>A), whereas the other eight polymorphisms (KC76441: exon 4; g1233G>A, exon 5; g1491C>T, g1548C>T, g1623C>T, g1626C>T, exon 6; g1879G>A, g1996G>A, exon 8; g2538C>T) were found to produce synonymous mutations. Based on the possible impact of the identified mutations on the enzyme's structural conformation or the effect of gene expression, seven polymorphisms were chosen to be genotyped in a population of 427 purebred Chios ewes. The g.134C>A SNP on exon 1, found to encode a non-synonymous mutation resulting to the change of the non-essential hydrophobic amino acid with cyclic structure (Pro) to an essential hydrophilic amino acid (Thr), had allelic frequencies of 0.985 and 0.015 for the g.134C and g.134A allele, respectively. The genotypic frequencies were found to

deviate from Hardy-Weinberg equilibrium ($P$ = 0.036). The genotypic frequencies for the mutations located in exon 10 and the 3' UTR were in Hardy-Weinberg equilibrium.

## 1. Introduction

A critical parameter for animal selection in the dairy industry is the knowledge of the genetic identity of each animal, which is associated with interesting characteristics (e.g., milk quality and quantity) affecting the farmers' income and at the same time, satisfying the consumers' demands. Nowadays, valuable information is being added on animals' genetic evaluation by exploitation of findings derived through the approach of quantitative trait loci (**QTL**) mapping of genetic loci, genome-wide associations and the identification of single nucleotide polymorphisms (**SNPs**) in candidate genes affecting important milk traits.

The *LIPG* gene encoding the enzyme endothelial lipase (**EL**/ endothelial cell-derived lipase: EDL) was firstly reported in 1999 as part of the triglyceride (**TAG**) lipase gene family including lipoprotein lipase (***LPL***) and hepatic lipase (***HL***) (Hirata et al., 1999; Jaye et al., 1999). LPL enzymes are found on the surface of mammary endothelial cells hydrolyzing the fatty acids (**FAs**), which are transported as TAG by very-low-density lipoproteins (**VLDL**) from the liver or chylomicrons from the intestine. Thus, these FAs derived from lipase hydrolysis and plasma FAs derived from body fat mobilization end up in the mammary gland (Noble 1978; Chilliard et al., 2000a). Due to the hydrolysis action of LPL on TAG (Bionaz and Loor 2008; Shingfield et al., 2010), via a selective cleavage at sn-1(3) position (Barber et al., 1997), FAs are released from the lipoprotein core. The LPL enzyme exhibits higher activity in the mammary gland compared to other tissues as is upregulated during lactation (Bionaz and Loor 2008; Shingfield et al.,

2010). The EL enzyme belongs in the LPL family and shows similarities to the rest of the family enzymes as it shares 44% and 41% molecular homology with the LPL and HL enzymes, respectively (Yu et al., 2018). However, it is the only lipase synthesized by vascular endothelial cells, compared to LPL enzymes synthesized by parenchymal cells. Additionally, EL exhibits the lowest triglyceride lipase to phospholipase ratio compared to the other lipase enzymes in the family. Thus, EL is an enzyme with much higher phospholipase activity rather than triglyceride lipase activity, with also a significant role in determining the high-density lipoprotein (**HDL**) metabolism in the body (Jaye et al., 1999; McCoy et al., 2002; Holmes et al., 2011; Yu et al., 2018). HDL is the preferred substrate of EL, therefore expression of EL is inversely correlated with the HDL levels in plasma (Ishida, Zheng et al., 2004; Yu et al., 2018). The use of recombinant human EL suggests that sphingomyelin regulates EL's activity and substrate specificity showing lower hydrolysis of low-density lipoproteins (**LDL**) sphingomyelin/phosphatidylocholine ratio compared to HDL sphingomyelin/phosphatidylocholine ratio (Yang et al., 2014). Under inflammatory conditions (e.g., atherosclerosis) EL overexpresses and is regarded as an enzyme with atherogenic action (reviewed by Yu et al., 2018; Elnaggar et al., 2019). These reports show that EL is a multitude function enzyme, which compared to the HDL, it is the bridging molecule for a more sufficient internalization of LDL and VLDL in the cell. This function leads to catabolism thus providing cell with precursors for lipid biosynthesis (reviewed by Yu et al., 2018). The different function that EL expresses is also determined by the different sequence between its homologous enzymes (LPL, HL) in the area of the catalytic site (Ishida et al., 2003; Yu et al., 2018). According to the study of Ishida, Zheng et al. (2004) three isoforms of the EL enzyme have been characterized in

human; EDL1a (68Kda), EDL2a (46Kda) and EDL2b (38Kda). The isoforms EDL2a and EDL2b derive from the gene's alternative splicing and contradictory to the EDL1a, which localizes on the endothelial cells surface bound to proteoglycans, the EDL2a and EDL2b locate in cells cytosol with no enzymatic action (Ishida, Zheng et al., 2004). The role of these two isoforms is unclear with a possible assumption to be involved in the cytoplasmic transportation or storage of lipid substrates (Ishida, Zheng et al., 2004; Yu et al., 2018).

The *LIPG* gene maps on *Ovis aries* chromosome (**OAR**) 23, spans 10 exons and 9 introns, and encodes for a 500 amino acid polypeptide chain. The gene is located where suggestive QTL for milk, fat, and protein yields were detected (Gutiérrez-Gil et al., 2009). Furthermore, the *LIPG* gene locates in a region just next to the *ACAA2* gene encoding for the enzyme acetyl-CoA acyltransferase which, except from its essential role in catalyzing the last step in FA β-oxidation, it is significantly associated with milk yield (Orford et al., 2012; **Chapter 4:** Miltiadou et al., 2017b), milk protein percentage, protein yield, fat yield (**Chapter 4:** Miltiadou et al., 2017b) and milk FA content (C9:0, C11:0, C12:1 cis-9, C13:0 and ω6/ω3 index: **Chapter 5:** Symeou et al., 2020) in Chios sheep.

Thus, based on the characterization of the OAR23 as the putative location of QTL associated with milk traits (Gutiérrez-Gil et al., 2009) and due to the functional significance of the enzyme encoded by the *LIPG* gene, this gene is regarded as a putative positional and functional candidate. Therefore, this study aims at the molecular characterization of the ovine *LIPG* gene and the identification of SNP in the coding region of the gene in Chios sheep enabling the conduction of an association study including *ACAA2-LIPG* haplotypes in the future.

## 2. Methods and materials

### *2.1. Animals and sampling*

For the current study, a total of 427 purebred Chios dairy ewes in mid-lactation were used. The experimental ewes were bred in four different small ruminant farms in Cyprus. All experimental procedures that involved animal handling were carried out according to the regulations of the national legislation (Animal Welfare Law, 1994) and international guidelines (Directive 2010/63/EU for animal experimentation) and approved by the corresponding departmental committee.

### *2.2. Primer design*

Since limited ovine genomic DNA sequence was available in the public domain, bovine exonic sequences were first blast searched against raw ovine sequence reads held in the ISGC sequence data depository (https://isgcdata.agresearch.co.nz/). Larger contiguous sequences were then constructed using the acquired data until the entire ovine exonic sequences, including some flanking intronic sequence, were constructed. According to the assembled sequences and the use of the program Primer3 (http://frodo.wi.mit.edu/primer3/), primers were subsequently designed (**Table 6.1**).

### *2.3. DNA extraction*

A jugular blood sample was obtained from all 427 ewes to perform genomic DNA extraction. The Genomic DNA Blood kit (Macherey-Nagel, Duren, Germany) was used for genomic DNA

isolation, according to the manufacturer's instructions. After extraction, DNA quality and quantity were estimated by UV absorption at 260 and 280 nm.

### *2.4. SNP identification and genotyping*

Subsequently to DNA extraction, all ten exons and the UTRs of the *LIPG* gene were PCR amplified, sequenced and genotyped from 20 randomly selected animals. PCR reactions were performed in 25-μl volumes containing 25 ng of genomic DNA, 0.5 U of Taq DNA polymerase (Qiagen Inc., Valencia, CA), 0.5 μM of each primer, 0.2 mM dNTPs and 1.5 mM $MgCl_2$ (2.5 Mm $MgCl_2$ for *LIPG* exon 2) for each primer pair. After an initial 5-min denaturation step at 94°C, the reaction mix was subjected to 30 cycles at 94°C for 30 sec, 56°C annealing temperature for 30 sec and 72°C for 30 sec with a final elongation step at 72°C for 5-min. For SNP identification and genotyping, a cost-effective direct DNA sequencing protocol was performed according to Miltiadou et al. (2017a) as described in **Chapter 4**; the obtained PCR products generated 96-well plates, which were precipitated by the addition of 5μL of 3M sodium acetate, pH 5.3, with 25μL of isopropanol. After incubation at -70C for 20 min and centrifugation at 4°C for 45 min (2,720 x g), the supernatant removal took place by inverting the plates on paper towels and briefly spinning the plates upside down for 2 s at 500 x g. A washing step followed by the addition of 100μL of 70% ethanol without mixing with the precipitated products. Then, another centrifugation carried on for 10 min at 2,720 x g at 4°C, where the supernatants were removed as before. The final step was a 5 min drying at 50°C completed with the addition of 40 μL of deionized water in each well to re-dissolve the purified DNA. For the sequencing cycle 10-μL volume reaction were set up with the use of 2 μL of the purified templates, 0.5 μL of Big Dye

**Table 6.1.** Primers for PCR used to amplify *LIPG* exons and single nucleotide polymorphisms (SNPs) identified

| Primer | Sequence 5' →3' | Amplicon size (bp) | Exon size | SNP | Nucleotide from the start of exon | Location in GenBank entry | Amino acid change |
|---|---|---|---|---|---|---|---|
| LIPG_exon1_F | GCTGGAAACTTCGAGAGGTG | 238 | 164 | C>A | 70 | KC764411:g134 | Pro – Thr |
| LIPG_exon1_R | GAAGCTTTGGGGGTGGTAAT | | (1-64: 5'UTR) | | | | |
| LIPG_exon2_F | AACAAGAACTGGACCCCAGA | 295 | 182 | | | | |
| LIPG_exon2_R | CCATGTAGAGCTGGAGAGCA | | | | | | |
| LIPG_exon3_F | GGACACCAGCTTGGACATTT | 491 | 180 | | | | |
| LIPG_exon3_R | TCCTAAAAGGGGTCGGATTC | | | | | | |
| LIPG_exon4_F | TGGCATGACTGCTGTTGAAT | 225 | 112 | G>A | 23 | KC764411:g1181 | Glu – Gly |
| LIPG_exon4_R | AGCATCCCTGAGTGAAGGAG | | | G>A | 75 | KC764411:g1233 | No change (Ala) |
| LIPG_exon5_F | AGTGCGGACGTCTTCATTCT | 375 | 222 | C>T | 62 | KC764411:g1491 | No change (Asp) |
| LIPG_exon5_R | GCTGATTCAGGAGGAGATGC | | | C>T | 119 | KC764411:g1548 | No change (ile) |
| | | | | C>T | 194 | KC764411:g1623 | No change (Asn) |
| | | | | C>T | 197 | KC764411:g1626 | No change (Asp) |
| LIPG_exon6_F | AGCTCAGTCCTGACGTCTCTG | 396 | 243 | G>A | 80 | KC764411:g1879 | No change (Pro) |
| LIPG_exon6_R | TCTGGTTTGCAACGATGAGA | | | G>A | 197 | KC764411:g1996 | No change (Arg) |
| LIPG_exon7_F | GACAAGCGAGGCTCACAAAT | 227 | 121 | | | | |
| LIPG_exon7_R | CCCTGAGCACCTCTCAGACT | | | | | | |
| LIPG_exon8_F | CAGAGACCAGTGTTCCTCTCC | 298 | 219 | C>T | 58 | KC764411:g2538 | No change (Asp) |
| LIPG_exon8_R | CCTCTGCTAACCACGGACAC | | | G>T | 159 | KC764411:g2639 | Val - Gly |
| LIPG_exon9_F | CCTGGAGCTTTGGCTACATC | 173 | 106 | | | | |
| LIPG_exon9_R | GAACTGCTGAGCACATCCTG | | | | | | |
| LIPG_exon10 | GAGCTGCGAAGCCAACTAAC | 555 | 465 | C>T | 63 | KC764411:g3140 | |
| (3'UTR)_F | | | (97-465:3'UTR) | G>A | 133 | KC764411:g3210 | |
| LIPG_exon10 | GAGTTTCTGCACCTCCCAGA | | | G>A | 208 | KC764411:g3285 | |
| (3'UTR)_R | | | | C>T | 215 | KC764411:g3292 | |
| | | | | G>A | 279 | KC764411:g3356 | |
| | | | | C>A | 296 | KC764411:g3373 | |

Terminator Reagent v.3.1 (Applied Biosystems), 0.5 µL of the reverse primer (5 µM), 1.75 µL of 5 x sequencing buffer, and 5.25 µL of distilled water. After an initial 2-min denaturation step at 96°C, the sequencing reactions were subjected to 30 cycles at 96°C for 15 s, 50°C for 15 s, and 60°C for 4 min. When the reaction ended, 10 µL of water were added, followed by 5 µL of 125 mM EDTA and 60 µL of 100% ethanol to precipitate the termination products. First, the plates were incubated at room temperature for 15 min in the dark, centrifuged at 2,720 × g at 4°C for 45 min, and the supernatants were removed as before. Following a wash with 250 µL of 70% ethanol, the plates were dried for 5 min at 50°C and the termination products were dissolved in 10 µL of formamide before reading on an ABI 3130 genetic analyzer (Applied Biosystems).

## 3. Results and Discussion

### *Genotype and allelic frequencies*

Multiple alignments among 20 sequenced ovine samples revealed the identification of seventeen polymorphic sites within the coding regions and the UTRs of the *LIPG* gene, whereafter, a more significant number of individuals was used for genotyping; one SNP in exon 1 (KC764411: g134C>A; 427 ewes genotyped), two SNPs in exon 4 (KC764411: g1181G>A, g1233G>A; 59 ewes genotyped), four SNPs in exon 5 (KC764411: g1491C>T, g1548C>T, g1623C>T, g1626C>T; 59 ewes genotyped), two SNPs in exon 6 (KC764411: g1879G>A, g1996G>A; 20 ewes genotyped), two SNPs in exon 8 (KC764411: g2538C>T, g2639G>T; 94 ewes genotyped), and six SNPs in the 3' UTR (KC764411: g3140C>T, g3210G>A, g3285G>A, g3292C>T, g3356G>A, g3373C>A; 427 ewes genotyped) (**Table 6.1**). **Table 6.2** presents the allele and genotypic frequencies for all identified SNPs. Alignment of the ovine sequences at exons 2, 3, 7

**Table 6.2.** Genotypic and allelic frequencies of *LIPG* gene

| SNP | Genotypes | Genotyped animal no. | N | Frequency | Allele | Frequency | Hardy-Weinberg equilibrium $\chi^2$ test |
|---|---|---|---|---|---|---|---|
| | | | ***LIPG* exon 1** | | | | |
| KC764411:g134 | CC | 427 | 415 | 0.972 | C | 0.985 | P=0.0036 |
| | AC | | 11 | 0.026 | A | 0.015 | |
| | AA | | 1 | 0.002 | | | |
| | | | ***LIPG* exon 4 SNP1** | | | | |
| KC764411:g1181 | GG | 59 | 18 | 0.31 | G | 0.551 | P=0.959 |
| | AG | | 29 | 0.49 | A | 0.449 | |
| | AA | | 12 | 0.20 | | | |
| | | | ***LIPG* exon 4 SNP2** | | | | |
| KC764411:g1233 | GG | 59 | 18 | 0.31 | G | 0.551 | P=0.959 |
| | AG | | 29 | 0.49 | A | 0.449 | |
| | AA | | 12 | 0.20 | | | |
| | | | ***LIPG* exon 5 SNP1** | | | | |
| KC764411:g1491 | CC | 59 | 13 | 0.22 | C | 0.44 | P=0.415 |
| | CT | | 26 | 0.44 | T | 0.56 | |
| | TT | | 20 | 0.34 | | | |
| | | | ***LIPG* exon 5 SNP2** | | | | |
| KC764411:g1548 | CC | 59 | 13 | 0.22 | C | 0.44 | P=0.415 |
| | CT | | 26 | 0.44 | T | 0.56 | |
| | TT | | 20 | 0.34 | | | |
| | | | ***LIPG* exon 5 SNP3** | | | | |
| KC764411:g1623 | CC | 59 | 23 | 0.39 | C | 0.6 | P=0.373 |
| | CT | | 25 | 0.42 | T | 0.4 | |
| | TT | | 11 | 0.19 | | | |
| | | | ***LIPG* exon 5 SNP4** | | | | |
| KC764411:g1626 | CC | 59 | 25 | 0.42 | C | 0.65 | P=0.843 |
| | CT | | 28 | 0.46 | T | 0.35 | |
| | TT | | 7 | 0.12 | | | |
| | | | ***LIPG* exon 8 SNP1** | | | | |
| KC764411:g2538 | TT | 94 | 61 | 0.65 | T | 0.72 | P=0.000 |
| | CT | | 14 | 0.15 | C | 0.28 | |
| | CC | | 19 | 0.2 | | | |
| | | | ***LIPG* exon 8 SNP2** | | | | |
| KC764411:g2639 | GG | 94 | 61 | 0.65 | G | 0.72 | P=0.000 |
| | GT | | 14 | 0.15 | T | 0.28 | |
| | TT | | 19 | 0.2 | | | |
| | | | ***LIPG* exon 10 SNP1** | | | | |
| KC764411:g3140 | CC | 427 | 2 | 0.005 | C | 0.07 | P=0.897 |
| | CT | | 52 | 0.122 | T | 0.93 | |
| | TT | | 373 | 0.874 | | | |

Table 6.2 **(*Continued*)**

| SNP | Genotypes | Genotyped animal no. | N | Frequency | Allele | Frequency | Hardy-Weinberg equilibrium $\chi^2$ test |
|---|---|---|---|---|---|---|---|
| | | | ***LIPG* exon 10 SNP2** | | | | |
| KC764411:g3210 | AA | 427 | 375 | 0.878 | A | 0.94 | P=0.592 |
| | AG | | 51 | 0.119 | G | 0.06 | |
| | GG | | 1 | 0.002 | | | |
| | | | ***LIPG* exon 10 SNP3** | | | | |
| KC764411:g3285 | AA | 427 | 413 | 0.967 | A | 0.984 | P=0.7305 |
| | AG | | 14 | 0.033 | G | 0.016 | |
| | GG | | 0 | | | | |
| | | | ***LIPG* exon 10 SNP4** | | | | |
| KC764411:g3292 | CC | 427 | 413 | 0.967 | C | 0.984 | P=0.7305 |
| | CT | | 14 | 0.033 | T | 0.016 | |
| | TT | | 0 | | | | |
| | | | ***LIPG* exon 10 SNP5** | | | | |
| KC764411:g3356 | GG | 427 | 413 | 0.967 | G | 0.984 | P=0.7305 |
| | AG | | 14 | 0.033 | A | 0.016 | |
| | AA | | 0 | | | | |
| | | | ***LIPG* exon 10 SNP6** | | | | |
| KC764411:g3373 | AA | 427 | 366 | 0.857 | A | 0.93 | P=0.8186 |
| | AC | | 59 | 0.138 | C | 0.07 | |
| | CC | | 2 | 0.005 | | | |

and 9 revealed no polymorphisms. Eight out of the seventeen identified SNP (located at exons 4, 5, 6 and 8) cause synonymous changes, while three other SNP located at exons 1, 4 and 8 cause non-synonymous changes. The human *LIPG* gene also spans 10 exons and encodes for a 500 amino acid polypeptide chain (Yu et al., 2018). The amino acid sequence between the human and ovine EL enzyme is the same except 67/500 amino acids (**Figure 6.1**). Nevertheless, the catalytic center necessary for lipid hydrolysis is fully conserved among the TG lipase family. It comprises of three amino acids, Serine 169, Aspartic acid 193 and Histidine 274 (Hirata et al., 1999; Jaye et al., 1999; Yu et al., 2018), and remains the same between the human and ovine EL with no alterations (**Figure 6.1**).

The g134C>A SNP in exon 1 leads to the substitution of a non-polar amino acid (Pro) to a polar uncharged amino acid (Thr) (**Table 6.3**). The specific non-synonymous mutation attracted our scientific interest due to the type of amino acid substitution since proline forms a ring-like structure (pyrolidene ring) which can lead to steric clashes affecting the enzyme's conformational stability. Furthermore, proline has a nonstandard structure with a distinctive side chain, which classifies proline as unique amino acid among the twenty naturally occurring amino acids, as its amino group comprises part of the side chain (Bajaj et al., 2007). Proline is a helix breaker with a disrupting role of the a-helix pattern in protein's secondary structure, and according to several studies, proline introduction is suggested to be an industrial method enhancing the proteins' thermostability (Luo and Horowitz, 1993; Yu et al., 2015; Xu et al.,

| Score | Expect | Method | Identities | Positives | Gaps |
|---|---|---|---|---|---|
| 907 bits(2344) | 0.0 | Compositional matrix adjust. | 431/498(87%) | 454/498(91%) | 0/498(0%) |

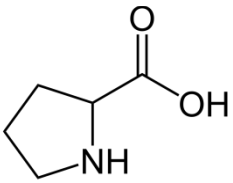
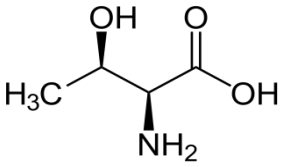
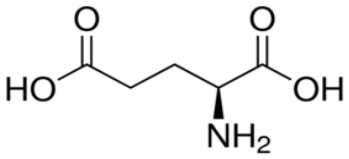
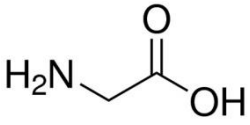
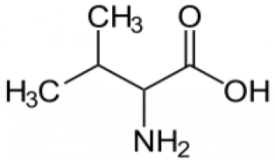
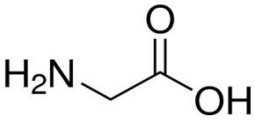
```
Query  1    MRNTVLQLCLWSAYCCFATGDPIPLGPEGRLQDEFDKSRVVPTAAKPPVRFNLRTSEDPE  60
Sbjct  1    .S.S.PL..F..LCY...A.S.V.F......E.KLH.PKATQ.EV..S........K...  60

Query  61   HEGCDLSLGRSQPLEDCGFNVTAKTFFIIHGWTMSGMFENWLYKLVSALQMREKGANVVV  120
Sbjct  61   ....Y..V.H.......S..M..............I.....H......HT...D.....  120

Query  121  VDWLPLAHQLYVDAVNNTRGVGLSIAQMLDWLQGKEDFSLENVHLIGYSLGAHVAGYAGN  180
Sbjct  121  ..........T.......V..H...R......E.D....G.....................  180

Query  181  FVKGTVGRITGLDPAGPLFEGADIHKRLSPDDADFVDVLHTYTRSFGLSIGIQMPVGHID  240
Sbjct  181  ...............M.....................................  240

Query  241  IYPNGGDFQPGCGLNDVLGSIAYGTIAEVLKCEHERAVHLFVDSLVNQDKPSFAFQCTDS  300
Sbjct  241  ........................T..V.....................  300

Query  301  NRFKKGICLSCRKNRCNSIGYNAKKTRNKRNSKMYLKTRAGMPFRVYHYQMKIHVFSYKN  360
Sbjct  301  ........................M.....................  360

Query  361  MGEMEPTFYVTLYGTNADSQVLPLEMVEQIGLNATNTFLVYTEEDLGDLLKIRLTWEGKS  420
Sbjct  361  ...I..............T....I..R.EQ..................Q.....A.  420

Query  421  QSWYNLWKELRSYLTQPRVSKQELHIRRIRVKSGETQQKLTFCAEDLENTLISPGQELWF  480
Sbjct  421  .........F....S...NPGR..N...........R.....T..P...S....R....  480

Query  481  HKCRDGWRMKNETSPTVE  498
Sbjct  481  R.................  498
```

**Figure 6.1.** Alignment between the ovine (Query: XP_011959069.2) and human (Sbjct: NP_006024.1) EL polypeptide chains. Dots resemble identical amino acids in the sequence, while the red letters resemble the amino acid change. The rectangles resemble the three identical amino acid positions between the ovine and human EL comprising the catalytic center (Serine 169, Aspartic acid 193, Histidine 274).

2020). According to literature, proline's rigid pyrrolidene ring is expected to rigidify flexible regions such as β-turns as it decreases the conformational freedom of Cα-N rotation (Hardy et al., 1993; Yu et al., 2015), while the missing amide hydrogen atom from proline destabilizes the protein due to the lack of hydrogen bonding (Bajaj et al., 2007). Within the 427 Chios ewes, C allele in exon 1 is the most common with frequencies of 0.985 and 0.015 for the C and A alleles, respectively, and the genotypic frequencies for CC (0.972), AC (0.026) and AA (0.002) classes deviate from Hardy-Weinberg equilibrium ($P$<0.05) (**Table 6.2**).

The g1181G>A SNP in exon 4 (allele frequencies; G: 0.551, A: 0.449), causes an amino acid change (Glu → Gly) by replacing the polar acidic amino acid, glutamic acid, with a smaller and non-polar amino acid, glycine (**Table 6.3**). Glycine is the smaller amino acid from a list of twenty naturally occurring amino acid residues used for protein composition. The specific non-polar amino acid has a hydrogen atom as a side chain, which makes the amino acid very flexible because there is a lot of free rotation around the α-carbon atom. It comprises an exception from the other amino acids as is the only one without an asymmetric carbon atom and the only non-polar amino acid not forming hydrophobic bonds in the tertiary structure. Glycine is one of the two amino acids, with proline, considered as helix breakers because of the introduction of kinks in the coiled polypeptide chain, thus affecting the enzyme's conformational stability. Glycine comprises a source of instability as it confers the highest conformational entropy, compared to the other amino acids. As a result, replacement of glycine decreases the unfolding entropy and increases the thermal stability (Matthews et al., 1987; Xu et al., 2020). In a genotyped population of 59 Chios ewes, the genotypic frequencies for GG (0.305), AG (0.491) and AA (0.203) classes were found to be in Hardy-Weinberg equilibrium (P>0.05) (**Table 6.2**).

**Table 6.3.** Non synonymous mutations in *LIPG* exons 1, 4 and 8 leading to amino acid change

| | |
|---|---|
| **Exon 1:** g134C>A Pro → Thr | |
| **Proline**<br>Non-polar, alkyl amino acid<br>  | **Threonine**<br>Polar, neutral amino acid<br>  |
| **Exon 4:** g1181G>A Glu → Gly | |
| **Glutamic acid**<br>Polar, acidic amino acid<br>  | **Glycine**<br>Non-polar, alkyl amino acid<br>  |
| **Exon 8:** g2639G>T Val → Gly | |
| **Valine**<br>Non-polar amino acid<br>  | **Glycine**<br>Non-polar, alkyl amino acid<br>  |

SNPs (g.2538T>C and g2639G>T) identified in exon 8, cause a synonymous and a non-synonymous substitution, respectively. The g2639G>T polymorphism in exon 8 causes an amino acid change (Val → Gly), both amino acids belong to the same non-polar amino group (**Table 6.3**), therefore, it would be expected that the particular amino acid change in protein's primary structure probably does not affect its conformational stability or its activity. However, valine is a bulkier amino acid, compared to glycine, while the second represents a bigger backbone conformational flexibility with greater configurational entropy rather than valine, thus probably resulting in lower thermal stability (Matthews et al., 1987; Xu et al., 2020). Both SNP appeared to have the same allelic and genotypic frequencies; the most prevalent alleles frequency (exon 8; SNP1: T, SNP2: G) are 0.72 and the least prevalent alleles (exon 8; SNP1: C, SNP2: T) are 0.28, while the genotypic frequencies of the homozygous for the prevalent allele, heterozygous and homozygous for the rarer allele are 0.65, 0.15 and 0.2, respectively (**Table 6.2**). Genotypic frequencies are found to deviate from the Hardy-Weinberg equilibrium ($P$<0.05) (**Table 6.2**).

Regarding the rest of the mutations in the 3'UTR, the frequency of the most common alleles was 0.93 (g3140T), 0.94 (g3210A), 0.984 (g3285A), 0.984 (g3292C), 0.984 (g3356G) and 0.93 (g3373A) and all genotypes were found to be consistent with the Hardy-Weinberg equilibrium ($P$>0.05) (**Table 6.2**). The 3'UTR attracts scientific interest due to its content in various regulatory regions post-transcriptionally affecting gene expression. Polymorphisms in the 3'UTR may influence the regulation of the mRNA level and protein expression through the effect on the binding of microRNAs (**miRNA**) to mRNA (reviewed by Chen et al., 2008). The complementary binding of the miRNA seed region with the mRNA, could due to SNP, either

eliminate or weaken a miRNA target or create a perfect sequence match to a microRNA seed region that had no previous association with the given mRNA. Thus, increase or decrease in microRNA binding would probably result in decrease or increase in protein translation (reviewed by Chen et al., 2008). Moreover, apart from miRNAs, SNP in the 3' UTR could affect binding elements sites namely AU-rich elements (**AREs**). RNA-binding proteins (**RBPs**) or RNAs bind the AREs, which interaction has crucial influence on the post-transcriptional control (reviewed by Day and Tuite, 1998; Matoulkova et al., 2012), regulating the transcript and protein stability (stabilization or destabilization), or the transcript transport outside the nucleus and the translational regulation (enhancement or depression) (reviewed by Matoulkova et al., 2012). Additionally, the AAUAAA region in the 3' UTR directs the poly (A) tail formation to the very end of the transcript. Poly (A) binding proteins (**PABP**) recognize and bind the poly (A) tail, where they prevent the 3' end from degrading by exonucleases attack, regulate mRNA translation, stability, export from the nucleus and translation. Thus, SNP in this region affects the interaction between the poly (A) tail-PABP complex with proteins in the 5' UTR which promote mRNA translation (reviewed by Day and Tuite, 1998; Kong and Lasko, 2012; Matoulkova et al., 2012).

The polymorphisms found within the *LIPG* gene exon 1 and the 3' UTR, have therefore attracted our interest due to the nature of the amino change in exon 1 and their position next and in the UTRs, as they may affect the protein's tertiary conformation or the gene expression, respectively. Therefore, this group of seven SNP was chosen to be genotyped for the entire purebred Chios sheep population under study consisting of 427 animals. Among the 427 Chios ewes, three and ten haplotypes were identified for exon 1 and 3'UTR (**Table 6.4**), respectively,

**Table 6.4.** Haplotype frequency of the single nucleotide polymorphisms (SNP) in exon 1 and the 3'UTR for the *LIPG* gene.

| ***LIPG* gene – exon 1** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Haplotype | SNP1- g134 | | | | | | Animal no. | F |
| 1 | CC | | | | | | 415 | 97.19 |
| 2 | AC | | | | | | 11 | 2.58 |
| 3 | AA | | | | | | 1 | 0.23 |
| ***LIPG* gene – 3' UTR** | | | | | | | | |
| Haplotype | SNP1- g3140 | SNP2- g3210 | SNP3- g3285 | SNP4- g3292 | SNP5- g3356 | SNP6- g3373 | Animal no. | F |
| 1 | TT | AA | AA | CC | GG | AA | 313 | 73.30 |
| 2 | TT | AA | AA | CC | GG | AC | 58 | 13.58 |
| 3 | CT | AG | AA | CC | GG | AA | 35 | 8.20 |
| 4 | CT | AG | AG | CT | AG | AA | 13 | 3.04 |
| 5 | CT | AA | AA | CC | GG | AA | 2 | 0.47 |
| 6 | TT | AA | AA | CC | GG | CC | 2 | 0.47 |
| 7 | CT | AG | AA | CC | GG | AC | 1 | 0.23 |
| 8 | CT | AG | AG | CT | AG | AC | 1 | 0.23 |
| 9 | CC | GG | AA | CC | GG | AA | 1 | 0.23 |
| 10 | CC | AG | AA | CC | GG | AA | 1 | 0.23 |

while the haplotypes for both regions together were thirteen (**Table 6.5**). The most frequent haplotype is observed in 310 ewes with 72.6 frequency and all SNPs are homozygous for the most frequent allele at each SNP in that haplotype (**Table 6.5**). Preliminary results, however, show no significant association between the haplotypes identified within the *LIPG* exon 1 and 3' UTR and milk fat percentage, protein percentage or milk fatty acid traits (unpublished data). Results from the approximate association of the polymorphisms in *LIPG* exon 1 and 3' UTR with linkage disequilibrium (**LD**), show high correlation between SNP1 and SNP2 (LD > 0.9), and SNP3, SNP4 and SNP5 (LD = 1) in the 3' UTR. The neighbor gene to *LIPG*, the *ACAA2*, already associated with dairy traits in Chios ewes shows no correlation with the polymorphisms in the *LIPG* gene (**Table 6.6**).

**4. Conclusions**

This study reports the molecular characterization of the complete coding region, including partial 5' and 3' UTR, of the ovine *LIPG* gene in a population of Chios ewes in Cyprus. Sequence revealed the presence of seventeen SNPs both in exonic regions and the 3' UTR. Preliminary association analysis of the SNPs with milk traits, suggests that both the non-synonymous SNPs affecting the enzymes tertiary structure, and the SNPs in the 3'UTR affecting the cis-acting elements are not associated with milk traits.

**Table 6.5.** Haplotype frequency of the single nucleotide polymorphisms (SNP) in the *LIPG* gene.

| | ***LIPG*-exon 1** | | | ***LIPG*-3'UTR** | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Haplotype | SNP1-g134 | SNP1-g3140 | SNP2-g3210 | SNP3-g3285 | SNP4-g3292 | SNP5-g3356 | SNP6-g3373 | Animal no. | F |
| 1 | CC | TT | AA | AA | CC | GG | AA | 310 | 72.60 |
| 2 | CC | TT | AA | AA | CC | GG | AC | 49 | 11.48 |
| 3 | CC | CT | AG | AA | CC | GG | AA | 35 | 8.20 |
| 4 | CC | CT | AG | AG | CT | AG | AA | 13 | 3.04 |
| 5 | AC | TT | AA | AA | CC | GG | AC | 8 | 1.87 |
| 6 | AC | TT | AA | AA | CC | GG | AA | 3 | 0.70 |
| 7 | CC | CT | AA | AA | CC | GG | AA | 2 | 0.47 |
| 8 | CC | TT | AA | AA | CC | GG | CC | 2 | 0.47 |
| 9 | CC | CT | AG | AA | CC | GG | AC | 1 | 0.23 |
| 10 | CC | CC | GG | AA | CC | GG | AA | 1 | 0.23 |
| 11 | CC | CC | AG | AA | CC | GG | AA | 1 | 0.23 |
| 12 | CC | CT | AG | AG | CT | AG | AC | 1 | 0.23 |
| 13 | AA | TT | AA | AA | CC | GG | AC | 1 | 0.23 |

**Table 6.6.** Pairwise linkage disequilibrium (LD) between 8 SNP, one SNP in the 5'UTR of the *ACAA2* gene, one SNP in exon of the *LIPG* gene, and six SNP in the 3' UTR of the *LIPG* gene.

| | | **ACAA2-3'UTR** | **LIPG-exon 1** | **LIPG-3'UTR** | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | SNP1-g2982 | SNP1-g134 | SNP1-g3140 | SNP2-g3210 | SNP3-g3285 | SNP4-g3292 | SNP5-g3356 | SNP6-g3373 |
| **ACAA2-3'UTR** | **SNP1-g2982** | **1** | **0.0008** | **0.0284** | **0.0267** | **0.0064** | **0.0064** | **0.0064** | **0.0322** |
| ***LIPG*-exon 1** | SNP1-g134 | | 1 | 0.0040 | 0.0039 | 0.0009 | 0.0009 | 0.0009 | 0.0747 |
| ***LIPG*-3'UTR** | SNP1-g3140 | | | 1 | **0.9403** | 0.1855 | 0.1855 | 0.1855 | 0.0124 |
| | SNP2-g3210 | | | | 1 | 0.2063 | 0.2063 | 0.2063 | 0.0115 |
| | SNP3-g3285 | | | | | 1 | **1** | **1** | 0.0009 |
| | SNP4-g3292 | | | | | | 1 | **1** | 0.0009 |
| | SNP5-g3356 | | | | | | | 1 | 0.0009 |
| | SNP6-g3373 | | | | | | | | 1 |

Values above the diagonal refer to the correlation between two polymorphisms. Bold numbers indicate high LD correlation.

# CHAPTER 7

# General Discussion

The milk lipid fraction is consisted of specific FA groups (e.g. MUFA and PUFA) and FAs (e.g. conjugated linoleic acid) with health related properties. The impact of these milk constituents on human health attracted the interest of research and industry alike towards the enrichment of milk and consequently cheese with beneficial FAs through feed supplementation since nutrition comprises the most crucial determinant controlling and altering the milk FA composition. The current study, in **Chapters 2 and 3**, demonstrates the use of an agro-industrial by-product of oil extraction from olives, the olive cake, in the diets of Chios sheep. Prior to inclusion in sheep diets, olive cake was subjected to ensiling, a cost-effective procedure allowing its safe maintenance throughout the year (Hadjipanayiotou, 1999; **Chapter 2**: Symeou et al., 2019). In **Chapter 2** and **Chapter 3**, three different inclusion rates of olive cake silage, 0%, 7% and 14% DM in the former study and 0%, 10% and 20% DM in the latter, were included in the diets of Chios ewes for four and three weeks, respectively. For milk FAs analysis, a rapid and non-destructive NMR technique was used as a tool for routine analysis of milk fat identifying major FA groups, minor FAs and cholesterol (**Chapter 2**), while gas chromatography was the analyzing tool for both milk and Halloumi cheese (**Chapter 3**). The olive cake silage resulted in the change of the milk FA content in the direction of improving health related aspects, as it caused an effective drop of the SFA in milk and concurrently increased the UFA (MUFA and PUFA) content and more specifically the main CLA isomer (C18:2 *cis*-9, *trans*-11), while reducing the atherogenicity and thrombogenicity indices, compared to the control. Olive cake silage was proved to be an effective feed supplement improving the milk and cheese FAs, as the reduction in the SFA content reduced their potential hypercholesterolemic effects, while increase in the CLA (i.e. rumenic acid) content confers antiobesity, antidiabetic, anti-atherosclerotic and, anti-

carcinogen effects and the ability to modulate the immune system (reviewed by Park, 2014; Jenkins et al., 2015; Kim et al., 2016; Gómez-Cortés et al., 2018). Moreover, the reduction of the atherogenicity and thrombogenicity indices lowers the risk of cardiovascular diseases. The obtained results are generally in concordance with studies including other (fresh or dried) forms of olive cake in feeds of sheep (Chiofalo et al., 2004; Vargas- Bello-Perez et al., 2013; Abbeddou et al., 2011a, 2011b and 2015), goats (Molina-Alcaide et al., 2010) and cows (Castellani et al., 2017; Neofytou et al., 2020), suggesting that ensiling didn't adversely affect the potentially beneficial effects of olive cake on ovine milk FA profile. Indeed, the ensiling technique has shown to increase the nutritional value of olive cake, as it helps avoiding rapid deterioration and since the ensiled olive cake is well preserved it can be then utilized throughout the year in ruminant feeding (Hadjipanayiotou, 1999; Molina-Alcaide & Yanez-Ruiz, 2008; Weinberg et al., 2008). Moreover, ensiling this particular mixture of skins, pulp, woody endocarp and seeds of olives is an attractive alternative for utilization as a supplement in ruminant diets, not only in terms of nutritional value, but also in terms of cost compared to other preservation methods (Hadjipanayiotou, 1999). Taking into consideration that the magnitude of the olive oil industry in Mediterranean countries has ended up having a negative impact at these areas due to the pollution with vast quantities of olive mill wastes (Roig et al., 2006), a solution for the agro-industrial by-product of oil extraction from olives is their incorporation in animal feeds (Molina Alcaide and Nefzaoui, 1996). This is the first study demonstrating that the inclusion of ensiled olive cake in dairy ewe diets improves the FA profile of ovine milk and consequently Halloumi cheese at rates up to 20% DM, and the first study using routine assessment of major FA groups and minor FAs in ruminant milk by NMR analysis

(**Chapter 2**: Symeou et al., 2019). However, the underlined mechanisms responsible for the observed improvement of the milk FA profile are largely unknown and therefore further studies investigating the observed effects at the transcriptome and proteomic level in liver, mammary gland and adipose tissue would cast light towards that direction. Moreover, further studies are necessary for the optimization of the NMR technique so it is directly used on fat extracted from raw instead of lyophilized milk, thus creating a fast extraction protocol. Also, the optimization of an NMR technique and mathematical equations for the discrimination and quantitation of the CLA isomers, especially between the isomers C18:2 *cis*-9, *trans*-10 and C18:2 *trans*-10, *cis*-12, would be very enlightening and thus the NMR analysis could be used independently from GCMS analysis.

Other than nutrition, the genetic factors comprise another important parameter affecting milk traits. Following the increased consumers' demands for dairy products of high textural and sensory quality, enriched with constituents positively associated with human health, this thesis aim was the study of several candidate genes which can affect economically important dairy traits. Five candidate genes were selected according (i) to their function implicated with lipid metabolism, (ii) their position in chromosomes where QTL for dairy traits were detected to map and (iii) their expression levels or association studies between variations or SNPs with milk production traits and fatty acid content in milk.

In **Chapters 4 and 5**, our study identified association of the g.2982T>C SNP in the 3'UTR of the *ACAA2* gene with important sheep traits in an extended population of Chios sheep from multiple flocks in Cyprus and in Greece. The SNP was associated with milk yield in both

populations without affecting milk fat percentage, with CC animals exhibiting the lowest and highest mean predicted values for the populations in Cyprus and Greece, respectively (**Chapter 4**). It was estimated that the g.2982T>C SNP in the Cypriot population had a stronger association with first-lactation milk yield which explained 2.25% of the phenotypic variance, while in the Greek population it explained 0.62% of the phenotypic variance of milk yield for all lactations and 2.34% of the animal variance. Additional evidence of association in the Cypriot population was also revealed with milk protein percentage and milk fat yield, where higher predicted protein percentage values were exhibited by CC animals and higher milk fat yield values were exhibited by CT and TT animals (**Chapter 4**: Miltiadou et al., 2017a). Furthermore, an association between the *ACAA2* g.2982T>C polymorphism was found with milk FAs with negative dominance effects on C9:0, C11:0, C12:1 *cis*-9 and C13:0 and negative additive effects on the ω6/ω3 index (**Chapter 5**: Symeou et al., 2020).

The *ACAA2* g.2982T>C SNP was firstly identified and associated with milk yield from our previous study (Orford et al., 2012) and we subsequently confirmed the association in **Chapter 4** in an extended population of Chios sheep from multiple flocks in Cyprus (Miltiadou et al., 2017a) and in Greece as the use of a bigger and different data set strengthens the evidence of the observed association (Sasaki et al., 2013). The allele frequencies obtained from the population in Greece were in Hardy-Weinberg equilibrium similarly to Orford et al. (2012), but opposed to the frequencies from the extended population in Cyprus. The differences could possibly reflect the direction of animal selection mainly based on milk yield and not lamb growth in commercial farms, as the meat prices during the last decade were reduced, whereas the selection indices used at the experimental flock in Orford et al. (2012) combine the

individual capacity of young stock for growth and ewe milk production (Mavrogenis and Constantinou, 1991). In **Chapter 4**, the use of two extended and independent sheep population in Cyprus and Greece has shown over-dominance of the CT genotype on milk yield as its phenotype lies outside the phenotypical range of both homozygous genotypes, while the CT genotype was found to be the superior and inferior genotype, respectively. The overall effects estimated from the population in Cyprus, however, were lower compared with Orford et al., (2012), which could be attributed to the use of multiple flocks managed in different ways, thus increasing the variation introduced, as a substantial fraction of the environmental variance for production traits is attributed to farm (Carta et al., 2009; Sasaki et al., 2013). The estimates obtained from the analysis in this study are regarded to be more representative indicating the true effect of the gene in the population. Further research is deemed necessary to elucidate the importance of the effects in the first lactation in the Cypriot population, compared to the rest of lactations. Considering the association outcomes from both Cyprus and Greece, it is possible that the SNP is not the causative mutation. However, since the association of *ACAA2* with milk traits is consistently detected and since there is strong functional evidence for allelic expression imbalance of the *ACAA2* gene (Miltiadou et al., 2017a), it is likely that the investigated SNP is in linkage or linkage disequilibrium with the causative SNP. For instance other cis-acting elements located in the promoter or other regulatory regions that differ in both populations, possibly due to founder effects could be responsible for the associations and functional effects observed. Indeed, the Greek population was endangered in the past (Rogdakis, 2002) and the Cypriot Chios sheep population has been established from a small number of less than 30 animals

(Mavrogenis A., personal communication), while it has suffered from a bottleneck effect due to selection for scrapie resistance (Brown et al., 2014).

The association found in **Chapter 4** with milk yield, protein percentage and yield, and fat yield, may explain part of the QTL for milk, fat, and protein yields observed in the region harboring the *ACAA2* gene (Gutiérrez-Gil et al., 2009). In the Chios sheep population in Cyprus, CC animals exhibited superior and inferior values for protein percentage and milk yield, respectively, compared to TT and CT animals, consistently with the negative genetic correlation between the two traits (Bencini and Pulina, 1997; Fuertes et al., 1998). Concerning protein yield, there were no differences between genotypic classes, thus the decrease in protein content could be probably explained by a dilution effect caused by the increase of milk yield (Emery, 1988). Furthermore, fat yields recorded for CC animals were significantly lower compared to TT and CT animals, a result probably attributed to decreased milk yield with similar fat content.

The association of the *ACAA2* gene with the FA contents of milk (**Chapter 5**: Symeou et al., 2020) could be either attributed to the SNP being the causative mutation, or because the SNP is in linkage or linkage disequilibrium with the causative locus or loci. Although the latter is more likely, since the acetyl-CoA produced by the ACAA2 enzyme is formed into malonyl-CoA, which is utilized for FA chain elongation, as well as because β-oxidation provides energy for such anabolic reactions, a functional role of the *ACAA2* affecting directly or indirectly the studied traits, cannot be excluded. The effect of *ACAA2* SNP on ω6/ω3 content, an index with well-

established effects in human health, could be exploited by incorporation in a selection scheme including other SNPs affecting the ω6/ω3 index toward improvement of milk FA quality.

In **Chapter 5**, molecular characterization of the untranslated regions of ovine *DGAT1* and *SCD1* genes was performed to identify polymorphisms putatively affecting milk traits due to the significant regulatory roles of those regions in gene expression (Xie et al., 2005; Sugimoto et al., 2015). The examined gene regions were found to be monomorphic (**Chapter 5**: Symeou et al., 2020) and did not allow further association analysis. In studies performed mainly in cattle, *DGAT1* and *SCD1* have been shown to affect the milk fat and FA content. Genotyping of the *DGAT1* gene in cows revealed the K232A polymorphism, where the A allele was associated with decreased contents of the C10, C12, C14, and C16 indices, and increased contents of C18 and CLA indices, compared to the K allele (Schennink et al., 2008). However, genotyping of the ovine *DGAT1* gene for the identification of SNPs revealed that the corresponding exonic region is monomorphic in sheep (Scata et al., 2009; Miltiadou et al., 2010). Moreover, the detection of SNPs in the non-coding regions (introns and 5'UTR) of the ovine *DGAT1* gene revealed a negative association with milk fat content (Scata et al., 2009), while a SNP located in exon 17 affected the milk FA contents and lactose (Dervishi et al., 2015). Regarding the *SCD1* gene, an A293V polymorphism in the bovine genome was associated with milk FA and FA indices (Mele et al., 2007; Moioli et al., 2007; Schennink, 2008; Conte et al., 2010; Bouwman et al., 2011; Buitenhuis et al., 2014; Palombo et al., 2018), while in sheep the gene was found to be located in a region harboring a QTL affecting the CLA/VA ratio (Carta et al., 2006), as well as a SNP identified in an intronic region was associated with the CLA/VA ratio (Miari et al., 2009). In goats, a deletion in the untranslated region of the gene showed association with the milk FA

profile (Zidi et al., 2010). Therefore, although there was a strong evidence of association of *DGAT1* and *SCD1* with the milk FA profile particularly in cattle, in the case of sheep, the coding regions have been previously reported to be either monomorphic or exhibit limited polymorphism with low frequencies for the minor allele (*DGAT1*: Scata *et al*., 2009; Dervishi *et al*., 2015; *SCD1*: García-Fernández *et al*., 2009; Miari *et al*., 2009) and in the Chios sheep breed the coding and important regulatory regions were found to be monomorphic and thus not associated with milk traits (Miltiadou *et al.,* 2010; **Chapter 5**: Symeou et al., 2020). This outcome could be possibly attributed to a founder effect due to the low number of sheep initially used to establish the Chios sheep population in Cyprus (Mavrogenis A., personal communication) and a bottleneck effect the Cypriot Chios sheep population has suffered due to the selection for scrapie resistance (Brown et al., 2014). It is also likely that other polymorphisms outside the examined regions could be responsible for associations with milk traits in a breed specific manner, and therefore, other regulatory such as intronic regions could be investigated. However taking into consideration the outcomes of the current and other studies in sheep, it is possible that there are significant differences in the genetic polymorphisms explaining milk traits between different ruminant species.

In the present study, molecular characterization of *FASN* exons identified a SNP on exon 31 (g.14777C>T SNP) (**Chapter 5**: Symeou et al., 2020), also identified by Crisa et al. (2010) in Altamurana and Gentile di Puglia breeds. The g.14777C>T SNP in the *FASN* gene exhibited a negative dominant effect mode on an odd-chain FA, the C13:0, which explained 0.32% of the total phenotypic variance (**Chapter 5**: Symeou et al., 2020). In the study of Crisa et al. (2010), the T allele of the same SNP in exon 31 showed a positive substitution effect on milk FAs; C10:0,

C10:1, C12:0 and C14:0 in both of the studied sheep breeds compared to the Sarda breed, which was found to be monomorphic for the C allele and proved to be a breed with a lower content of the medium-chain FA in milk. The *FASN* gene has been studied in cattle and was found to affect the milk fat percentage (Roy et al., 2006; Schennink et al., 2009) and the milk FA content such as C14:0, C18:1 *cis*-9, C18:2 *cis*-9, *cis*-12 (Zhang et al., 2008; Schennink et al., 2009). In ovine, the QTL detected in the same or neighboring area with the gene were associated with C14:0 and C16:0 (Carta et al,. 2008), C10:0 and the PUFA content of milk (García-Fernández et al., 2010b), while the identification of SNPs showed to affect the milk FA profile of the medium-chain FA (C10:0, C10:1, C12:0, C14:0) (Crisa et al., 2010), stearic acid and CLA (Moioli et al., 2012) in sheep and C14:0, C16:0 and C18:2 *cis*-9, *cis*-12 in goats (Haile et al., 2016). Therefore, other studies suggest association of the *FASN* gene mainly with even medium chain FAs consistently with the function of *FASN* gene product (Crisa et al., 2010), while in our study *FASN* was correlated with an odd-chain FA. The odd-chain FAs, such as the C13:0 are found in low percentage in milk, compared to the even-chain FAs, which were found to be associated with the particular *FASN* SNP also studied by Crisa et al. (2010). A proportion of odd-chain FAs are likely to be *de novo* synthesized in the udder of ruminants from propionate (Vlaeminck et al., 2006). In addition, it is suggested that *FASN* may be involved in the synthesis of the odd-chain FAs, as a much larger proportion of their variance is explained by genetic rather than herd parameters (Heck et al., 2012). Therefore, an involvement of *FASN* in odd-chain FAs cannot be excluded. However, most of the odd-chain FAs are synthesized in the rumen by bacteria (Vlaeminck et al., 2006). Moreover the association detected in **Chapter 5** explains a very small portion of the phenotypic variance and therefore, it is more likely that the

correlation detected could be due to linkage or LD with other loci affecting the trait (Andersson, 2001), rather than due to a causative gene effect.

In **Chapter 6**, our study identified seventeen SNPs through molecular characterization of the complete coding region and partial UTRs of the ovine *LIPG* gene of a purebred population of Chios ewes in Cyprus. Three of the SNPs located in exonic regions encoded non-synonymous mutations (g134C>A: Pro→Thr, g1181G>A: Glu → Gly, g2639G>T: Val → Gly) and six of the SNPs were located in exon 10 (3' UTR), while the rest of the mutations were synonymous. The polymorphisms in exon 1 and 3'UTR were genotyped in a population of 427 purebred Chios ewes due to their possible impact on the enzyme's structural conformation and their effect on gene expression, respectively. The *LIPG* gene locates on OAR23, which was characterized as the putative location of QTL associated with milk traits such as milk, fat and protein yields (Gutiérrez-Gil et al., 2009). Furthermore, *LIPG* gene is in the vicinity of the *ACAA2* gene, which was associated with milk yield (Orford et al., 2012; **Chapter 4**: Miltiadou et al., 2017a), milk protein percentage, protein yield, fat yield (**Chapter 4**: Miltiadou et al., 2017a) and milk FA content (C9:0, C11:0, C12:1 cis-9, C13:0 and ω6/ω3 index: **Chapter 5**: Symeou et al., 2020) in Chios sheep. Thus, according to its position and function, *LIPG* gene is regarded as a putative positional and functional candidate. The g.134C>A SNP on exon 1, was found to encode a non-synonymous mutation resulting to the change of the non-essential hydrophobic amino acid (Pro) to an essential hydrophilic amino acid (Thr). The cyclic structure of proline alters the secondary structure (Hardy et al., 1993; Yu et al., 2015) and destabilizes the protein (Bajaj et al., 2007). Regarding the 3'UTR, it contains regulatory regions post-transcriptionally affecting gene expression, thus polymorphisms in the region influence the regulation of the mRNA and protein

expression. Such regions are microRNA (miRNA) targets on the mRNA (reviewed by Chen et al., 2008), AU-rich elements (AREs) affecting the interaction with RNA-binding proteins (RBPs) (reviewed by Day and Tuite, 1998; Matoulkova et al., 2012) and the AAUAAA region of the 3'UTR, which directs the formation of poly (A) tail (reviewed by Day and Tuite, 1998; Kong and Lasko, 2012; Matoulkova et al., 2012). This study reports the identification of non-synonymous SNPs that can affect the tertiary structure of the enzyme and SNPs in the 3'UTR affecting the cis-acting elements regulating gene expression. Thirteen haplotypes were identified for the SNPs of both regions, however, preliminary association analysis with milk traits, suggested no association with milk fat percentage, protein percentage or milk FA traits.

Overall, studies in cows have yielded important findings for consistent and well established gene effects on milk traits and the milk FA profile. This kind of knowledge would be useful for the small ruminant industry as it could possibly facilitate the improvement of milk and cheese FA composition. In the case of dairy sheep, a large number of locally adapted breeds are commonly used in different countries and, therefore, breed specific associations have been detected. The effect of *ACAA2* SNP on milk traits and on ω6/ω3 content, an index with well-established effects in human health, could be possibly incorporated in a selection scheme including other SNPs affecting milk traits and the ω6/ω3 index toward improvement of milk FA quality (Miltiadou et al., 2017a; Symeou et al., 2020). Given the consistently significant correlations of the *ACAA2* gene with milk traits, the identified SNP has been incorporated into the sheep HD SNP chip developed by the International Sheep Genomics Consortium (ISGC) aiming to be used for functional studies (James Kijas, Csiro, Brisbane, Australia, personal

communication). Further genomic and functional analysis could identify the causative mutation responsible for the associations and functional effects observed.

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## Thesis Deliverables

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**Symeou S**., D. Miltiadou, C. Constantinou, P. Papademas, and O. Tzamaloukas. **2021**. Feeding olive cake silage up to 20% of DM intake in sheep improves lipid quality and health-related indices of milk and ovine halloumi cheese. **Tropical Animal Health and Production** 53 (2), 1-7.

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