Cyprus University of Technology

Department of Agricultural Sciences,
Biotechnology and Food Science

Doctoral Dissertation

Feeding By-product Feedstuffs Affected Milk Fatty Acid Profile and the Expression of Lipogenic Genes in Ruminants

Marina C. Neofytou

Limassol, April 2021

CYPRUS UNIVERSITY OF TECHNOLOGY

FACULTY OF GEOTECHNICAL SCIENCES AND
ENVIRONMENTAL MANAGEMENT

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Approval Form

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Presented by

Marina C. Neofytou

Thesis principal supervisor:

Dr. Ouranios Tzamaloukas, Assistant Professor, Cyprus University of Technology

Thesis advisory committee:

Dr. Despoina Miltiadou (co-supervisor), Associate Professor, Cyprus University of Technology

Dr. Ariadne-Loukia Hager-Theodorides (member), Assistant Professor, Agricultural University of Athens

Ph.D. thesis examining committee:

Dr. Styliani E. Chadio-Mantzari (chairperson, external examiner), Professor, Agricultural University of Athens

Dr. Sokratis Stergiadis (member, external examiner), Associate Professor, University of Reading

Dr. Ouranios Tzamaloukas (member, principal supervisor), Assistant Professor, Cyprus University of Technology

Cyprus University of Technology

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ACKNOWLEDGMENTS

A journey full of emotions has been come to an end. However, reaching at this point you realize that this journey would not have been possible without the contribution of specific people.

Primarily, I would like to express my special thanks of gratitude to my supervisor, Assistant Professor, Ouranio Tzamalouka, for his valuable guidance, keen interest and encouragement at various stages of my PhD. Thank you for everything!!!

At the same time, I am very much thankful to the members of my advisory committee, the Associate Professor, Despoina Miltiadou, from the Department of Agricultural Sciences, Biotechnology and Food Science of the Cyprus University of Technology, who guided and advised me in molecular biology aspects during the process of elaboration of the present doctoral thesis, and the Assistant Professor, Ariadne-Loukia Hager-Theodorides, from the Department of Animal Production Science of the Agricultural University of Athens for her advice and suggestions and the warm hospitality she gave me during my two-month mobility through ERASMUS program in the Laboratory of Animal Breeding & Husbandry. I would also like to thank her students, Mrs. Lena Sfakianaki and Mrs. Katerina Politi for their valuable help and friendship we developed.

Additionally, I would like to thank Mr Dionysis Sparaggis and the technical stuff of the Agricultural Research Institute’s farm for the management of the animals’ herd.

Then, I would like to thank my colleagues and friends for their support, collaboration and the beautiful moments we shared all these years. More specifically, I would like to thank the girls, Mrs. Simoni Symeou and Mrs. Constantina Constantiou, from our Animal Nutrition and Genetics group, for the friendship and collaboration we have developed. I would also like to thank the PhD students of the Department of Agricultural Sciences, Biotechnology and Food Science, mainly, Ms. Androula Varnava, Mr. Nikolas Markantonis, Mr. Christodoulos Michael, Ms. Panagiota Xylia, Ms. Smaragda Spanou, Mr Panayiotis Mousikos and Marina Christofi, for the funny moments we shared all these years. Moreover, a special thank to Dr. Nikolaos Nikoloudakis for the excellent collaboration we had all these years.

I also acknowledge with a deep sense of reverence, my gratitude towards my family in Greece and Cyprus, who always supported me morally.

Last but not least, I would like to thank my Dimitris, for the unconditional love and support he gave me all this period. You were always by my side when I needed you and you lifted me up when I fell. Thank you for everything!!!

ABSTRACT

The present thesis aimed to evaluate industrial by-products, ensiled olive cake (OC), and dried distillers' grains with solubles (DDGS) as diet components to ruminants. Since nutrition is the primary environmental factor regulating milk's concentration and composition, particularly milk fat, feeding industrial by-products rich in unsaturated fatty acids (FA), like DDGS and OC, is a reasonable attempt to improve the nutritional quality of milk and dairy products.

In this regard, the effect of dietary inclusion of 10% (on diet DM) of ensiled OC, as a replacement of forages, was evaluated in two experiments, investigating the effects on milk yield, composition and FA profile of cow milk (**Chapter 2 and 3**) and related Halloumi cheese (**Chapter 3**). Moreover, for examining any possible impact of this oil rich by-product feeding on bovine lipid metabolism, changes in the expression of selected genes involved in mammary and adipose lipid metabolism were assessed (**Chapter 3**). The results of these studies showed that the inclusion of 10% (DM) of ensiled OC did not affect milk yield and improved the quality of milk and Halloumi cheese by reducing saturated lipids and increased unsaturated ones including oleic (C18:1 *cis*-9), vaccenic (C18:1 *trans*-11) and conjugated linoleic (CLA *cis*-9, *trans*-11; rumenic acid) acids, all related to positive effects to human health. Milk FA differences observed were not associated with alterations in the mammary expression of genes involved in FA synthesis, uptake, translocation, and lipogenesis regulation. However, an upregulation of *SREBF1* mRNA expression in perirenal fat of cows fed the OC diet was demonstrated.

Furthermore, to evaluate the use of this by-product on other ruminant species, an additional study was implemented in lactating goats testing the effects of 10 and 20% inclusion rates (DM) of ensiled OC supplementation (OC10 and OC20 groups,

respectively) on milk yield, composition and FA profile (**Chapter 4**). The expression of genes related to mammary and adipose lipid metabolism of Control and OC20 groups was also evaluated for examining the effect of OC feeding on mammary and adipose tissue metabolism. The results of this study confirmed those observed in cows. More specifically, all major saturated FA between C4:0 to C16:0 were reduced, and consequently, the atherogenic index was diminished, while the content of long-chain (>16 carbons; LCFA) and mono-unsaturated FA (MUFA) were enhanced in the caprine milk of both OC groups. Among individual UFA, increments of oleic, C18:1 *trans*-10, and vaccenic acids were demonstrated in both OC groups, as well as in the concentration of rumenic acid, which was increased by 11 and 21% ($P < 0.001$) with OC10 and OC20 diets, accordingly, compared to Control. Additionally, the supplementation of goat diets with 20% (DM) of ensiled OC altered the mammary expression of *SLC2A1* ($P < 0.05$), *VLDLR* ($P < 0.01$), *FABP3* ($P < 0.01$) and elevated the *SLC2A1* ($P < 0.05$) and *FASN* ($P < 0.01$) genes in the adipose tissue.

Finally, another by-product that was also studied was the wheat based-DDGS included at rates of 6% and 12% (DM) in diets of dairy ewes, as a replacement of concentrate mix, investigating its potential effects on milk production and content, as well as on milk FA composition (**Chapter 5**). The results showed that milk yield, milk protein percentage, protein, and fat yield did not differ between groups. In contrast, milk fat percentage decreased only in the group contained the highest inclusion rate of DDGS compared to the Control group. The milk FA profile of ewes fed both DDGS diets improved significantly. In particular, the concentration of total SFA was reduced, while long-chain, mono-unsaturated, and poly-unsaturated FA content was increased in the milk of DDGS groups. Additionally, the content of linoleic (C18:2n-6) and rumenic acids was

elevated in both DDGS groups compared to control. Changes in FA profile resulted in a decline in the atherogenic index of milk by 20% and 35% in the groups contained 6 and 12% (DM) of DDGS, respectively, compared to control.

Overall, the findings of the present dissertation, which are summarized and discussed in **Chapter 6**, are very promising and fully support the use of these by-products (OC and DDGS) in ruminant diets for improving the quality of milk and derived Halloumi cheese, opening new insights and perspectives for their practical application and future research.

Keywords: *olive cake, wheat distillers' grains with solubles, rumenic acid, fatty acids, gene expression, dairy cows, ewes, goats, Halloumi cheese, milk production*

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LIST OF ABBREVIATIONS

2P/3P:	Two-Phase/ Three-Phase
AA:	Arachidonic Acid
ACACA:	Acetyl-CoA Carboxylase
ADF:	Acid Detergent Fiber
ADL:	Acid Detergent Lignin
AI:	Atherogenic Index
ALA:	a-Linolenic Acid
aNDF:	amylase-Neutral Detergent Fiber
ARI:	Agricultural Research Institute
BH:	Biohydrogenation
cDNA	Complementary DNA
CLA:	Conjugated Linoleic Acid
CP:	Crude Protein
CVD:	Cardiovascular Disease Risk
DDGS:	Dried Distillers’ Grains with Solubles
DHA:	Docosahexaenoic Acid
DI:	Desaturation Index
DM:	Dry Matter
DMI:	Dry Matter Intake
EPA:	Eicosapentanoic Acid
FA:	Fatty Acids
FABP3:	Fatty Acid Binding Protein 3
FAME:	Fatty Acids Methyl Esters

FASN:	Fatty Acid Synthase
FAT/CD36:	Fatty Acid Translocator
FFA:	Free Fatty Acids
G6PDH:	Glycose 6 Phosphate Dehydrogenase
LA:	Linoleic Acid
LCFA:	Long-Chain Fatty Acids
LPL:	Lipoprotein Lipase
MCFA:	Medium-Chain Fatty Acids
MEC:	Mammary Epithelial Cells
MFD:	Milk Fat Depression
MUFA:	Mono-Unsaturated Fatty Acids
NEFA:	Non-Esterified Fatty Acids
OA:	Oleic Acid
OC:	Olive Cake
PPARγ:	Peroxisome Proliferator Activated Receptor gamma
PUFA:	Poly-Unsaturated Fatty Acids
RA:	Rumenic Acid
RT-qPCR:	Reverse-Transcriptase quantitative Polymerase Chain Reaction
SCD1:	Stearoyl-Coa Desaturase 1
SCFA:	Short-Chain Fatty Acids
SFA:	Saturated Fatty Acids
SLC2A1:	Solute Carrier family 2-member 1
SNF:	Solids Non-Fat
SREBF1:	Sterol Regulatory Element Binding Transcriptional Factor 1
TAG:	Triacyglycerols

UFA:	Unsaturated fatty acids
VA:	Vaccenic Acid
VFA:	Volatile Fatty Acids
VLDL:	Very Low-Density Lipoprotein
VLDLR:	Very Low-Density Lipoprotein Receptor
DWGS:	Wet Distillers’ Grains with Solubles

CHAPTER 1: General Introduction

Nowadays, the estimated world milk production has reached an average of 828 million tonnes, which is extensively increased since its first report in 1987 (522 million tonnes) (FAO, 2019). Over 85% of the total milk production is coming from cattle, while the remaining 15% is derived from other species like buffalo (11%), goat (2.3%), sheep (1.4%), and camel (0.2%). In Cyprus, though, milk production has increased from 55,880 tonnes in 1968 to 235,340 tonnes in 2017, from which 80% is represented by milk from cows, followed by ovine and caprine milk, and this rate is annually growing at an average of 3.37% (FAO, 2019).

Milk is the secretion of the normally functioning mammary gland of the females of all mammals, which is produced temporally following parturition for the nourishment of the young of the species during the initial period of growth (McDonald et al., 1975). It contains considerable nutrients like fat, proteins, lactose, vitamins, minerals, as well as cells, hormones, enzymes, and immunoglobulins (Balthazar et al., 2017). Each one of these parameters contributes to the essential organoleptic characteristics (i.e. flavour, colour and taste) of milk and its dairy products. Milk composition is affected by several factors, including the different species, breed, age, stage of lactation, parity, diet, farming system, season and physical environment (Linn, 1988). Among ruminants, ovine milk is richer in protein and fat than milk from cows and goats, while bovine and caprine milk composition is similar (**Table 1,** Balthazar et al., 2017). Moreover, the average fat globule size is smaller in milk from small ruminant species (ewe and goat; diameter: 3.5 μm) compared to cow (4.0 μm), which partly contributes to the higher digestibility of ovine and caprine milk (Bernard et al., 2018).

Table 1. Proximate composition of bovine, caprine, and ovine milk

Item	Bovine milk	Caprine milk	Ovine milk
Moisture, (g/100g)	87.9 ± 0.5	87.6 ± 0.7	82.9 ± 1.4
Fat, (g/100g)	3.3 ± 0.2	3.8 ± 0.1	5.9 ± 0.3
Ash, (g/100g)	0.7 ± 0.1	0.8 ± 0.1	0.9 ± 0.1
Lactose, (g/100g)	4.7 ± 0.4	4.1 ± 0.4	4.8 ± 0.4
Protein, (g/100g)	3.4 ± 0.1	3.7 ± 0.1	5.5 ± 1.1
Casein, (g/100g)	3.0 ± 0.1	2.4 ± 0.1	4.7 ± 0.5
αs1- Casein, (%)^a	39.7	5.6	6.7
αs2 - Casein, (%)^a	10.3	19.2	22.8
β - Casein, (%)^a	32.7	54.8	61.6
κ - Casein, (%)^a	11.6	20.4	8.9

Adapted from Balthazar et al., (2017). ^{a} Expressed as a percentage of total casein

Milk and dairy products are among the most well-known and consumed food sources in human diets (Lock and Bauman, 2004). Nowadays, 6 billion people globally consume milk and dairy products, especially in developed countries (Kapaj and Deci, 2017). It is essential to mention that the development of technology led to the production of milk products with varying fat content available in the market and consumed worldwide (Lordan et al., 2018). Among the nutrients that are constituents of milk and its dairy products, milk fat is responsible for many of their sensory, physical, and manufacturing properties (Chilliard et al., 2000).

Nutritionally, milk fat is a dense energy source consisted mainly of triacylglycerols (**TAG**, ca. 98% of total milk lipids) and small amounts of 1,2-diacylyglycerides and monoacylglycerides (0.02%), free fatty acids (0.22%, **FFA**), and retinol esters, whereas milk TAG contain over 400 different fatty acids (**FA**), most of these are present in traces (Jensen, 2002). Generally, the most abundant FA in milk have chain lengths between 4 - 18 carbon atoms **(Table 2)**. In addition, individual FAs can be either saturated or unsaturated. The saturated fatty acids (**SFA**) present in ruminants' milk accounts for approximately 70 g/100g of total fat, while 25 g/100 g of total fat is mono-unsaturated FA (**MUFA**) (Jensen, 2002; MacGibbon and Taylor, 2006; Shingfield et al., 2010). Oleic acid (C18:1 *cis*-9, **OA**) is the main FA among MUFA found in milk fat, typically at contents between 20% and 25% of total FA, while about 5 to 15% of total C18:1 are *trans* FA with one *trans*-double bonds, from which vaccenic acid (C18:1 *trans*-11, **VA**) is present in the highest proportion (Chilliard et al., 2003). Poly-unsaturated fatty acids (**PUFA**) constitute about 2.3 g/100g of total FAs of milk, from which linoleic acid (**LA**) and a-linolenic (**ALA**) acids being the most plentiful (Lindmark Månsson, 2008). Moreover, ruminants' fat contains certain FAs with an odd number of carbons, such as pentadecanoic (C15:0) and heptadecanoic (C17:0) acids, which are synthesized by the bacterial flora in the rumen (Shingfield et al., 2010). Among ruminant species, milk fat from goats and ewes contains higher medium-chain FAs (**MCFA**) concentrations, especially FA with carbon lengths from 6 to 10 compared to cow milk fat (**Table 2**). Indeed, the FA known as caproic (C6:0), caprylic (C8:0), and capric (C10:0) are so termed due to the goat milk in which they are preferentially found and form up to 15–18% of total milk FA, compared to 5–9% in cow milk (Balthazar et al., 2017; Bernard et al., 2018).

Table 2. Major fatty acid content (means values expressed as a percentage of total fatty acid methyl esters) of bovine, caprine, and ovine milk

Item	Bovine milk	Caprine milk	Ovine milk
C4:0	3.90	2.18	3.51
C6:0	2.39	2.90	2.50
C8:0	1.50	2.73	2.64
C10:0	3.20	9.97	7.82
C12:0	3.60	4.99	4.38
C13:0	0.19	0.15	0.17
C14:0	11.12	9.81	10.40
C14:1 *cis*-9	0.80	0.18	0.28
C15:0	1.20	0.71	0.99
C16:0	27.9	28.2	25.9
C16:1 *cis*-9	1.50	1.59	1.03
C17:0	0.60	0.72	0.63
C18:0	12.20	8.88	9.57
Σ *trans* C18:1	4.25	2.55	2.35
Σ *cis* C18:1	21.70	21.70	22.45
C18:2 *n-6*	1.40	3.19	3.21
CLA total	1.10	0.70	1.60
C18:3 *n-3*	1.00	0.42	0.80
C20:0	0.35	0.15	0.45

Adapted from MacGibbon and Taylor, (2006); Balthazar et al., (2017); Bernard et al., (2018).

Milk fat secretion and FA composition are of great interest with regard to human nutrition (Parodi, 2004; Chilliard et al., 2007; Ferlay et al., 2017). Indeed, the different lipid and FA compounds presented in ruminants' milk fat could be potentially positive or negative factors for human health (Parodi, 2004). Milk and dairy products are high in SFA and *trans* fats, and their consumption has long been thought to contribute to cardiovascular disease risk (**CVD**) and in the aetiology of other chronic conditions. However, particular SFA have different effects on human health (Shingfield et al., 2008, 2013). For instance, lauric (C12:0), myristic (C14:0), and palmitic (C16:0) acids have been associated with elevated serum LDL-cholesterol concentrations in humans (Ferlay et al., 2017), while stearic acid (C18:0) has been shown to be essentially neutral (Shingfield et al., 2008).

Nevertheless, ruminants' milk fat is the richest natural common source of conjugated linoleic acid (**CLA**) isomers, which have attracted scientific interest due to their potential positive effects regarding human health (Parodi, 2004; Palmquist et al., 2005). CLA is a class of geometric and positional isomers of octadecadienoic acid, containing a conjugated double bond. Among them, rumenic acid (CLA *cis*-9, *trans*-11, **RA**) is the predominant CLA isomer accounting for between 75-90% of total CLA, followed by the CLA *trans*-7, *cis*-9 and CLA *trans*-10, *cis*-12 isomers (Bauman and Griinari, 2003). Several studies have been published showing RA potential beneficial properties by highlighting their anticarcinogenic effects (Pariza et al., 2001), their activity in minimizing the development of atherosclerosis (Lock et al., 2004), and accumulation of body fat (Evans et al., 2002), in enhancing bone mineralization (Platt et al., 2012), in modulating the immune system (Song et al., 2005), influencing glucose and lipid metabolism (Belury et al., 2003), and more recently their promising results on inflammatory bowel disease (Kim et al., 2016). The findings concerning the relation between RA and health effects in humans are commonly stated in the literature, although a closely future target is to elucidate the specific underline mechanism of RA in human health (Kim et al., 2016).

Besides CLA, other PUFA within the omega-3 (n-3) class in ruminants' milk fat have different impacts on human health (Stupin et al., 2019). For instance, ALA (C18:3 *cis*-9, *cis*-12, *cis*-15) is the essential *n-3* PUFA that is the substrate for elongation and desaturation to eicosapentaenoic acid (**EPA**; C20:5 *cis*-5, *cis*-8, *cis*-11, *cis*-14, *cis*-17) and docosahexaenoic acid (**DHA**; C22:6 *cis*-4, *cis*-7, *cis*-10, *cis*-13, *cis*-16, *cis*-19) (Burdge and Calder, 2005). As shown in **Table 2**, ruminants' milk fat includes low amounts of *n-3* PUFA (≤1% total FAs) and is mainly composed of ALA, with about 5% of total milk *n-3* PUFA being EPA, whereas DHA is rarely detected in milk. Although ALA is widely more under-studied than EPA and DHA, there is evidence that ALA provides health benefits beyond just conversion to EPA and DHA (Stupin et al., 2019).

By taking into account the beneficial effects of *n-3* FA on human health, it is also important to consider the amount of omega-6 (*n-6)* FA in a diet such as LA (C18:2 *cis*-9, *cis*-12), as ALA and LA compete for the same enzymes in elongation and desaturation to long-chain *n-3* and *n-6* PUFA, respectively (Simopoulos, 2002). Excessive LA intake intervenes with the conversion of ALA to EPA and produces a series of pro-inflammatory signaling molecules synthesized from the long-chain *n-6* PUFA, arachidonic acid (C20:4 *cis*-5, *cis*-8, *cis*-11, *cis*-14, **AA**), while the series of molecules produced from EPA are anti-inflammatory (Simopoulos, 2016). For this reason, and due to *n-3* and *n-6* PUFA cannot be interconverted by mammals (Nguyen et al., 2019), the dietary ratio *n-6* to *n-3* ratio is an essential factor to be considered when it comes to health outcomes, and generally, it is recommended to be between 4:1 and 1:1 in the human diet (Simopoulos, 2002).

Furthermore, another FA that has been associated with positive effects for human health is OA (C18:1 *cis*-9), which, as mentioned above, is the principal MUFA found in milk fat (Shingfield et al., 2010). The OA represents 49% to 83% of total FA in olive oil (Servili et al., 2014) and its by-products (Tzamaloukas et al., 2021), and its consumption has been related to the prevention of many diseases, including CVD, rheumatoid arthritis, and cancer (reviewed by Piccinin et al., 2019). Additionally, improved pancreas and liver secretory activity and reduced gastric-duodenal ulcers risk by OA have been indicated by Bermudez et al., (2011). Another beneficial role of OA has been to prevent SFA-induced inflammation (Camell and Smith, 2013). Indeed, in a previous study implemented in mice fed a high fat diet, OA administration ameliorated insulin sensitivity, reduced pro-inflammatory cytokines levels (Interleukin-1β, Interleukin-6 and Tumor Necrosis Factor-α), and upregulated the anti-inflammatory ones (Interleukin-10 and adiponectin levels) (Holland et al., 2011). Furthermore, OA displays the peculiar beneficial role in the brain's development, which is the organ with the highest lipid content followed by the white adipose tissue. Given that lipids are essential for the homeostasis and alterations in lipid metabolism in this organ, OA is the only FA synthesized by astrocytes acting as a neurotrophic factor for neurons (Bento-Abreu et al., 2008). All these data confirm the essential role of OA in human health, and further studies and clinical trials should be conducted to elucidate the role of OA in other diseases.

However, ruminats’ fat contains C18:1 *trans* FA that have been associated with increased LDL, triglycerides and insulin levels and reduced beneficial high-density lipoproteins (HDL) (Dhaka et al., 2011). *Trans* FA could be either formed during industrial partial hydrogenation of vegetable oil, a process widely commercialized to produce solid fats or through the biohydrogenation (**BH**) in ruminants (Gebauer et al., 2011). Nevertheless, a blanket statement cannot be applied to the wide variety of these FA. For instance, VA is the most abundant *trans* FA present in ruminants’ milk fat and there are data suggesting that the consumption of VA may provide health advantages regarding CVD, cancer, immune function and inflammation (Field et al., 2009; Gebauer et al., 2011).

1.1 Milk Fat Synthesis

In ruminants, the FA that compose milk TAG are either *de novo* synthesized in the mammary gland (ca. 40%), or they are imported from the plasma (ca. 60%) (Chilliard et al., 2000). The *de novo* FA are synthesized from acetate and 3-hydroxybutyrate, produced by ruminal fermentation of carbohydrates and by rumen epithelium from absorbed butyrate, respectively, thus resulting in short-chain FA (**SCFA**) and MCFA (C4:0 to C16:0). Acetate has been estimated to contribute between 70% and 80% of acetyl-groups for lipogenesis in adipose tissue, 15–30% in intramuscular depots (Smith and Crouse, 1984), and it is the major precursor for *de novo* FA synthesis in the mammary gland (Bauman et al., 2006). The FA which are imported from the plasma, are either released by the enzyme lipoprotein lipase (***LPL***) (Lock and Bauman, 2004) from triglycerides circulating in chylomicra or very low-density lipoprotein (***VLDL***) or derived from the plasma non-esterified FA (**NEFA**) that circulate bound to albumin, for long-chain 18-C FA as well as around half of the C16:0, depending on the diet composition (Lock and Bauman, 2004; Ferlay et al., 2017). These long-chain FA (**LCFA**) originate mainly from dietary lipid absorption from the digestive tract (with the dietary FA undergoing total or partial hydrogenation in the rumen) and from body reserves mobilization (especially at the beginning of lactation; McDonald et al., 1975). Commonly, mobilization of body fat accounts for less than 10% of milk FA. This proportion increases in ruminants in negative energy balance directly related to the extent of the energy deficit (Bauman and Griinari, 2001).

Furthermore, FA may be desaturated in the secretory mammary epithelial cells (**MEC**) (Chilliard et al., 2000). Indeed, MEC contain the stearoyl-CoA desaturase (***SCD***) complex, often referred to as Δ9-desaturase, an enzyme that catalyzes the oxidation of fatty acyl CoA esters resulting in the introduction of a *cis* double bond between carbon atoms 9 and 10 (Shingfield et al., 2010). The activity of *SCD* in the ruminant mammary gland is thought to occur as a mechanism to maintain and regulate the fluidity of milk to ensure efficient ejection from the mammary glands (Shingfield et al., 2010). The preferred substrate of *SCD* is C18:0, leading to OA synthesis (Palmquist et al., 2005; Ferlay et al., 2017). Moreover, several other saturated acyl CoA serve as substrates for *SCD*, including C10:0, C12:0, C14:0, C15:0, and C17:0 (Shingfield et al., 2010).

Additionally, endogenous synthesis via the action of *SCD* on VA is responsible for 64% to 97% of RA, while a small amount of RA is synthesized through the BH of unsaturated FA (**UFA**) by rumen bacteria (Palmquist et al., 2005). In the study of Shingfield et al., (2009), the abomasal infusion of a mixture of C18:1 FAs, including VA (administration: 92.1 g/d), in cows enhanced milk VA and RA concentrations. In particular, the concentration ratios of product versus product plus substrate for *SCD* indicated that 27.5% of the administered VA was converted to RA, similarly to previous estimates of 24.6 to 28.9% (Mosley et al., 2006; Shingfield et al., 2007; Tyburczy et al., 2008). This indicates a close agreement in the estimates of endogenous RA synthesis and provides further support that the action of Δ9-desaturase on VA converted to RA is essentially constant under most conditions in the lactating cow (Griinari et al., 2000; Shingfield et al., 2007a; Glasser et al., 2008), despite differences between individual animals, breeds, and experimental approaches.

1.2 Factors Affecting Milk Fat Synthesis

In general, milk fat can be affected by biological factors including breed (Soyeurt et al., 2006), genetic variation (Bobe et al., 2008), lactation stage (Stoop et al., 2009) and parity (Kelsey et al., 2003), as well as by environmental factors such as the rearing type (Tzamaloukas et al., 2015a), seasonal feed changes (de la Fuente et al., 2009), nutrition (Larsen et al., 2010) and the interactions between them (Macdonald et al., 2008; Stergiadis et al., 2013) **(Fig. 1)**. Although the breeding selection and improvements in genetic merit can be used to effect long-term changes in milk fat content, nutrition is the key factor regulating milk fat synthesis representing a practical tool to alter its yield and composition (Chilliard et al., 2000, 2003; Ferlay et al., 2017; Hanus et al., 2018).

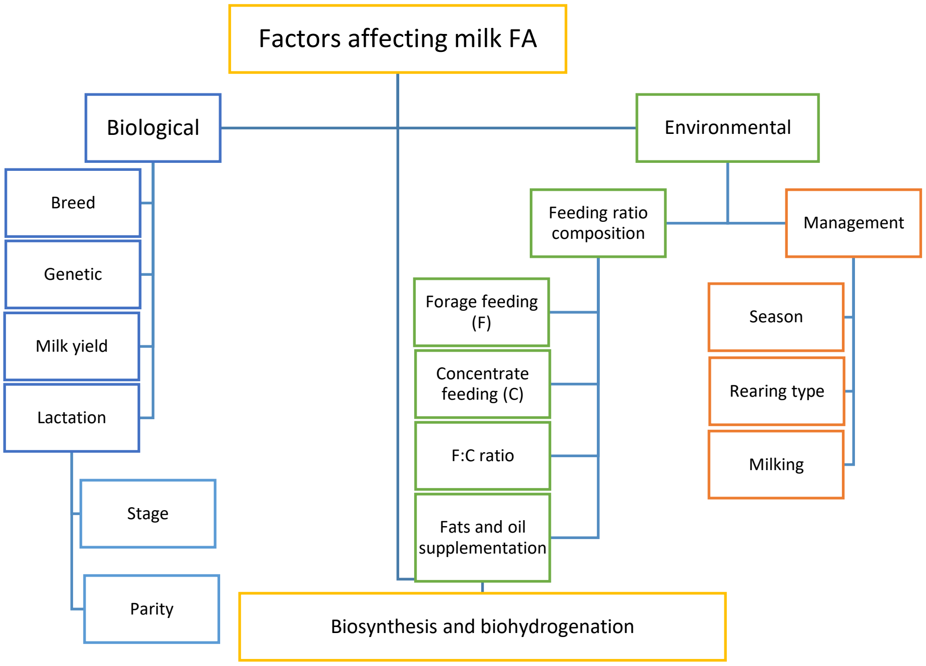

Fig. 1. Diagram of the sources of variability in the fatty acid profile of milk (adapted from Hanus et al., 2018 with slight modifications)

As mentioned above, diet is the most crucial factor affecting the metabolic processes occurring in the rumen as well as the composition of the rumen microbiome (Chilliard 2000). Several diets offered in ruminants, including different types of forage, lipid supplements, the forage to concentrate ratio, and the associated starch level and/ or their interaction, contribute to changes in duodenal flow and the proportion of each FA (Bernard et al., 2018; Hanus et al., 2018).

In general, there are two feeding strategies for the intensive production of milk. The first one referred to the year-round indoor feeding based on preserved feeds, while the second one is based on seasonal feeding depended on grazing during summer combined with indoor feeding during winter. The preserved feeds include mainly ensiled grass, corn, legume, grass-legume silages, or a combination of them, all supplemented with concentrates, a usual practice followed in areas with forage shortages such as Cyprus (Papachristoforou and Tzamaloukas, 2011). The FA profile of plants used for feeding purposes depends mainly on local soil and climatic conditions (Chilliard et al., 2007). It has been reported that the milk fat of grazing cows contains higher concentrations of beneficial FA with regards to human health (OA, ALA, VA, and RA) and lower percentages of SFA compared to milk from confined cows (Ferlay et al., 2017).

Similar to cows, the milk FA content with beneficial effects on human health increased in small ruminants due to pasture grazing. More specifically, the RA content in milk fat of grazing goats and/or sheep fed with spring-pasture (early growth stage of grass) increased significantly compared to indoor diets (Tsiplakou et al., 2006; Dervishi et al., 2012). Furthermore, studies with grazing goats showed prominent levels of ALA, C20:5n-3, and total *n-3* PUFA or VA and RA (Renna et al., 2012; Pajor et al., 2014). Additionally, studies comparing the FA profile of ewes fed hay indoors to those grazing fresh low mountain pastures showed that the ewes fed the fresh pastures had higher milk content of RA and LCFA (Dervishi et al., 2012).

The use of oilseeds in dairy diets is a common nutritional strategy for improving the FA profile of milk and has been recently reviewed by Bernard et al., (2018). There are different types of oilseeds, with soybean and rapeseed or linseed products being the most commonly used due to their high-quality protein and energy content (Hanus et al., 2018). Additionally, in the last decades, there is an increasing interest in the use of camelina or marine products such as algae in animal nutrition (Bernard et al., 2018). The extent to which oilseeds alter milk FA composition is based on many factors like the percentage of oil included in the diet, FA profile of the lipid supplement, the form of lipid in the diet, and/or processing of oilseeds and the composition of the basal diet (Chilliard et al., 2007). According to Shingfield et al., (2013), the use of oilseeds in ruminants' diet results in decreases in the milk concentration and mammary secretion of *de novo* FA, with no significant differences in SCFA (C4:0 and C6:0) concentration responses to supplements enriched with OA, LA or ALA lipids. Such changes were also accompanied by increases in the concentrations of stearic acid, OA and total C18:1 *trans* percentages.

1.3 Fatty Acids Metabolic Origins

Irrespective of the dietary source, all lipids entering the rumen, either as TAG (neutral lipids) in concentrates or as glycolipids or phospholipids (polar lipids) in forage, are strongly altered due to two lipolysis and BH of UFA from microbial action (Dewhurst et al., 2006; Lock et al., 2006; Ferlay et al., 2017).

Lipolysis of dietary lipids is predominantly achieved through rumen bacteria and microbial lipases, which are extracellular enzymes, and secondly through plant lipases (Bauman and Griinari, 2003). Bacteria in the rumen split off the FA from the glycerol backbone, and the released glycerol is fermented to volatile FA (**VFA**) (Chilliard et al., 2000). Moreover, VFA are produced through the fermentation of sugars in the rumen. In general, lipolysis occurs quite rapidly as the lipids are exposed during rumination to bacterial digestion of feed particles and is essentially complete so that no monoglycerides or diglycerides pass to the lower digestive tract (Chilliard et al., 2000, 2007; Shingfield et al., 2010; Ferlay et al., 2017).

The FA released in the rumen pass to the abomasum and then to the small intestine, which is the primary site for absorption of the FA. UFA hydrogenated rapidly in the rumen and it is suggested to do so to protect the rumen microbiome due to UFA toxicity to many of the ruminal bacteria, particularly to those which are involved in fiber digestion (Bestwick et al., 2010). In particular, this pathway converts UFA to C18:0 (final product of BH) via isomerisation into *trans* FA intermediates, followed by BH of the double bonds (Ferlay et al., 2017). In cattle diets, more than 90% of the UFA are hydrogenated to produce C18:0 that flow to the small intestine (Ferlay et al., 2017).

The major rumen pathways for the BH of ALA and LA, which are the most abundant FA in forages and concentrate feeds, are shown in **Fig. 2**. Although most of these FA are entirely hydrogenated for C18:0, rumen outflow also contains small quantities of BH intermediates absorbed and incorporated into the body and milk fat (Ferlay et al., 2017). Alternative pathways during BH of LA may occur when starch and/ or PUFA contents in the diet are high, and the rumen pH is low, which induced changes in the rumen bacterial populations with the production of CLA *trans* - 10, *cis* - 12 and

C18:1 *trans*-10, instead of RA and VA, respectively (Collomb et al., 2006; Wallace et al., 2007; Shingfield et al., 2010). Additionally, these energy-rich diets can exert adverse effects on milk fat, causing the low milk fat depression (**MFD**), which characterized by a reduction of up to 50% in milk fat yield with no change in the yield of milk or other milk components (Bauman et al., 2011). Several theories have been proposed to explain the causes of MFD, with the BH theory being the most universal (Bauman and Griinari, 2003; Shingfield and Griinari, 2007b). This theory attributes diet-induced MFD to inhibition of milk fat synthesis by specific FA intermediates of the *trans*-10 pathway produced during ruminal BH of dietary PUFA (Bauman and Griinari, 2003; Shingfield et al., 2010). In support of this hypothesis, CLA *trans*-10, *cis*-12, a metabolite of linoleic acid metabolism in the rumen (**Fig.2**), has been established to inhibit milk fat synthesis by post ruminal infusion studies conducted on lactating cows (Baumgard et al., 2001; Peterson et al., 2003, 2004). In addition, other studies have provided further evidence that CLA *cis*-10, *trans*-12 (Sæbø et al., 2005), CLA *trans*-9, *cis*-11 (Perfield et al., 2007), and C18:1 *trans*-10 (Shingfield et al., 2009), which are also ruminal BH intermediates of LA (Wallace et al., 2007), exert antilipogenic effects, as well.

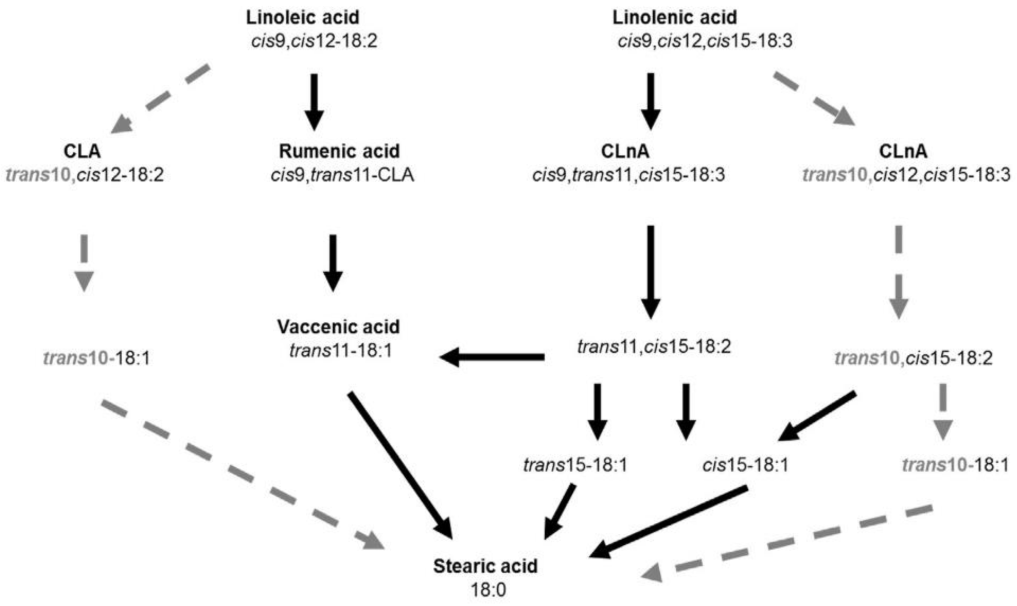

Fig. 2. The main pathways of microbial biohydrogenation of linoleic and linolenic acids in the rumen (adapted from Ferlay et al., 2017). Arrows with solid lines represent the classical processes that occurred in BH, while arrows with dashed lines describe the alternative pathways of BH.

1.3.1 Fatty Acid Absorption and Transport

The FA absorption occurs predominantly in the small intestine's jejunum region (Lock et al., 2006). The lipids that leave the rumen are mainly FFA (85-90%) and phospholipids (10-15%) found as part of microbial cell membranes (Chilliard et al., 2007). In the rumen, most of the FFA are found as potassium, sodium, or calcium salts of FA due to its near-neutral pH (6.0 – 6.8). After passing through the acid conditions (pH ~2.0) of the abomasum, the FA salts are dissociated, and the FFA are adsorbed to the surface of small feed particles that have passed through the digestive contents (Lock et al., 2006). The FA making up the FFA portion are mainly SFA (80-90%).

The key to the absorption of FA in the intestine is the formation of complexes called micelles, which are bi-layer disks consisting of bile salts (secreted in bile from the liver), phospholipids, and insoluble lipids. Micelles are needed to transport the FA to the surface of the intestinal cells, where they can be absorbed into the cells (Bauman and Griinari, 2003; Lock et al., 2006).

When FA are absorbed into intestinal cells, they are reconverted to TAG by combining with glycerol produced during blood glucose metabolism. TAG are packaged into lipoprotein particles (chylomicrons or *VLDL*) in combination with cholesterol, phospholipids, and specific proteins (Chilliard et al., 2000, 2007), and they are secreted into the lymph. Through the blood stream, the lipoprotein particles are transported to various organs of the body such as the mammary gland, muscle, and heart that can use the TAG (Sampath and Ntambi, 2005). TAG in chylomicrons or *VLDL* are split off to FFA by the enzyme *LPL,* an enzyme found in these tissues' capillaries. Then, the FFA entering the cells can be reconverted into TAG (such as milk fat) or burned to release energy that can fuel cell functions (such as contraction in skeletal or heart muscle) (Lock et al., 2006).

1.4 Feeding Strategies to Improve the Milk FA Profile of Ruminants

1.4.1 The Use of By-Products in Ruminant Diets

During the last decades, research has focused on examining the impacts of nutrition by using different types of diets or various sources of fats in order to improve milk FA composition with regards to human health and disease prevention (Parodi, 2004; Palmquist, 2006; Ferlay et al., 2017). The inclusion of different UFA in the diet of dairy animals is a reasonable attempt to improve milk FA composition for human consumption (Ferlay et al., 2017). Moreover, due to livestock production is growing faster than any other sector (FAO, 2019), and feedstuffs are the most crucial input in all livestock production systems in terms of cost, the availability of low-priced, high-quality feeds is essential for livestock production. In this regard, the inclusion of agro-industrial by-products, rich in UFA, in ruminant diets is of great interest for improving milk FA profile, reducing feeding costs, and diminishing environmental impacts associated with by-products accumulation (Correddu et al., 2020). The total amount of agro-industrial by-products in the European Union is estimated to be 16 million tons, with Germany (3 million tons), the UK (2.6 million tons), Italy (1.9 million tons), France (1.8 million tons), and Spain (1.6 million tons) be the main producers (FAO, 2019).

By-products such as gluten and germ from corn, and soybean hulls (mainly consisting of the outer covering of soybean), corn gluten feed (resulting from the extraction processes of the starch), beet pulp (resulting from the sugar manufacturing process), corn gluten meal, soybean meal, cottonseed meal, linseed meal and sunflower meal (obtained by grinding the material resulting after oil extraction), have been frequently used in the animal feed industry (Correddu et al., 2020). Besides these by-products, there are others derived from oilseed processing or distillery and biofuel production that could be promising alternative feedstuffs and could have a profit for the farmers (Salami et al., 2019).

1.4.1.1 By-Products of Oilseed Processing

The Mediterranean countries, which produce 98% of olive oil worldwide (IOOC, 2015), generate large amounts of by-products (Molina-Alcaide and Yáñez-Ruiz, 2008; Tzamaloukas et al., 2021). Those by-products presented **in Fig. 3** include the crude olive cake (**OC)**, obtained by mechanical extraction and contains residual oil and stones; the exhausted OC, which is the residue obtained after oil extraction from the crude olive cake by a solvent, usually hexane; the partly destoned OC, which is the result of partially separating the stone from the pulp by screening or ventilation; the olive pulp, which is produced when the stone has been separated before oil extraction; and other residues resulting from the cleaning operations (Sansoucy et al., 1985). The high moisture content in those by-products is one of the greatest challenges for their practical use because it may cause storage problems and affect, as mentioned above, the environment as a result of their rapid deterioration (Owaimer et al., 2004). Ensiling is an adequate and cost-effective technology to preserve high moisture feedstuffs, enhance their nutritional value, and increase palatability, mainly when applied in olive oil by-products (Hadjipanayiotou, 1999).

OC is the most abundant agro-industrial by-product and contains the olive kernel shell crushed into fragments, the skin, and the crushed pulp. About 50% of OC is water and includes a remaining quantity of oil. Both water and oil make OC subject to rapid spoilage (Molina-Alcaide and Yáñez-Ruiz, 2008; Tzamaloukas et al., 2021). The nature and type of OC produced depend on the olive oil extraction processing that may be used (Molina-Alcaide and Yáñez-Ruiz, 2008). Two different oil extraction procedures have been used extensively worldwide; the three-phase (**3P**) and two-phase (**2P**) system (**Fig. 2**). The main difference between these two systems is the higher moisture, and the lower oil content of the by-products produces from the 2P procedure, compared to the traditional system (3P). Thus, the main difference between the 2P and 3P - OC produced is that the latter is more solid and less humid than the 2P-OC (Alburquerque et al., 2004).

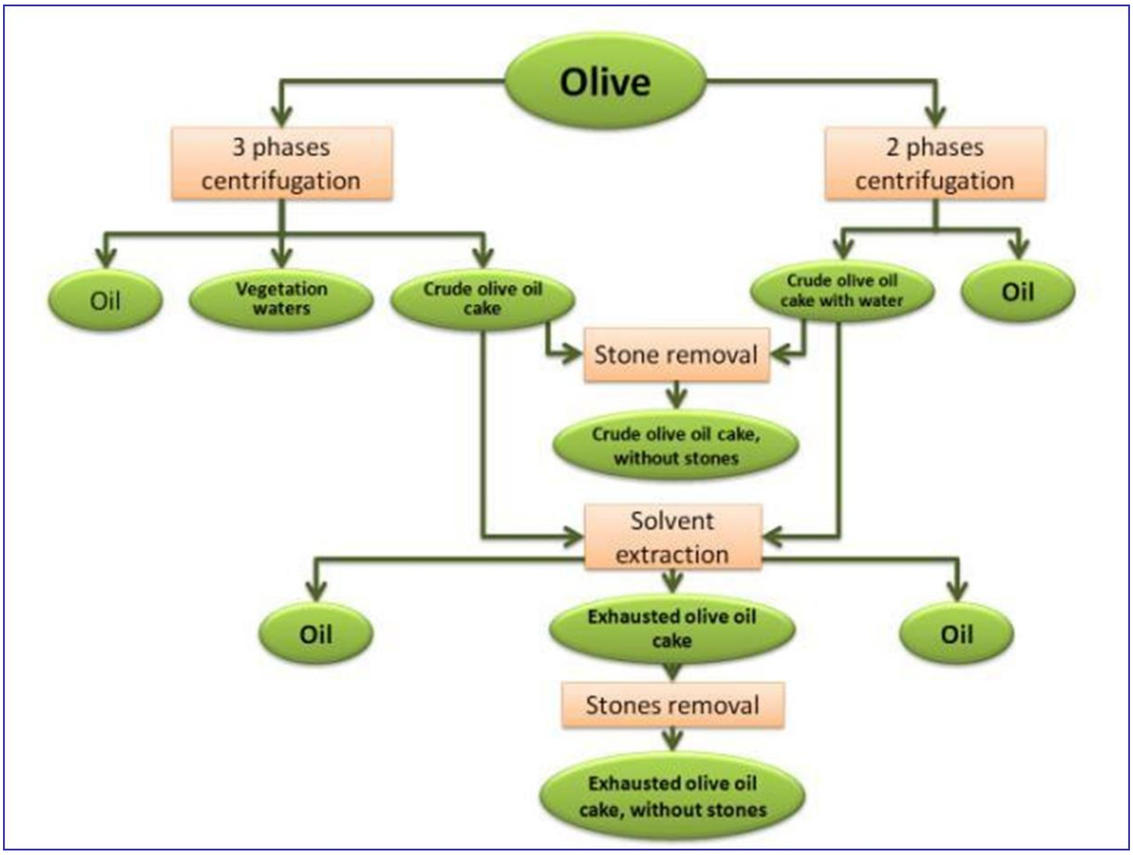

Fig. 3. The nature/type of olive cake produced according to the different processing methods used [adapted from Heuzé et al., (2015)].

Although OC is characterized by a low oil percentage (3 - 4% of DM) (Abbeddou et al., 2011b), it contains a high level of OA that could improve the milk lipid profile of ruminants (Chiofalo et al., 2002). The OC has been mainly investigated in sheep diets (Molina-Alcaide and Yáñez-Ruiz, 2008). Feeding trials including dried (Abbeddou et al., 2011a, b, 2015), partly destoned dried (Vargas-Bello-Pérez et al., 2013), partly destoned fresh (Chiofalo et al., 2004), or ensiled (Symeou et al., 2019, 2021) OC decreased SFA and increased MUFA content. A limited number of studies examining the effect of processed OC on FA composition of milk and/or related cheese is available in the case of goats (Molina-Alcaide et al., 2010) and cattle (Castellani et al., 2017) showing almost similar results.

1.4.1.2 By-Products of Distillery and Biofuel Production

The rapid growth of processing plants that utilize grains to produce bioethanol has increased the availability of by-products that can be used as alternative feedstuffs in dairy animals and, nowadays, have become mainstream commodities (USDA, 2010; Salami et al., 2019). In the U.S., corn is the main source of starch (glucose) used to produce bioethanol. With the exception of sugar cane, corn provides the highest ethanol yields, followed by barley and wheat yields (U.S. Grains Council, 2012; **Table 3**).

Table 3. Starch content and ethanol yield of various feedstocks

Feedstock	Moisture (%)	Starch (%)	Ethanol Yield (L/MT)[a]
Starch	-	100.0	720
Sugar cane	-	-	654
Barley	9.7	67.1	399
Corn	13.8	71.8	408
Oats	10.9	44.7	262
Wheat	10.9	63.8	375

[a] expressed as liter per metric ton [adapted from U.S. Grains Council, (2012)]

There are two different bioethanol production processes from grains that generate sufficient amounts of by-products; the wet milling and dry grind technology, from which the latter is the main process used (USDA, 2010). During the dry-milling process **(Fig. 4)**, the grains are either ground or milled to reduce their particle size and produce bran-free flour. Then, water and recycled stillage are added to the ground/milled grain, which act as conditioners to begin leaching of soluble protein, sugars, and non-starch bound lipids (liquefaction step) (U.S. Grains Council, 2012). Cooking is then used to hydrolyze starch into glucose (saccharification step) along with the addition of amylolytic enzymes for yeast (*Saccharomyces cerevisiae*) to convert glucose to ethanol. Complete hydrolysis of the starch polymer requires a combination of enzymes, with amylases being the most widely used (Sarikaya et al., 2000). After fermentation, ethanol is collected using distillation columns. The water and solids remaining after distillation of ethanol are called whole stillage and can be centrifuged to separate coarse solids from liquid to yield wet grains and thin stillage (U.S. Grains Council, 2012). Wet grains can be offered to livestock directly, or they can be dried to produce dried distillers' grain. Moreover, the thin stillage can be dehydrated to produce condensed distillers solubles (also called syrup) which are mixed with distillers' grain form the feeds called wet or dried distillers' grain with solubles (**DWGS** or **DDGS,** respectively) (Pezzanite et al., 2006; USDA, 2010).

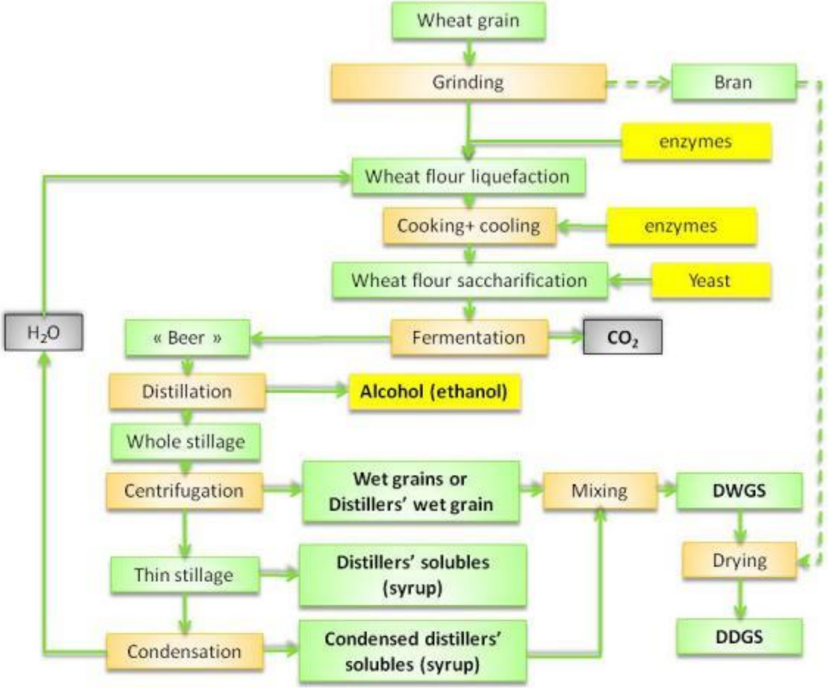

Fig. 4 The bioethanol manufacturing process (dry milling) produced from maize. DWGS: wet distillers' grain with solubles; DDGS: dried distillers' grain with solubles [adapted from Heuzé et al., (2017)]

DDGS represents an interesting supplement for ruminants due to its high protein, fat, and energy content (crude protein: from 28.9 to 30.9% on DM; fat: 11.2%; energy: from 12.5 to 13.1 MJ ME/kg DM, U.S. Grains Council, 2012; Schingoethe et al., 2009). The high levels of ruminal undegradable protein, in combination with the beneficial fibre that contains, make DDGS a foodstuff that does not threaten animal health due to rumen acidosis (Pecka-Kiełb et al., 2017). Moreover, its richness in PUFA makes it a cost-effective by-product that can be used in dairy animals for enhancing the quality of milk fat (Klopfenstein et al., 2008; Schingoethe et al., 2009).

Previous studies in dairy cattle included DDGS up to 20% of DM intake (Anderson et al., 2006; Kleinschmit et al., 2006; Sasikala-Appukuttan et al., 2008; Chibisa et al., 2012; Kurokawa et al., 2013) or even up to 30% of DM (Janicek et al., 2008), showing that neither production nor fat percentage of milk were adversely affected, particularly, when diets contained adequate levels of forage fibre (Schingoethe et al., 2009). A decrease in milk fat content has been noticed in cows when either DDGS-supplemented diets contained less effective fibre (Leonardi et al., 2005; Benchaar et al., 2013) or high levels of fat (8 % fat) were offered (Abdelqader et al., 2009); however, fat yield and production of milk were unaffected in those studies. Moreover, concerning studies examining the effects of DDGS on milk FA composition, it has been reported that including DDGS up to 20% (DM) in cow diets led to decreased SFA content with concomitant increased levels of MUFA and PUFA, including RA (Leonardi et al., 2005; Anderson et al., 2006; Sasikala-Appukuttan et al., 2008; Abdelqader et al., 2009; Kurokawa et al., 2013; Gaillard et al., 2017a, b).

A limited number of studies investigating the effect of using DDGS in diets of small ruminants is available for fattening sheep (McKeown et al., 2010; Charles et al., 2012; Alshdaifat and Obeidat, 2019), while in the case of lactating ewes and goats the information is even more scarce (Cais-Sokolińska et al., 2015; Alshdaifat and Obeidat, 2019). The few data existing showed that 20 and 30 % (DM) of DDGS inclusion in the diets of dairy Awassi ewes increased yield of milk, protein and fat, whereas percentages of milk fat and protein were unaffected (Alshdaifat and Obeidat, 2019), while the milk FA content was not tested in this study. Additionally, Cais-Sokolińska et al., (2015) indicated that when DDGS was included at 3.5% (DM) in the diets of Polish dairy sheep and goat breeds, it did not affect fat and protein percentages of milk. In this study, milk FA profile was determined, and increased levels of total *trans* - MUFA and PUFA were reported in the milk of ewes and goats, while RA content was either affected favorably or not affected in caprine and ovine milk, respectively, with DDGS supplementation.

1.5 Regulation of Milk Fat Synthesis

Synthesis of milk fat requires coordination of enzymes involved in metabolite transport, *de novo* lipogenesis in the mammary gland, FA uptake, translocation, desaturation, and esterification, and the formation, transport, and excretion of the milk lipid droplet (Bauman et al., 2011). However, a deep understanding of mammary physiology and molecular adaptations to diet and/or physiological state is needed to efficiently manipulate milk fat synthesis and development of dairy products with specific characteristics (e.g., more UFA and CLA) (Bionaz and Loor, 2008a).

In this regard, nutrigenomics is an umbrella tool that refers to the impact of dietary components on physiological processes by altering gene expression, epigenetic mechanisms, proteins, or metabolites (Bauman et al., 2011). Until recently, ruminant functional genomics studies were not feasible. However, the invention and improvement of quantitative polymerase chain reaction (**qPCR**) (Mullis et al., 1986; Mullis, 1990), accompanied by the discovery of the synthesis of complementary DNA (**cDNA**) from mRNA using a reverse-transcriptase (**RT**) (VanGuilder et al., 2008), allowed the study of the expression of mRNA, usually present in cells in extremely low amounts. The onset of

RT-qPCR was an essential step toward a better understanding of the transcriptional control of milk fat synthesis. This was exemplified by a study where the expression of several genes thought to be involved in all steps of milk fat synthesis was measured with high precision in mammary tissue of dairy cows from pregnancy to the end of subsequent lactation (Bionaz and Loor, 2008a). Among the genes reported in the study of Bionaz and Loor, (2008a), acetyl-CoA-carboxylase (***ACACA***), fatty acid synthase (***FASN***), and glucose 6 phosphate dehydrogenase (***G6PDH***) are key genes encoding enzymes involved in *de novo* FA synthesis, while the very low density lipoprotein receptor (***VLDLR***) in concert with *LPL* take up and hydrolyze TAG. Translocators involving the solute carrier family 2 member 1 (***SLC2A1***) and the fatty acid translocator (***FAT/CD36***) play a significant role in FA transport, while the fatty acid binding protein 3 (***FABP3***) provides substrates to the steroyl-CoA-desaturase 1 (***SCD1***), the most abundant gene in mammary gland decreasing the saturation content of FA in ruminant milk. Additionally, the sterol regulatory element binding transcriptional factor 1 (***SREBF1***) and the peroxisome proliferator activated receptor gamma (***PPARγ***) constitute regulators of milk lipid metabolism (Osorio et al., 2016).

Nowadays, experiments examining the effect of different ruminant diets on FA composition also sought to understand the impact of dietary treatment on the expression of lipogenic genes in mammary and adipose tissue through mRNA abundance and/or enzyme activity measurements (Thering et al., 2009; Angulo et al., 2012; Bernard et al., 2012; Bichi et al., 2013b; Vahmani et al., 2014; Castro-Carrera et al., 2015; Toral et al., 2017). These studies, in their attempt to understand the underlying mechanisms driving milk fat synthesis and its regulation, have been mainly tested diets supplemented with plant and/or marine lipids or with specific FA, such as CLA isomers which are all known to induce MFD (Osorio et al., 2016). The corresponding responses of genes of the major mammary lipogenic pathways (*de novo* synthesis, FA uptake and transport, TAG synthesis, and FA desaturation) as well as their regulatory elements (transcription factors) were investigated in studies that have been previously reviewed (Shingfield et al., 2010, 2013; Bauman et al., 2011; Osorio et al., 2016; Bernard et al., 2018).

1.5.1 Regulation of mammary lipogenic gene expression by dietary factors

Previous in vivo studies demonstrating the nutritional regulation of lipogenic gene expression in the mammary gland of lactating cows, testing milk fat-depressing diets (Piperova et al., 2000; Ahnadi et al., 2002; Peterson et al., 2003; MacH et al., 2011; Vahmani et al., 2014), or abomasal/intravenous infusion of CLA *trans*-10, *cis*-12 (Baumgard et al., 2002; Harvatine and Bauman, 2006; Gervais et al., 2009; Vyas et al., 2013; Harvatine et al., 2018) indicated that, reductions in *de novo* FA (FA with C < 16) secretion have been associated with a downregulation of the mRNA abundance of key genes involved in milk fat synthesis like *ACACA*, *FASN*, *SCD1* and *SREBF1* due to increased levels of CLA *trans*-10, *cis*-12 and/ or C18:1 *trans*-10, FA that cause MFD (Shingfield et al., 2009; Bauman et al., 2011). This coordinated inhibition of genes involved in lipogenesis has been associated with reduced expression of *SREBF1* and proteins involved in the activation and distribution of *SREBF1* towards the nucleus (Harvatine and Bauman, 2006), which is a mechanism demonstrated in bovine mammary cells and has been confirmed by many studies reviewed by Bauman et al., (2011) and Osorio et al., (2016), suggesting that this transcriptional factor plays a central role in lipogenesis.

Nevertheless, information referring to nutrigenomics and mammary lipid metabolism in small ruminants is scant and contradictory (Shingfield et al., 2013). For instance, some studies support that CLA *trans*- 10, *cis*-12 -induced MFD in lactating ewes involves the *SREBF* family and a coordinated downregulation of genes related to mammary lipid synthesis (e.g., *ACACA, FASN, SCD1, or SREBF1*) (Hussein et al., 2013; Carreño et al., 2016). However, other *in vivo* studies in dairy sheep (Bichi et al., 2013a; Castro-Carrera et al., 2015; Toral et al., 2017) or dairy goats (Bernard et al., 2005, 2009, 2012; Toral et al., 2013) seem to indicate that there is no a clear link between changes in milk FA production and/or mammary mRNA abundance of key candidate genes and transcription factors involved in lipid metabolism. The *in vitro* study of Zhang et al., (2018) reported that this specific CLA *trans*-10, *cis*-12 isomer, reduced the mRNA abundance of *FASN*, *SCD1*, and *ACACA* in caprine mammary gland epithelium. The reason behind such contrasting results, is still to be clarified.

Moreover, direct comparison studies between dairy cows and goats revealed that the goats are less sensitive to the diets known to induce MFD (high starch and plant oil or fish oil diets) than cows (Toral et al., 2015). Whether this finding depends on differences in the two species' responsiveness, or species-specific lipogenic genes pathways is still unclear. Recent more complete comparison studies conducted with dairy cows and goats fed various lipid supplements known to induce MFD or conversely to increase fat content in the bovine milk revealed inter-species differences and species-by-diet interactions, suggesting that different molecular mechanisms other than direct *trans*-intermediate activity may take place (Bernard et al., 2017; Fougère et al., 2018; Fougère and Bernard, 2019). In the same studies, changes in milk fat content and FA secretion were not associated with alterations in the mammary expression of any selected genes known to be involved in the major lipogenic pathways (Bernard et al., 2017; Fougère and Bernard, 2019). Therefore, those authors suggested other regulatory mechanisms such as posttranscriptional or posttranslational linked to changes in milk fat content and FA secretion may be involved.

1.5.2 Regulation of adipose lipogenic gene expression by dietary factors

Adipose tissue metabolism in pregnant and lactating dairy animals is essential in establishing and supporting lactational efficiency (Sumner and McNamara, 2007; Rocco and McNamara, 2013). Specific adaptations in enzymes controlling lipogenesis and lipolysis during lactation help to supply FA to the mammary gland and other organs (Shirley et al., 1973). Although the physiological adaptations of the adipose tissues occurring during lactation have been well described in ruminants (Vernon, 2005), adipose tissue's possible contribution to the regulation of mammary lipogenesis still needs to be documented.

Up to date, the *in vivo* studies investigating the effects of nutrition on gene expression in adipose tissue in dairy animals are scarce. In some studies, testing plant oil supplementation in cows (Thering et al., 2009; Vahmani et al., 2014) and in small ruminant (Bernard et al., 2009, 2012; Bichi et al., 2013a; Toral et al., 2013; Castro-Carrera et al., 2015), no significant effect or a slight variation on the adipogenic expression was shown. In contrast, Harvatine et al., (2009) reported that intravenous infusion of CLA *trans*-10, *cis*-12 upregulated *FASN, SREBF1, LPL, SCD1,* and *PPAR*γ in adipose tissue of dairy cows. It has been hypothesized that this particular CLA isomer increases the adipogenic expression of enzymes involved in *de novo* synthesis, uptake, and desaturation of FA as well as the transcriptional factors such as *SREBF1* and *PPARγ* (Bauman et al.

2011), suggesting energy partitioning towards adipose tissue fat stores during MFD in cows (Harvatine et al., 2009).

1.6 Outline of the thesis

Based on the aforementioned aspects, the main objective of the present thesis was to evaluate the effects of different by-products derived from oilseed processing like ensiled OC and biofuel production, like DDGS, on the milk yield and composition, on the FA profile of milk and Halloumi cheese and on the expression of specific genes involved in lipid metabolism of mammary and adipose tissue in ruminants to elucidate likely mechanisms of action when oil rich feed by-product is added to the ruminant diets. In this sense, in **Chapter 2**, the inclusion of 10% (DM) of ensiled OC in cow diets for a feeding period of up to 21 days was examined to exemplify the possible effects of using this by-product for a short-term period on milk production, composition, and FA profile. Subsequently, in **Chapter 3,** the effect of dietary inclusion of 10% (DM) of ensiled OC, in an experiment that lasted 8 weeks, on milk yield, composition, and FA profile of cow milk and related Halloumi cheese, was evaluated. Moreover, for examining any possible impact of dietary supplementation of ensiled OC on bovine lipogenic gene expression, changes in the expression of selected genes involved in mammary and adipose lipid metabolism were assessed. An additional study was conducted on lactating goats testing the effects of 10 and 20% (DM) of ensiled OC supplementation on milk yield, composition and FA profile (**Chapter 4**). In the same study, the dietary impact of the 20% (DM) of OC on the mRNA abundance of selected genes involved in caprine mammary and adipose tissue lipid metabolism, was investigated too. Another by-product that was also studied (**Chapter 5**) was the wheat based-DDGS included at rates of 6%

and 12% (DM) in diets of dairy ewes, showing its potential effects on milk production and content, as well as on milk FA composition. Finally, in **Chapter 6**, the main findings of the present thesis were summarized and discussed thoroughly, while some crucial future perspectives were risen up.

CHAPTER 2: Feeding ensiled olive cake in lactating cows for a short-term period

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A slightly modified version of this chapter has been submitted for publication

Neofytou, M.C., Miltiadou, D., Symeou, S., Sparaggis, D., Tzamaloukas, O. Short-term forage substitution with ensiled olive cake increases beneficial milk fatty acids in lactating cows. Tropical Animal Health and Production (accepted).

ABSTRACT

This study aimed to evaluate the effect of short-term forage substitution with ensiled OC on yield, composition and FA profile of cows' milk. Mid-lactating Holstein - Friesian cows were randomly assigned for 21 days to two isoenergetic and isoproteic feeding treatments (12 animals per treatment), containing 0 and 10% DM of ensiled OC (CON and OC groups, respectively). Milk yield was recorded daily, and milk samples were collected at 14 and 21 days of the trial for analyzing the fat, protein and FA profile of milk. No significant differences were observed in milk yield, protein and fat nor in protein and fat percentage of milk between groups. However, dietary supplementation with ensiled OC modified the FA profile of cow milk. Feeding cows with ensiled OC resulted in a significant decline of MCFA, while LCFA and MUFA were risen in milk ($P < 0.05$). Among individual SFA, palmitic was particularly reduced, while among individual MUFAs, increments of OA were demonstrated with the OC treatment ($P < 0.05$). Although total PUFA were decreased, the concentration of RA tended to be elevated with OC feeding ($P = 0.06$). Overall, short-term forage substitution with ensiled OC improved, beneficially to human health, the lipid profile of milk without adversely affecting milk yield or milk composition of lactating cows.

2.1 INTRODUCTION

The inclusion of different products rich in UFA in the diets of lactating ruminants alters milk FA composition beneficially for human health (Ferlay et al., 2017). Crude OC, which is the most abundant by-product of olive oil production, represents an alternative forage substitution and could be useful for tropical and Mediterranean areas with a shortage of roughages (Costa et al., 2019). Nevertheless, the inclusion of OC in ruminants' feed is now rare because of its low nutritional value, rapid spoilage, due to rancidity, and seasonal availability (Hadjipanayiotou, 1999; Owaimer et al., 2004), while the process of drying or destoning add extra costs to this by-product. Considering the disadvantages of using fresh or dried OC, the ensiling method applied in Cyprus is a cost-effective alternative to overcome those difficulties, allowing the use of OC in ruminants throughout the year. Moreover, OC may improve the lipids of milk due to its richness in OA. Studies performed in dairy sheep demonstrated a decrease in SFAs and an increase in MUFAs and, in some cases, increased specific beneficial UFAs, such as RA content of milk by the inclusion of dried (Abbeddou et al., 2011a, b, 2015), partly destoned fresh (Chiofalo et al., 2004) or ensiled OC (Symeou et al., 2019, 2021). Recent studies in dairy cows reported similar results. Castellani et al., (2017) showed increased MUFAs and RA and decreased SFA content in milk of cows fed dried olive pomace for a long feeding period of 64 days. To date, no study has examined the effect of OC diet inclusion as a forage substitution for a short-term feeding period in lactating cows. This information would be useful in farming practices of specific areas during periods of forage shortages or to produce a special dairy product. Therefore, the objective of this study was to assess the short-term inclusion of 10% (DM) of ensiled OC into the diets of lactating dairy cows, on milk production, protein and fat content, as well as on milk FA composition.

2.2 MATERIALS AND METHODS

2.2.1 Animals, Management and Experimental Diets

The experiment was performed on the Agricultural Research Institute farm (ARI; Athalassa, Nicosia) in Cyprus. All experimental procedures were carried out according to the national legislation regulations (Animal Welfare Law, 1994) and international guidelines (Directive, 2010/63/EU for animal experimentation) and approved by the corresponding departmental committee of the Cyprus University of Technology.

Twenty - four and mid-lactating Holstein Friesian cows were distributed in 2 separate groups of 12 animals each, with each group divided into 3 pens (4 cows in each), homogenous for age, milk yield (mean ± SEM: 29.31 ± 1.31 kg per head per day), and body weight (mean ± SEM: 582.0 ± 11 kg). Two different iso-energetic and iso-nitrogenous feeding treatments were assayed for a period of 21 days. Throughout the experiment, 3 pens were supplemented with a conventional diet (CON), and 3 pens were supplemented with the conventional diet in which part of the forages were replaced with 5kg per cow per day of ensiled OC (fresh OC with DM content of 47.7%) (OC group), obtaining a concentrate to forage ratio of 64:36 in both treatments. In detail, the diets contained 0 and 10% (DM) of ensiled OC and this specific inclusion rate was chosen based on previous studies in Cyprus (Hadjipanayiotou 1999) and unpublished pre-experimental data (Tzamaloukas’ personal communication) in which is suggested that 10% (DM) of OC can be included on ruminants’ diets without adverse effects on milk traits.

The OC constituted from a mixture of skins, pulp, woody endocarp, and seeds obtained after extraction of olive oil from locally cultivated olives (Ladolia and Kalamon cv.). The OC was collected by a three-stage oil mill and ensiled according to the method developed as follows: after olive oil extraction, crude OC was obtained immediately and accumulated on the surface of a clean and hard floor of the silos. The pile of OC, which did not contain any additives or other feed materials for helping the fermentation process, was covered with a black polyethylene film (8mm thick) and was firmly closed. The film covering the pile was stretched for expelling the air, and soil was used for covering the edges of the film. The ensiled OC was fermented in silos for 3 to 4 months before use.

Animals of both groups were fed individually with automatic feeders (Westfalia, Albersdorf, Germany), offering the concentrates 4 times per day, while forages were divided and offered manually to each group after morning (0430 h) and evening (1630 h) milkings. The OC supplement was offered directly after morning milking, and cows consumed it entirely within about 15 min. Water was provided ad libitum. Dry matter intake (**DMI**) was monitored daily by manually weighing the offered diets and collected orts. Representative feed samples of ensiled OC, alfalfa, barley hay, barley straw, and concentrate mix were collected at the beginning and the end of the trial, mixed per treatment, and samples were taken for analysis. The chemical composition of the two treatments is presented in **Table 4**. Dry matter (**DM**), ash, crude fat, and crude protein (**CP**) were determined as described by AOAC International, (2005). Crude fibre, acid detergent fiber (**ADF**), acid detergent lignin (**ADL**), and amylase-neutral detergent fibre (**aNDF**) were measured according to van Soest et al., (1991).

Table 4. Ingredients and chemical composition of dietary treatments and the olive cake silage used

Item	Treatment[1]		Ensiled OC
	CON	OC	
Ingredient composition			
Ensiled OC	-	10	
Alfalfa	7	7.07	
Barley hay	18	13	
Barley straw	11	6.	
Concentrate mix [2]	64	64	
Chemical composition, (% DM)			
Dry matter, %	92.9	83.83	47.58
Crude protein, % DM	17.24	17.22	5.45
Crude fat, % DM	1.75	2.29	6.93
Crude fiber, % DM	17.35	18.72	49.00
Ash, % DM	6.56	6.02	2.51
aNDF, % DM	35.74	36.44	71.46
ADL, % DM	3.5	5.62	28.25
ADF, % DM	21.12	22.4	54.62
Metabolized Energy (MJ/kg)[3]	9.2	9.41	-

[1]CON = control group, OC = olive cake group; [2]Concentrate mix = 16% barley, 21% maize, 17.8% soybean meal 48%, 13% sunflower cake, 10% wheat bran, 18% sugar beet pulp, 4.24% mineral and vitamin mix; [3]Values from NRC (2001)

2.2.2 Measurements and Milk Sampling

All animals were machine milked (Fulwood, Shropshire, UK) twice daily (at 0430 h and 1630 h), and milk yields were recorded electronically (AfiMilk MPC Milk Meter, Kibbutz, Israel) at each milking. Raw milk samples for the determination of the lipid profile were collected at 14 and 21 days from each cow during the two consecutive milkings (morning and evening) and were stored at - 80°C for further analysis. Measurements for total fat and protein were obtained by the use of combined thermo-optical procedures (Lactostar 3510, Funke Gerber, Berlin, Germany) calibrated previously for protein with the Lowry protein assay and fat, with the Gerber method

989.05 (AOAC International, 2005), while solids non-fat (**SNF**) measured through the method described by Tzamaloukas et al., (2015a).

2.2.3 Lipid Analysis

The milk fat isolation was carried out according to the rapid double centrifugation method of Feng et al., (2004) with slight modifications. Briefly, 20 mL aliquots of fresh milk were first centrifuged at 17800 × g for 30 min at 4°C. The resulting fat cakes were removed, placed in new tubes, and allowed to melt at room temperature for 20 min. The samples were then recentrifuged at 19500 × 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.

For the feed fat isolation, the method used was as follows: after crude fat determination (Soxtec method), 2 ml of hexane was added to the residue in the aluminum collection pots and transferred to a glass tube (pre-weighed). Then, the residue was dried down under a gentle stream of nitrogen gas, and the glass tube was reweighed. Subsequently, 1 ml of hexane was added for re-dissolving the residue, and then an aliquot containing 20-25 mg of crude fat was removed and put into fresh tubes.

Fatty acid methyl esters (**FAME**) of milk and feed lipids were prepared by transesterification with methanolic potassium hydroxide according to the ISO (2002) method. Essentially, 40 μL of transesterification reagent (11.2% KOH in methanol) were added to the tubes containing extracted lipid from milk and feeds and shaken vigorously for 1 min. The tubes were then allowed to incubate for a further 5 min at room temperature before the addition of 0.1 g of sodium bisulfate. The particulate material was then removed by centrifugation at 350 × g for 3 min at room temperature, and aliquots of the clear organic phase transferred to amber gas chromatography vials and stored at −70°C until analyzed. FA profiles were generated by analyzing the FAME samples on a GCMS-QP2010 Plus Gas Chromatography Mass Spectrometer (Shimadzu, Duisburg, Germany) equipped with an HT280T autosampler (HTA, Brescia, Italy) following the method of Kramer et al., (2008). Typically, 1 µl aliquots of FAME were separated with a split ratio of 1:20 using an Agilent CP-Sil 88 fused silica capillary column (100 m × 0.25 mm internal diameter × 0.2 µm film thickness). The column was held for 4 min at 70°C after injection, increased at 13°C/min to 175°C and then held at that temperature for a further 27 min. The temperature was then raised to 215°C at 4°C/min, at which it was held for a further 36 min. Helium was the carrier gas at 1 mL/min, with both injector and interface temperatures of 225°C. Chromatographic profiles were analyzed using Shimadzu GCMS Postrun Solution software where individual peaks were identified by comparison of their retention indices to those of commercially available authentic standards (Supelco 37-FAME standard mix, CLA *cis*-9, *trans*-11, CLA *trans*-10, *cis*-12, C18:1 *trans*-11; 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). All FAME peaks identified were quantitated by peak integration and individual FAME expressed as a percentage of the total fat (Butler et al., 2011).

Milk atherogenic index (**AI**) was determined using the formula proposed by Ulbricht and Southgate, (1991): $AI = (C12:0 + 4 \times C14:0 + C16:0) / (\Sigma MUFAs + \Sigma PUFAs)$ and the desaturation index (**DI**) was determined using the formula suggested by Garnsworthy et al., (2010): $DI = (C14:1\ cis\text{-}9 \times 100) / (C14:0 + C14:1\ cis\text{-}9)$.

2.2.4 Statistical Analysis

All data were subjected to an analysis using a mixed-effects design with repeated measurements using SAS (version 9.4. SAS Institute Inc., Cary, NC). The model included the fixed effects of time (T), experimental diet (D) and their interaction (T x D), and the random effect of cows and pen. Time was considered a repeated factor for each analyzed variable. Statistical significance declared at $P < 0.05$. P-values between > 0.05 and ≤ 0.10 were interpreted as trending towards significance. Normality of data was assessed both visually and through the normality test of Shapiro-Wilk using using the SPSS 20 software (StatSoft Inc., Tulsa, OK, USA).

2.3 RESULTS

2.3.1 OC Silage and Diets

The FA composition of diets and OC silage is shown in **Table 5**. By included ensiled OC in cow diets, the most FA were affected significantly, with OA being the major FA found, around 34% of diet lipids. The OC diet showed a significantly lower palmitic and linoleic acid content, while an increased proportion of stearic was demonstrated compared to the CON diet.

Table 5. Fatty acid composition (expressed as a percentage of total fatty acid methyl esters) of the dietary treatments used and ensiled olive cake (OC)

	Treatments[1]				
Item	CON	OC	SEM	P-value[2]	Ensiled OC
C16:0	26.48	23.58	0.81	**	13.45
C16:1 *cis*-9	0.32	0.61	0.07	**	1.48
C18:0	1.91	2.55	0.12	***	4.15
C18:1 *cis*-9	21.81	34.00	2.73	***	63.58
C18:2n-6	23.55	21.66	0.51	**	13.12
C18:3n-3	2.14	1.99	0.18	NS	1.46

[1]CON = control group, OC = olive cake group ; [2]Probability of significant effects = *P < 0.05; **P < 0.01; ***P < 0.001; NS: Non significant

2.3.2 Performance Variables of Cows

Table 6 shows the DMI, milk production and yield, fat and protein yield, and the composition of the milk samples after 21 days of supplementation. OC did not influence the DMI, the yield of milk, protein, and fat yield, nor the milk content of fat, protein, and SNF of the cows during the trial.

Table 6. Milk production and chemical composition of milk from cows fed diets with 0 or 10 % (DM) of ensiled olive cake

	Treatments[1]			P-value		
Item	CON	OC	SEM	D	T	D x T
DMI, kg/d	22.87	22.90	1.40	NS	NS	NS
Yield, kg/day						
Milk	25.91	25.54	1.93	NS	NS	NS
Fat	0.84	0.86	0.06	NS	NS	†
Protein	0.91	0.91	0.06	NS	NS	†
Milk composition, %						
Fat	3.26	3.41	0.07	NS	NS	†
Protein	3.51	3.61	0.06	NS	†	NS
SNF[3]	9.37	9.39	0.09	NS	NS	NS

[1] CON = control group; OC = olive cake group, [2] Probability of significant effects due to diet (D); time (T); and their interaction (D x T), *P < 0.05; **P < 0.01; ***P < 0.001; † P < 0.1: tendency, [3]SNF = Solids Non-Fat

2.3.3 Milk Fatty Acid Composition

The analyzed FA and their aggregated groups determined in milk collected from CON and OC treatments are given in **Table 7**. Feeding OC significantly increased OA and total MUFA content at the expense of a significant decrease of MCFA in the milk of cows. A tendency of decreased levels was demonstrated in total SFA ($P=0.01$), while individual SFA like C4:0 and C16:0 were significantly reduced with OC feeding. In contrast, C18:0 was the only SFA that increased by the addition of OC in the cow diets. Total MUFA were risen and among individual 18-C MUFA, increased levels of OA ($P < 0.05$) or a tendency of elevated concentration of the sum of C18:1 *trans*-10 and *trans*-11 were observed in the milk of OC group compared to the CON group. On the contrary, the contents of C14:1 *cis*-9, C16:1 *cis*-9, and C10:1 *cis*-9 acids were not affected by the inclusion of OC in the cow diets.

Although the content of total PUFA was reduced significantly by OC feeding, a tendency ($P = 0.06$) of increased concentration of RA was observed in the milk of the OC group. No differences between treatments were observed in ALA and AA, while the OC inclusion reduced LA and the AI levels. No diet effect was indicated in the DI.

Table 7. Fatty acid composition (expressed as a percentage of total fatty acid methyl esters) of milk from cows fed a diet with 0 or 10 % (DM) of ensiled olive cake

	Treatments[1]			P-value[2]		
Item	CON	OC	SEM	D	T	D x T
C4:0	3.14	2.89	0.1	***	NS	**
C6:0	1.90	1.90	0.05	NS	NS	NS
C8:0	1.22	1.16	0.04	NS	†	NS
C10:0	2.85	2.48	0.16	NS	*	NS
C10:1, *cis*-9	0.35	0.31	0.02	NS	NS	NS
C12:0	3.29	2.80	0.17	NS	NS	NS
C14:0	11.19	10.45	0.31	NS	NS	NS
C14:1, *cis*-9	1.37	1.30	0.05	NS	NS	NS
C15:0	1.06	0.80	0.04	NS	NS	NS
C15:0 iso	0.25	0.27	0.02	NS	NS	NS
C16:0	32.64	29.66	0.53	***	NS	**
C16:1, *cis*-9	1.90	1.66	0.08	NS	*	NS
C17:0	0.48	0.43	0.03	NS	*	NS
C17:0 ant/iso	0.48	0.41	0.01	NS	NS	NS
C18:0	9.13	11.26	0.43	†	NS	NS
C18:1, *trans*-10/*trans*-11[3]	0.60	0.77	0.08	†	NS	NS
C18:1, *cis*-9	19.48	23.16	0.91	*	***	NS
C18:1, *cis*-11	0.49	0.56	0.04	NS	***	NS
C18:2n6	3.91	3.45	0.20	*	NS	NS
C18:3n3	0.64	0.41	0.07	NS	NS	NS
CLA - *cis*-9, *trans*-11	0.50	0.52	0.03	†	†	NS
C20:3n6	0.74	0.64	0.06	†	†	NS
C20:4n6	0.95	0.80	0.06	NS	NS	NS
SCFA[4]	9.33	9.11	0.22	NS	*	†
MCFA[5]	52.22	47.37	0.88	***	NS	NS
LCFA[6]	37.41	42.42	1.28	**	**	NS
<C16	26.76	24.92	0.71	NS	NS	NS
>C16	37.85	42.81	1.29	**	**	NS
SFA	67.95	65.12	0.75	†	NS	NS
MUFA	24.86	28.15	0.93	*	***	NS
PUFA	7.12	5.89	0.37	*	†	NS
AI[7]	2.64	2.25	0.12	*	*	NS
DI[8]	10.95	10.58	0.46	NS	NS	NS

[1] CON = control group, OC = olive cake group; [2] Probability of significant effects due to diet (D), time (T), and their interaction (D x T); *P < 0.05; **P < 0.01; ***P < 0.001; † P < 0.1: tendency, [3] Sum of C18:1 *trans*-10 and *trans*-11, [4] SCFA = Short Chain Fatty Acids; C4:0 to C10:1 *cis*-9, [5] MCFA = Medium Chain Fatty Acids; C12:0 to C16:1 *cis*-9, [6] LCFA = Long Chain Fatty Acids; C17:0 to C20:4n-6, [7] Atherogenic index = (C12:0 + 4 * C14:0 + C16:0)/ (ΣMUFA + ΣPUFA), [8] Desaturation index = (C14:1 *cis*-9 / C14:0 + C14:1 *cis*-9) * 100

2.4 DISCUSSION

The forage substitution with ensiled OC in the diets of dairy cows for a short-term period in our study had a marked effect on milk FA composition by increasing MUFA and reducing SFA, particularly MCFA. Previously studies implemented in cows (Castellani et al., 2017), ewes (Chiofalo et al., 2004; Abbeddou et al., 2011a,b, 2015; Vargas-Bello-Pérez et al., 2013; Symeou et al., 2019, 2021), and goats (Molina-Alcaide et al., 2010) reported a linear decline in SFA content with concomitant increased levels of MUFA by supplemented diets with various forms of processed OC. It is likely that MUFA of feed, escaping rumen BH, are transferred to milk FA content through mammary uptake from the plasma dietary FA, contributing to the higher MUFA content of milk (Shingfield et al., 2010). In addition, feeding unprotected oils rich in 18-C UFA increases C18:1 isomers in milk arising from ruminal metabolism and mammary desaturation of C18:0 produced in the rumen (Chilliard et al., 2007). Thus, milk OA can be elevated either through the action of mammary Δ9-desaturase with the substrate being stearic acid or by direct transfer from feed (Chilliard et al., 2007). In this regard, since the Δ9-desaturation index in the mammary gland did not differ between groups in the current study, it can be assumed that the increase of OA and MUFA is related more to the diet rather than to Δ9-desaturase activity.

Furthermore, the current study showed a reduction in MCFA when OC was fed. This may occur due to either a higher uptake of LCFA from the blood, as stated earlier, or/ and a lower *de novo* synthesis of MCFA in the mammary gland (Dorea and Armentano, 2017). *De novo* FA secretion can be inhibited by bioactive FA, including CLA isomers and *trans*-monoenes such as C18:1 *trans*-10 and *trans*-11 (Bauman et al., 2011; Dorea and Armentano, 2017), which tended to be elevated in the present study.

A tendency of increased levels of RA observed in the milk fat of cows fed the OC diet in the present study has been reported previously as a significant increase of RA in studies with cattle (Castellani et al., 2017) and contradictory results observed in sheep fed processed OC (Abbeddou et al., 2011b, 2015). RA can be either synthesized endogenously in the mammary gland through the action of mammary Δ9-desaturase with the substrate being VA or through the BH of UFA by rumen bacteria such as the *Butyrivibrio fibrisolvens* group (Chilliard et al., 2007). Thus, the difference in RA content in ruminant milk after OC feeding may be attributed to the different OC generated processes, other feed ingredients, or different supplementation periods.

Concerning other UFA, the LA concentration in milk was decreased with the supplementation of OC in the cow diets. This is in line with the study of He et al., (2012), who indicated reduced levels of LA after the inclusion of a high proportion of 18-C UFA in cow diets. In addition, similar results were observed in the milk fat of ewes after the inclusion of olive oil (Gómez-Cortés et al., 2008; Bodas et al., 2010) or processed OC (Abbeddou et al., 2011b, 2015) into their diets. However, previous studies in cows (Castellani et al., 2017) or small ruminants (Chiofalo et al., 2004; Molina-Alcaide et al., 2010; Vargas-Bello-Pérez et al., 2013) reported no effect in the concentration of LA in milk fat after OC feeding. The decreased LA levels observed in the milk fat of the OC group could be attributed to the lower LA content contained in the OC treatment compared to the CON diet or other feeding factors.

The forage substitution with ensiled OC at rates of 10 % in diet DM of lactating cows for a short-term period of 21 days did not affect milk yield as well as the fat and protein content or yield by using isoenergetic and isonitrogenous diets, similarly to previous studies feeding OC for a long-term period (Hadjipanayiotou, 1999; Meo Zilio et al., 2014; Castellani et al., 2017). It has been shown by a meta-analysis study evaluating the effect of adding oil supplements in cow diets that the impact of dietary lipid profile can be either positive or negative or without any significant difference in milk fat yield, although affecting the FA profile of milk (Dorea and Armentano, 2017).

2. 5. CONCLUSIONS

In conclusion, the use of ensiled OC in the diet of lactating dairy cows, for a short-term period of 21 days, modified the quality of milk by increasing the content of beneficial FA related to positive effects to human health. Those results show that this abundant by-product can be used as a cost-effective forage alternative in cow diets without adverse consequences in milk fat content or milk production.

CHAPTER 3: The use of ensiled olive cake in the diets of lactating cows

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A slightly modified version of this chapter has been published

Neofytou, M.C., Miltiadou, D., Sfakianaki, E., Constantinou, C., Symeou, S., Sparaggis, D., Hager-Theodorides, A.L, and Tzamaloukas, O. (2020) The use of ensiled olive cake in the diets of Friesian cows increases beneficial fatty acids in milk and Halloumi cheese and alters the expression of SREBF1 in adipose tissue. Journal of Dairy Science 103, 8998-9011

ABSTRACT

This study aimed to evaluate the effect of dietary inclusion of ensiled OC, a by-product of olive oil production, on milk yield and composition and FA profile of milk and Halloumi cheese from cows. Furthermore, the effect of OC on the expression of selected genes involved in mammary and adipose lipid metabolism was assessed in a subset of animals. Twenty - four dairy cows in mid-lactation were allocated in two iso-nitrogenous and iso-energetic feeding treatments, named the control diet (CON) and the olive cake diet (OC), in which part of the forages (alfalfa, barley hay, and barley straw) were replaced with ensiled OC as 10% of dry matter, according to a 2 × 2 crossover design with two 28-d experimental periods. At the end of the second experimental period, mammary and perirenal adipose tissue samples were collected from 3 animals per group for gene expression analysis by qRT-PCR. The expression of 11 genes involved in FA synthesis (*ACACA, FASN, G6PDH*), FA uptake or/and translocation (*VLDLR, LPL, SLC2A1, CD36, FABP3*), FA saturation (*SCD1*), and transcriptional regulation (*SREBF1, PPARγ*), was evaluated. No significant differences were observed between groups concerning milk yield, fat percentage, protein percentage and protein yield (kg/day), while milk fat yield (kg/day) increased in the OC group. Dietary supplementation with ensiled OC modified the FA profile of milk and Halloumi cheese produced. There was a significant decrease in the concentration of *de novo* synthesized FA, SFA, and the AI, while LCFA and MUFA concentration was increased in both milk and cheese. Among individual saturated FA, only stearic was elevated, while amongst individual mono-unsaturated FA, increments of OA and the sum of C18:1 *trans*-10 and *trans*-11 acids were demonstrated in milk and Halloumi cheese produced. Although no diet effect was reported on total PUFA, the concentration of RA was increased in both milk and Halloumi

cheese fat of the OC group. The expression of the genes tested was unaffected apart from an observed upregulation of *SREBF1* mRNA abundance in perirenal fat of cows fed the OC diet. Milk FA differences observed were not associated with alterations in the mammary expression of genes involved in FA synthesis, uptake, translocation, and lipogenesis regulation. Overall, the inclusion of ensiled OC in cow diets for a four-week period improved, beneficially for human health, the lipid profile of bovine milk and Halloumi cheese produced without adversely affecting milk yield and composition or the expression of genes involved in lipid metabolism of mammary and adipose tissues in cows.

3.1 INTRODUCTION

Milk FA composition has been associated with human health and disease prevention (Parodi, 2004; Palmquist, 2009; Ferlay et al., 2017). The inclusion of different UFA in the diet of lactating cows is a reasonable attempt to improve milk fat composition for human consumption (reviewed in Chilliard et al. 2000, 2003; Ferlay et al. 2017). Among all the different sources of unsaturated fats that have been used, the utilization of agro-industrial by-products could be a cost-effective way of enhancing the quality of milk and its products. Crude OC, a by-product of olive oil production, which is available in appreciable quantities in the Mediterranean basin, represents an interesting supplement for ruminants as a forage replacement that may improve the lipids of milk (Molina-Alcaide and Yáñez-Ruiz, 2008).

OC supplementation has been mainly used in sheep diets (Molina-Alcaide and Yáñez-Ruiz 2008). Feeding trials including dried (Abbeddou et al. 2011a,b, 2015), partly destoned dried (Vargas-Bello-Pérez et al., 2013), partly destoned fresh (Chiofalo et al., 2004), or ensiled (Symeou et al., 2019, 2021) OC showed decreased SFA and increased MUFA content in dairy ewes' milk. A limited number of studies examining the effect of processed olive cake on FA composition of milk and/ or cheese produced is available in the case of goats (Molina-Alcaide et al., 2010; Arco-Pérez et al., 2017) and cattle (Castellani et al., 2017). Castellani et al., (2017) showed an increase in MUFA and a decrease in SFA content as well as in atherogenic and thrombogenic indices in the milk and cheese of cows fed dried olive pomace.

During the last decade, experiments examining the effects of ruminant diets on FA composition also sought to understand the effects of dietary treatments on adipose tissue and mammary gland lipid metabolism and their contribution to changes in milk

FA; by looking more specifically at the relation between milk FA composition and expression of lipogenic genes (Ferlay et al., 2017; Fougère and Bernard, 2019). Among the complex network of genes coordinating milk fat synthesis and secretion, *ACACA*, *FASN*, and *G6PDH* are key genes encoding enzymes involved in *de novo* FA synthesis, while the *VLDLR*, in concert with *LPL*, take up and hydrolyze TAG (Bionaz and Loor, 2008a). Translocators, including *SLC2A1* and *FAT/CD36*, play a major role in FA transport, while *FABP3* provides substrates to *SCD1*, the most abundant gene in the mammary gland decreasing the saturation content of FA in ruminant milk (Bionaz and Loor, 2008a). Additionally, *SREBF1* and *PPARγ* constitute regulators of milk lipid metabolism (Osorio et al., 2016).

The inclusion of OC in ruminants' feed is quite limited because of its low nutritive value, seasonal availability, and its spoilage when stored without preservation for a long time (Hadjipanayiotou, 1999; Owaimer et al., 2004). Despite these disadvantages, OC is characterized by a high oil percentage and a high concentration of OA that could improve the milk lipid profile (Chiofalo et al., 2002). Research studies in Cyprus have proposed an ensiling method as an attempt to overcome the limitations of using fresh or dried OC, allowing the use of this by-product in animal nutrition throughout the year (Hadjipanayiotou, 1999). The effect of the supplementation of cow diets with ensiled OC for a short-term period (3-weeks) on the milk production and composition as well as on FA profile (**Chapter 2**) was examined recently, giving very promising results regarding the improvement of the quality of cow milk. So far, the effect of ensiled OC supplementation, for a longer period, on the FA content of bovine milk and Halloumi cheese has not been studied, neither the possible impact of any form of OC supplementation on bovine lipogenic gene expression.

The objective of the present study was to assess the effects of the addition of ensiled crude OC in the diet of mid-lactation Holstein-Friesian cows on (i) the FA composition of milk and Halloumi cheese and (ii) the expression of 11 genes involved in FA synthesis (*ACACA, FASN, G6PDH*), FA uptake and/ or translocation (*VLDLR, LPL, SLC2A1, CD36, FABP3*), FA saturation (*SCD1*) and transcriptional regulation (*SREBF1, PPARγ*) of lipid metabolism in the mammary and adipose tissues.

3.2 MATERIAL AND METHODS

3.2.1 Animals, Management and Experimental Diets

The experiment was performed on the ARI farm in Cyprus. All experimental procedures were carried out according to the national legislation regulations (Animal Welfare Law, 1994) and international guidelines (Directive, 2010/63/EU for animal experimentation) and approved by the corresponding departmental committee of the Cyprus University of Technology. No animals were euthanized as the experiment was adjusted to the scheduled program of slaughtering for renewing the herd of the ARI farm.

Twenty – four Holstein Friesian mid-lactating cows, in their fourth lactation were divided into 6 groups of 4 animals each, homogenous for age, milk yield (mean ± SEM: 29.31 ± 1.31 kg per head per day), and body weight (mean ± SEM: 582.0 ± 11 kg) and allocated randomly in two feeding treatments (3 groups per treatment). Animals were used in a replicated 2 × 2 Latin square to test the effects of the two treatments during two 28-days experimental periods. Cows were housed indoors and assigned to two iso-energetic and iso-nitrogenous feeding regimes (**Table 8**). The control group (CON) was fed on a conventional diet, whereas the olive cake group (OC group) received the conventional diet in which part of the forages were replaced with 5kg per cow per day of ensiled OC (fresh OC with DM content of 47.7%), obtaining a concentrate/forage ratio of 64:36 in both treatments. In detail, the diets contained 0 and 10% (DM) of ensiled OC and this specific inclusion rate was chosen based on previous studies in Cyprus (Hadjipanayiotou 1999) and unpublished pre-experimental data (Tzamaloukas' personal communication), in which is suggested that 10% (DM) of OC can be included on ruminants' diets without adverse effects on milk traits.

The OC constituted from a mixture of skins, pulp, woody endocarp, and seeds obtained after extraction of olive oil from locally cultivated olives (Ladolia and Kalamon cv.). The OC was collected by a three-stage oil mill and ensiled according to the method developed as follows: after olive oil extraction, crude OC was obtained immediately and accumulated on the surface of a clean and hard floor of the silos. The pile of OC that did not contain any additives or other feed materials to help the fermentation process was covered with a black polyethylene film (8mm thick) and was firmly closed. The film covering the pile was stretched for expelling the air, and soil was used for covering the edges of the film. The ensiled OC was fermented in silos for 3 to 4 months before use.

DMI was monitored daily during the last week of each experimental period. Animals of both groups were fed individually with automatic feeders (Westfalia, Albersdorf, Germany), offering the concentrates 4 times per day, while forages were divided and offered manually to each group after morning (0430 h) and evening (1630 h) milkings. The OC supplement was offered directly after morning milking, and cows consumed it entirely within about 15 min. Feeds were collected at the beginning and the end of each experimental period, mixed per treatment, and a final sample was taken for analysis. Water was offered ad libitum. The ingredient and chemical composition of the two treatments are presented in **Table 8**. DM, ash, crude fat, and CP were determined as described by (AOAC International, 2005). Crude fiber, ADF, ADL, and aNDF were measured according to van Soest et al., (1991).

Table 8. Ingredients and chemical composition of dietary treatments and the olive cake silage used

Item	Treatment[1]		Ensiled OC
	CON	OC	
Ingredient composition			
OC	-	9.22	
Alfalfa	7.1	7.07	
Barley hay	17.7	13.26	
Barley straw	10.9	6.36	
Concentrate mix [2]	64.3	64.3	
Chemical composition, (% DM)			
Dry matter, %	96.3	95.83	47.74
Crude protein, % DM	18.95	18.87	5.32
Crude fat, % DM	1.82	2.34	6.88
Crude fiber, % DM	17.49	18.89	49.53
Ash, % DM	7.35	6.82	2.71
aNDF, % DM	35.82	36.54	71.56
ADL, % DM	3.8	5.82	28.40
ADF, % DM	21.12	22.4	54.93
Metabolized Energy (MJ/kg)[3]	9.60	9.38	-

[1]CON = control group, OC = olive cake group; [2]Concentrate mix = 16% barley, 21% maize, 17.8% soybean meal 48%, 13% sunflower cake, 10% wheat bran, 18% sugar beet pulp, 4.24% mineral and vitamin mix; [3]Values from NRC (2001)

3.2.2 Measurements and Milk Sampling

All animals were machine milked (Fulwood, Shropshire, UK) twice daily (at 0430 h and 1630 h), and milk yields were recorded electronically (AfiMilk MPC Milk Meter, Kibbutz, Israel) at each milking, but only measurements taken the last week of each experimental period were analyzed statistically. Raw milk samples for the determination of the lipid profile were collected from each cow during the two consecutive milkings (morning and evening) at the end of each experimental period (namely week 4 and week 8) and were stored at – 80°C for further analysis. Measurements for total fat, protein, and SNF were obtained using combined thermo-optical procedures (Lactostar 3510, Funke Gerber, Berlin, Germany) calibrated previously for protein with the Lowry protein assay and fat with the Gerber method 989.05 (AOAC International, 2005).

At the end of the second experimental period (week 8), bulk milk obtained from cows of each treatment (CON and OC) was converted into seven different Halloumi cheeses respectively (14 samples in total), according to the method of Papademas and Robinson (1998). Cheese samples were collected (250–300 g) from each barrel, placed in plastic bags, and vacuum-stored at – 20°C until analysis.

3.2.3 Lipid Analysis

The milk fat isolation was carried out according to the rapid double centrifugation method of Feng et al., (2004) with slight modifications. Briefly, 20 mL aliquots of fresh milk were first centrifuged at 17800 × g for 30 min at 4°C. The resulting fat cakes were removed, placed in new tubes, and allowed to melt at room temperature for 20 min. The samples were then recentrifuged at 19500 × 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.

For the feed fat isolation, the method used was as follows: after crude fat determination (Soxtec method), 2 ml of hexane was added to the residue in the aluminum collection pots and transferred to a glass tube (pre-weighed). Then, the residue was dried down under a gentle stream of nitrogen gas, and the glass tube was reweighed.

Subsequently, 1 ml of hexane was added for re-dissolving the residue and then, an aliquot containing 20-25 mg of crude fat was removed and put into fresh tubes.

The cheese fat was extracted with diethyl ether as follows: 50 ml of diethyl ether and 0.5 g of Na_2SO_4 were added into 30-40 g of cheese samples and mixed vigorously. After 15 min, the mixture was filtered through filter paper (Whatmann No:4) from a funnel. The filtrate was centrifuged for 2 min at 6000 rpm to remove the undesired particles that originated from cheese. The liquid phase of diethyl ether and oil was taken into a centrifuge test tube, and diethyl ether was removed using a rotary evaporator at 40 ± 1°C. Then, the sample was flushed with nitrogen to remove remained ether from the oil.

FAME of milk, Halloumi cheese, and feed lipids were prepared by transesterification with methanolic potassium hydroxide according to the ISO (2002) method. Essentially, 40 µL of transesterification reagent (11.2% KOH in methanol) were added to the tubes containing extracted lipids from milk, cheese, and feeds and shaken vigorously for 1 min. The tubes were then allowed to incubate for a further 5 min at room temperature before the addition of 0.1 g of sodium bisulfate. The particulate material was then removed by centrifugation at 350 × g for 3 min at room temperature, and aliquots of the clear organic phase transferred to amber gas chromatography vials and stored at −70°C until analyzed. Fatty acid profiles were generated by analyzing the FAME samples on a GCMS-QP2010 Plus Gas Chromatography Mass Spectrometer (Shimadzu, Duisburg, Germany) equipped with an HT280T autosampler (HTA, Brescia, Italy) following the method of Kramer et al., (2008). Typically, 1 µl aliquots of FAME were separated with a split ratio of 1:20 using an Agilent CP-Sil 88 fused silica capillary column (100 m × 0.25 mm internal diameter × 0.2 µm film thickness). The column was held for 4 min at 70°C after injection, increased at 13°C/min to 175°C and then held at that temperature for a further 27 min. The temperature was then raised to 215°C at 4°C/min at which it was held for a further 36 min. Helium was the carrier gas at 1 mL/min, with both injector and interface temperatures of 225°C. Chromatographic profiles were analyzed using Shimadzu GCMS Postrun Solution software where individual peaks were identified by comparison of their retention indices to those of commercially available authentic standards (Supelco 37-FAME standard mix, CLA *cis*-9, *trans*-11, CLA *trans*-10, *cis*-12, C18:1 *trans*-11; 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). All FAME peaks identified were quantitated by peak integration and individual FAME expressed as a percentage of the total fat (Butler et al., 2011).

AI was determined in milk and Halloumi cheese using the formula proposed by Ulbricht and Southgate (1991): AI = (C12:0 + 4 x C14:0 + C16:0)/ (ΣMUFA + ΣPUFA) and the DI was determined using the formula suggested by Garnsworthy et al., (2010): DI = (C14:1 cis-9 x 100)/ (C14:0 + C14:1 cis-9).

3.2.4 Tissue Sampling for Expression Analysis

At the end of the second experimental period (week 8), three cows per treatment, randomly selected from each group, were transferred to an adjacent abattoir immediately after milking, and 50 g of perirenal and 50 g of mammary gland tissues were collected under sterile conditions (12 samples in total). All samples were snap-frozen in liquid N_2 immediately and stored at – 80°C until RNA extraction.

3.2.5 Primer Design, RNA Extraction, Synthesis of cDNA and RT- PCR

Primer design was performed using the Primer-BLAST tool at the National Centre for Biotechnology Information (**NCBI**) platform using mRNA (preferably reference where available) sequences for each target or housekeeping gene obtained from the NCBI database (**Table 9**).

Total RNA from perirenal adipose (25 mg) and mammary gland (50 mg) tissue was isolated using NucleoZOL reagent (MACHEREY-NAGEL GmbH & Co. KG) following the manufacturer's instructions. Briefly, 25 or 50 mg of tissue were homogenized in 500 μL NucleoZOL reagent. Adipose tissue samples were centrifuged at 12000 x g for 5 min, and supernatant below the fatty layer was transferred into a new tube. 200 μL RNAse free water were added, samples were vortexed and incubated at room temperature for 15 min, then centrifuged at 12000 x g for 15 min. Supernatants (500 μL) were mixed with 500 μL isopropanol incubated at room temperature for 10 min, then centrifuged at 12000xg for 10 min. Pellets were washed twice with 75% Ethanol and centrifuged at 8000xg for 3 min. Finally, pellets were air-dried and diluted in 60 μL RNAse free water, and samples were stored at −80°C for further analysis. RNA purity (260/280 and 260/230) and concentration (ng/μL) were assessed using a micro-volume UV spectrophotometer (Quawell Spectrophotometer 3000). The cDNA was synthesized from 1 μg total RNA in 20 μL reactions using the PrimeScript RT-PCR Reagent Kit (TAKARA Bio INC, Japan) according to the manufacturer's instructions, and samples were stored at – 20°C for further analysis.

The mRNA abundance of 11 candidate genes, shown in **Table 9** was assessed via RT – qPCR. To account for variation in RNA integrity, RNA quantification, and cDNA synthesis, the mRNA abundance was normalized using the geometric mean of 3 reference genes [ubiquitously expressed transcript (*UXT*), ribosomal protein S9 (*RPS9*), and ribosomal protein S15 (*RPS15*)], which have been recommended as the most suitable reference genes in the bovine mammary gland during lactation (Bionaz and Loor, 2007).

Table 9. Sequences (5′ to 3′) and efficiencies of the primers used in Real-Time quantification (q)PCR

Symbol	Name	Forward (F) and Reverse (R)	Access number	amplicon (bp)	R^2	Efficiency (%)
ACACA	Acetyl-Coa-Carboxylase A	F: ATGACGAAGACAAGGTCCCC R: ACCAGGAAAGTAAGGCGTCG	NM_174224.2	169	0.997	100
FASN	Fatty Acid Synthase	F: CACACTCCATCCTCGCTCTC R: GCCATATAGTCCGGCCTTCC	NM_001012669.1	206	0.996	86
G6PDH	Glucose 6 Phosphate Dehydrogenase	F: CCATGAGACCTGCATGAGCC R: AGATCCTGTTGGCAAACCTCAG	XM_024987752.1	200	0.996	87
SREBF1	Sterol Regulatory Element Binding Transcription Factor 1	F: ACATCTCTTGGAGCGAGCAC R: CTGAGAACTCCTTGTCCCCC	NM_001113302.1	83	0.997	95
VLDLR	Very Low Density Lipoprotein Receptor	F: AGAGGACAATGGCCGAGAGT R: GACAGCAAGAGGGGAAGGATG	NM_174489.2	114	0.999	86
LPL	Lipoprotein Lipase	F: GGAAGATCAGAGTAAAGGCAGGA R: CCAAGTTTCAGCCAGACTTTCTATT	NM_001075120.1	150	0.998	86
SLC2A1	Solute Carrier Family 2 Member1	F: GTCGTGTCGCTATTTGTGGTG R: GCCACAATGCTCAGGTAGGA	NM_174602.2	149	0.991	94
FAT/CD36	Fatty Acid Translocase	F: ACTGCGGATGGAATTTACAAAG R: ATGAGGCTGCATCTGTACCATTA	NM_001278621.1	142	1	96
FABP3	Fatty Acid Binding Protein 3	F: AGACCACAGCAGATGACAGG R: TGGGTGAGTGTCAGAATGAGTTT	NM_174313.2	142	0.997	91
PPARγ	Peroxisome Proliferator Activated Receptor γ	F: TTGTGAAGGATGCAAGGGTTTCT R: CATGCGCCCAAACCTGATG	NM_181024.2	175	0.998	94
SCD1	Stearoyl-Coa Desaturase 1	F: ACATTGATCCCCACCTGCAA R: CAAAAACGTCATTCTGGAACGC	NM_173959.4	184	0.999	93
Reference genes						
UXT	Ubiquitously Expressed Transcript	F: CTCAGCGACAACCTCACCAA R: TGTAGCTCTCTAAGCCCCTCT	NM_001037471.2	80	0.994	99
RPS9	Ribosomal Protein S9	F: AGGATTTCTTGGAGAGACGCC R: TGCTTGCGGACCCTGATG	NM_001101152.2	112	0.994	97
RPS15	Ribosomal Protein S15	F: GATGGCGGAAGTGGAACAGA R: CTGCATCAGTTGCTCATAGGAC	NM_001024541.2	109	0.991	109

RT – qPCR reactions were performed in an ABI 7500 Real-Time PCR system (Applied Biosystems, Darmstadt, Germany) using FastGene IC Green 2x Universal Mix (Bioline, London, UK). Each 10 μL reaction contained 1.0 μL of cDNA (synthesized from c.a. 12.5ng total RNA), 400 nM of forward and reverse primer, and 5.0 μL of IC Green Master Mix. The RT-qPCR analyses of each studied gene were performed using cDNA from 6 biological replicates, with 3 technical replicates per biological replicate. The qPCR thermal protocol used was as follows: 1 cycle of 95°C for 2 min, 40 cycles of 95°C for 5 sec, 60°C for 30 sec, and 95°C for 30 sec. A melt curve analysis followed this to ensure specific amplification. Real-time PCR runs with efficiencies between 86, and 110% were considered acceptable and used for data analysis.

Data were analyzed using the 7500 Fast Software option for the quantitation-relative standard curve with SYBR green reagents (Version 2.3; Applied Biosystems). Threshold cycle **(C_T)** values were obtained for each reaction using the automatic C_T option, in which the software calculates the baseline start and end values and the threshold in the amplification plot for a set of reactions. The software uses the baseline and threshold to calculate the C_T for each reaction. To perform relative quantitation of mRNA levels for each gene, a set of standards, consisting of 5-point serial dilutions (1:1, 1:3, $1:3^2$, $1:3^3$, and $1:3^4$) of cDNA prepared from a pool of cDNA from adipose and mammary tissue, were used along with the samples at every qPCR run. Results from the standards reactions (triplicates for each dilution point) were used to generate the relative standard curve for each gene under study. The corresponding standard curve was identified as the best-fit regression line of C_T (dependent variable, values obtained from the reactions as described above) on the log(Qty) [independent variable with values log(1), log(1/3), $\log(1/3^2)$, $\log(1/3^3)$ and $\log(1/3^4)$] described by the regression line formula: C_T = m [log

(Qty)] + b, where m is the slope, b is the y-intercept, and Qty is the relative mRNA level of the gene (quantity). Relative mRNA levels for each gene and biological sample were estimated as the mean Qty of three technical replicates, obtained using the regression formula mentioned above and applying the C_T values obtained from the reactions described above.

3.2.6 Statistical Analysis

All data at the animal level from the two 28-d experimental periods were subjected to an analysis of a 2 × 2 AB/BA crossover design (Kaps and Lamberson, 2009) using the SAS software (version 9.4, SAS Institute Inc., Cary, NC) to test the effects of OC feeding treatment. The statistical model included the fixed effects of period, experimental diet, and sequence (the order in which treatments were allocated to each animal). Differences between the diet means were tested based on least-squares mean difference and declared significant when the P was less than 0.05. P-values within 0.05 and 0.10 were interpreted as a trend toward significance. Normality of data was assessed both visually and through the normality test of Shapiro-Wilk using using the SPSS 20 software (StatSoft Inc., Tulsa, OK, USA).

The FA content in Halloumi cheese and feeds results were subjected to a two-group t-test with 7 and 3 replications, respectively.

The normalized relative mRNA expression data were subjected to non-parametric one-way ANOVA using the SAS Studio software (University edition version 3.8, SAS Institute Inc., Cary, NC, USA) as data were not normally distributed. For each gene, the dependent variable was the relative normalized mRNA expression level (12 biological replicates, 6 per treatment group), and the classification variable was the treatment group (two treatment groups, CON and OC groups). Differences between treatments' means were tested based on Wilcoxon scores followed by the Kruskal-Wallis test with DF = 1 and were declared significant when P < 0.05.

3.3 RESULTS

3.3.1 OC Silage and Diets

The FA composition of OC silage and diets is shown in **Table 10.** The inclusion of OC in the diets had significant effects on most FA, with OA being the major FA found at a concentration of around 30% of the dietary lipids of the OC supplemented diet. Palmitic (C16:0) and linoleic (C18:2n-6) acids were also abundant, whereas stearic (C18:0) and ALA acids were found in lesser quantities in both treatments. The OC diet showed a significantly lower content of palmitic and linoleic acids compared to the CON diet. In contrast, an increased proportion of stearic acid in the OC diet was demonstrated.

Table 10. Fatty acid composition (expressed as a percentage of total fatty acid methyl esters) of the dietary treatments used and ensiled olive cake (OC)

	Treatments[1]				
Item	CON	OC	SEM	P-value[2]	Ensiled OC
C12:0	1.05	0.68	0.09	*	-
C14:0	1.55	0.85	0.19	NS	-
C16:0	26.83	23.35	0.81	**	13.45
C16:1 *cis*-9	0.25	0.57	0.07	**	1.48
C18:0	2.04	2.58	0.12	***	4.15
C18:1 *cis*-9	21.16	33.30	2.73	***	63.58
C18:2n-6	23.52	21.39	0.51	**	13.12
C18:3n-3	2.23	1.68	0.18	NS	1.46

[1] CON = control group, OC = olive cake group, [2] Probability of significant effects = *P < 0.05; **P < 0.01; ***P < 0.001; NS: Non significant

3.3.2 Performance Variables of Cows

Table 11 shows the DMI, milk production and yield, fat and protein yield, as well as the composition of the milk samples after 4-week supplementation. OC did not influence the milk and protein yield nor the milk composition of the cows during the trial. However, the fat yield was greater ($P < 0.05$) in the OC group compared to the fat yield of the CON group, while the fat content tended to be higher in the OC group ($P = 0.087$). No differences were observed in total solids, milk production, or DMI measurements.

Table 11. Milk production and chemical composition of milk from cows fed diets with 0 or 10 %, on DM basis, the inclusion of ensiled olive cake for a 4-week period

Item	Treatments[1]		SEM	P-value[2]
	CON	OC		
DMI, (kg/d)[3]	25.58	25.56	1.55	NS
Yield, (kg/d)				
Milk	24.48	25.27	1.29	NS
Fat	0.80	0.85	0.05	*
Protein	0.86	0.89	0.04	NS
Milk composition, (%)				
Fat	3.29	3.40	0.08	†
Protein	3.52	3.53	0.02	NS
SNF	n	8.63	0.03	NS

[1] CON = control group, OC = olive cake group, [2] Probability of significant effects: * $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$; NS: Non significant; † $P < 0.1$: tendency, [3] DMI = Dry matter intake, [4] SnF = Solids non fat

3.3.3 Milk Fatty Acid Composition

The percentages of analyzed FAs and their aggregated groups determined in milk collected from CON and OC treated animals are given in **Table 12**. The inclusion of OC into the diet of lactating cows significantly increased the 18-C FA content at the expense of a decrease in de novo FA (C < 16) concentration. Total SFA and individual SFA, like C14:0 and C16:0, were decreased while C18:0 was increased by the addition of OC in the cow diets. Concentrations of the 18-C MUFA, like OA, and the sum of C18:1 *trans*-10 and *trans*-11 percentages, were significantly higher in the milk of cows that consumed OC compared to the milk of the CON group. Regarding other MUFA, the content of C14:1 *cis*-9 and C16:1 *cis*-9 acids was reduced, while the C10:1 *cis*-9 was not affected by the inclusion of OC in the cows' diets.

Although no differences in the concentrations of total PUFA were observed between treatments, it is important to mention that milk from the OC group contained a significantly higher level of RA (CLA *cis*-9, *trans*-11) compared to the CON group. However, similar FA content between treatments was observed in the CLA *trans*-10, *cis*-12, as well as in LA (C18:2n-6), ALA (C18:3n-3), and AA (C20:4n-6). The OC inclusion reduced the AI while the DI did not differ between treatments.

Overall, the differences in the concentrations of individual FA are reflected in differences between the short, medium, and long-chain FA, in which OC supplementation reduced MCFA and increased LCFA compared to the CON group, while no diet effects were observed in SCFA percentages of milk fat.

Table 12. Fatty acid composition (expressed as a percentage of total fatty acid methyl esters) of milk from cows fed a diet with 0 or 10 %, on a DM basis, the inclusion of ensiled olive cake for a 4-week period

Item	Treatments[1]		SEM	P-value[2]
	CON	OC		
C4:0	3.27	3.23	0.14	NS
C6:0	2.03	2.01	0.05	NS
C8:0	1.27	1.22	0.06	NS
C10:0	2.96	2.65	0.16	NS
C10:1 *cis*-9	0.33	0.29	0.01	NS
C12:0	3.46	3.00	0.18	NS
C13:0 iso	0.14	0.12	0.009	NS
C14:0	11.50	10.22	0.27	*
C14:1 *cis*-9	1.44	1.17	0.06	*
C15:0	0.99	0.86	0.04	†
C15:0 iso	0.25	0.27	0.03	NS
C16:0	34.77	30.88	0.50	**
C16:1 *cis*-9	1.96	1.51	0.06	**
C17:0 ant/iso	0.49	0.39	0.04	†
C17:0	0.49	0.48	0.03	NS
C18:0	8.61	11.76	0.29	***
C18:1 *cis*-9	18.32	22.17	0.41	**
C18:1 *cis*-11	0.46	0.61	0.03	*
C18:1 *trans*-10/*trans*-11[3]	0.66	0.92	0.07	*
C18:2n-6	3.55	3.56	0.32	NS
CLA *cis*-9, *trans*-11	0.59	0.65	0.006	*
CLA *trans*-10, *cis*-12	0.26	0.31	0.06	NS
C18:3n-3	0.64	0.47	0.16	NS
C20:3n-6	0,71	0.65	0.08	NS
C20:4n-6	0.92	0.85	0.08	NS
Sum				
SCFA[4]	9.68	9.41	0.21	NS
MCFA[5]	54.81	48.09	0.15	***
LCFA[6]	34.55	41.68	0.12	***
<C16:0	27.81	25.07	0.60	*
>C16:0	34.99	41.97	0.08	***
SFA	70.54	67.07	0.38	**
MUFA	23.12	26.49	0.62	**
PUFA	6.31	6.29	0.42	NS
AI [7]	2.93	2.33	0.06	***
DI [8]	11.13	10.06	0.39	NS

[1] CON = control group, OC = olive cake group, [2] Probability of significant effects = *P < 0.05; **P < 0.01; ***P < 0.001; NS: Non significant; †P < 0.1: tendency, [3] Sum of C18:1 *trans*-10 and *trans*-11, [4] SCFA= Short Chain Fatty Acids; C4:0 to C10:1 *cis*-9, [5] MCFA= Medium Chain Fatty

Acids; C12:0 to C16:1 *cis*-9, [6] LCFA= Long Chain Fatty Acids; C17:0 to C20:4n-6, [7]Atherogenic index = (C12:0 + 4 * C14:0 + C16:0) / (ΣMUFA + ΣPUFA), [8] Desaturation index = (C14:1 *cis*-9 / C14:0 + C14:1 *cis*-9) * 100

3.3.4 Fatty Acid Composition of Halloumi Cheese

The mean percentages of the different FA in Halloumi cheese and the aggregated groups of FA are presented in **Table 13**. Results showed that Halloumi FA composition was similar to milk FA composition and likewise affected by treatment. Halloumi cheese produced using OC milk contained more 18-C FA compared to the cheese of the CON group, while the percentage of total SFA was decreased ($P < 0.01$) by the addition of olive by-product since the OC group appeared to have lower values than the CON treatment.

There was no diet effect on C16:1 *cis*-9, C14:1 *cis*-9, and MCFA, whereas SCFA were decreased in Halloumi cheeses produced by the milk of the OC group. In accordance with milk FA results, MUFA of Halloumi cheese was increased by the OC inclusion. Among individual MUFA, the OC type of diet increased OA (C18:1 *cis*-9) and the sum of C18:1 *trans*-10 and *trans*-11 acids, while it decreased the content of C10:1 *cis*-9 acid.

Regarding PUFA, the average percentage of LA and RA in Halloumi cheese from cows of the OC-supplemented group was increased compared to the CON group. In line with these results, LCFA in Halloumi cheese increased with OC addition compared to the CON group ($P < 0.05$). In contrast, the OC inclusion did not modify the percentages of ALA (C18:3n-3), AA (C20:4n-6), C20:3n-6, and total PUFA. Finally, a reduced proportion in the atherogenic index was observed in the Halloumi cheese produced by the OC milk.

Table 13. Fatty acid composition (expressed as a percentage of total fatty acid methyl esters) of Halloumi cheeses made with raw milk from cows fed a diet with 0 or 10 %, on a DM basis, the inclusion of ensiled olive cake for a 4-week period

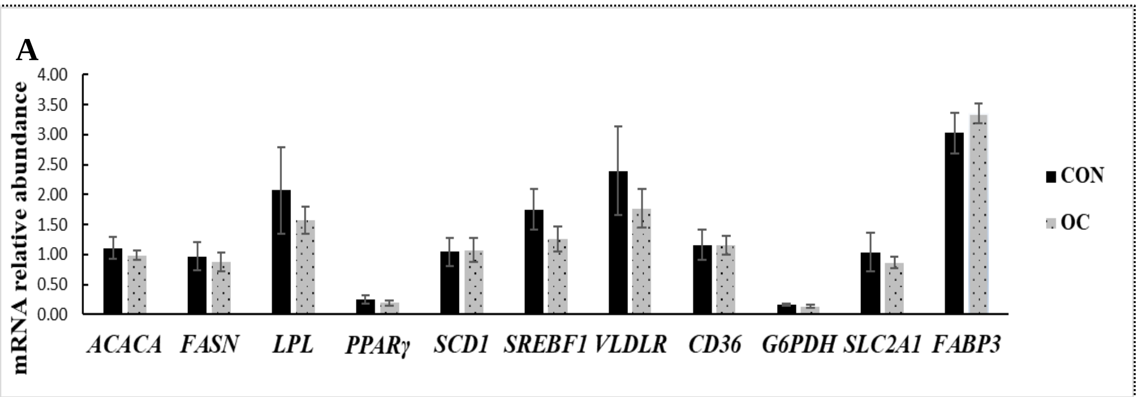
Item	Treatments[1]		SEM	P-value[2]
	CON	OC		
C4:0	3.48	2.34	0.18	**
C5:0	0.11	0.05	0.05	NS
C6:0	3.69	2.4	0.08	***
C8:0	3.03	1.32	0.06	*
C9:0	0.14	0.06	0.03	NS
C10:0	4.41	2.27	0.06	*
C10:1 *cis*-9	0.72	0.44	0.01	*
C12:0	3.70	3.71	0.03	NS
C12:1 *cis*-9	0.11	0.12	0.01	NS
C13:0	0.48	0.37	0.06	NS
C14:0	9.31	9.32	0.16	NS
C14:1 *cis*-9	1.58	1.38	0.07	NS
C15:0 iso	0.21	0.52	0.12	NS
C15:0	2.40	1.85	0.02	*
C16:0	25.13	26.49	0.19	NS
C16:1 *cis*-9	2.59	2.37	0.09	NS
C17:0	0.74	0.66	0.12	NS
C17:0 ant/iso	0.66	0.53	0.10	NS
C17:1 *cis*-9	1.28	1.13	0.11	NS
C18:0	7.17	10.18	0.02	***
C18:1 *trans*-10/*trans*-11[3]	0.46	0.91	0.02	**
C18:1 *cis*-9	18.53	21.53	0.67	***
C18:2n-6	2.65	3.26	0.21	*
CLA *cis*-9, *trans*-11	0.36	0.66	0.01	*
C18:3n-3	0.56	0.57	0.02	NS
C20:3n-6	0.17	0.17	0.02	NS
C20:4n-6	0.26	0.31	0.02	NS
Sum				
SCFA[4]	15.81	8.93	0.67	**
MCFA[5]	45.60	45.87	0.47	NS

LCFA[6]	33.15	40.06	1.56	***
SFA	64.90	61.85	0.88	*
MUFA	25.47	27.90	0.55	***
PUFA	4.10	4.88	0.20	NS
AI [7]	2.24	2.06	0.04	*

[1] CON = control group, OC = olive cake group, [2] Probability of significant effects: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS: Non significant, [3] Sum of C18:1 *trans*-10 and *trans*-11, [4] SCFA= Short Chain Fatty Acids; C4:0 to C10:1 *cis*-9, [5] MCFA = Medium Chain Fatty Acids; C12:0 to C16:1 *cis*-9, [6] LCFA = Long Chain Fatty Acids; C17:0 to C20:4n-6, [7] Atherogenic index = (C12:0 + 4 * C14:0 + C16:0) / (ΣMUFA + ΣPUFA)

3.3.5 Gene Expression

The mRNA abundance of candidate genes is presented in **Fig. 5 (A)** and **(B)** for mammary gland and adipose tissues, respectively. In adipose tissue, the mRNA expression of *SREBF1* was upregulated in cows fed with the OC supplemented diet. No significant effects were observed in the expression of any of the other genes examined in perirenal fat, neither in the transcriptional expression of any of the 11 genes analyzed in mammary tissues.



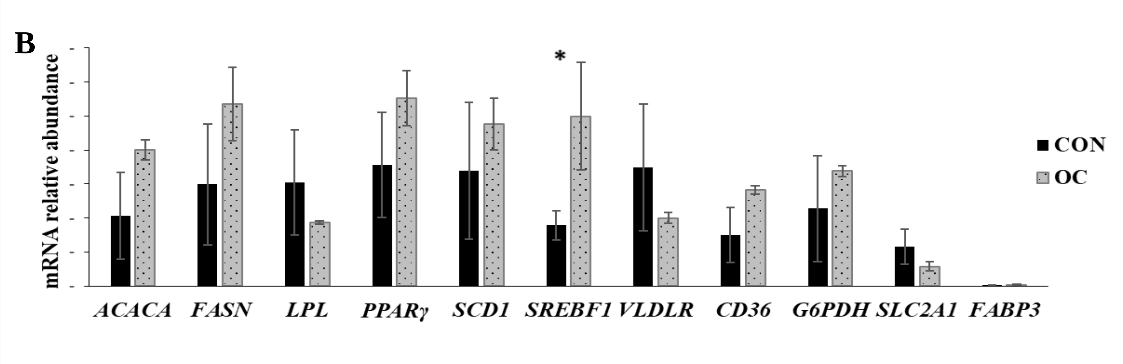


Fig. 5. mRNA relative abundance (mean ± SEM) of lipogenic genes and transcription factors of the mammary tissue **(A)** and adipose tissue **(B)** in dairy cows fed a control (CON) diet (plain bar) or the conventional diet supplemented with 10% (DM) of ensiled olive cake (OC; dotted bar).

mRNA levels are expressed as abundance relative to the geometric mean of *UXT, RPS9* and *RPS15* mRNA. Within each gene, *indicates significant differences (P < 0.05) between the experimental treatments. *ACACA* = acetyl-CoA-carboxylase A; *FASN* = Fatty acid synthase; *G6PDH* = glucose 6 phosphate dehydrogenase; *SREBF1* = sterol regulatory element binding transcription factor 1; *VLDLR* = very low density lipoprotein receptor; *LPL* = lipoprotein lipase; *SLC2A1* = solute carrier family 2 member 1; *CD36* = fatty acid translocase; *FABP3* = fatty acid binding protein; *PPARγ* = peroxisome proliferator activated receptor γ; *SCD1* = stearoyl-CoA desaturase 1.

3. 4 DISCUSSION

The current study was designed to investigate whether the partly replacing forages in cow diets with 10% OC silage (on DM) for a 8-week period, based on our previous expertise (**Chapter 2**), would give promising results in terms of improving milk fat quality, as well. Indeed, supplementing the diets of lactating cows with OC did not significantly affect milk fat content similar to our previous (**Chapter 2**) and other studies, while, in contrast, an increase in fat yield was demonstrated. In cattle, OC feeding up to 15% on a DM basis has not significantly affected milk fat yield (Hadjipanayiotou, 1999; Meo Zilio et al., 2014). However, in accordance with our study, Castellani et al., (2017) found a tendency of increased fat percentage. Although results for fat yield were not reported in that study, higher levels of fat content could have increased fat yield due to the similar milk production reported. In addition, a meta-analysis study evaluating the impact of adding free fat or oil supplements in cow diets showed that the effect of dietary lipid profile could be either positive or negative or without any significant difference in milk fat yield, although affecting the FA profile of milk (Dorea and Armentano, 2017).

The inclusion of OC in the diets of lactating cows in the present study did not affect milk yield, as expected by using isoenergetic and isonitrogenous diets and reported previously (Hadjipanayiotou, 1999; Meo Zilio et al., 2014; Castellani et al., 2017; **Chapter 2**). Furthermore, there was no significant effect on the protein content or yield similar to our previous expertise (**Chapter 2**) and others (Hadjipanayiotou, 1999; Meo Zilio et al., 2014). Although Castellani et al., (2017) observed that milk protein content was influenced by dietary dried olive pomace integration into cow diets, the authors attributed the unexpected results to the difference in the forage to concentrate ratio of the diets.

The addition of ensiled OC in the diets of dairy cows, in our study, altered the milk FA composition by affecting individual FAs with positive effects on human health. Levels of LCFA and particular FA with 18 carbon atoms such as OA and RA were increased, while total SFA, and particularly C14:0 and C16:0, and consequently the AI were decreased significantly.

The proportion of FA with less than 16 carbons ($C < 16$) was significantly reduced by replacing part of the forages with ensiled OC. Those results are in line with our previous work examining a similar inclusion rate of ensiled OC in cow diets (**Chapter 2**) and with other studies which reported reduced *de novo* FA content, particularly decreased concentration of MCFA and increased levels of LCFA by addition of a high level of unsaturated 18-C FA in cow diets (reviewed in Dorea and Armentano, 2017), as is the case of rich in oleic acid diets in the present and previous studies (Castellani et al. 2017; He et al. 2012; Weld and Armentano, 2018; **Chapter 2**). This could be attributed to either a higher uptake of long-chain FA from the blood and/ or a lower de novo synthesis of FA in the mammary gland (Chilliard et al., 2000; Dorea and Armentano, 2017). FA including *trans* monoenes and CLAs like CLA *trans*-10, *cis*-12 (Bauman et al., 2006, 2011), which was not significantly different between the two examined groups in the present study, could inhibit the *de novo* FA synthesis. The decreased MCFA and C < 16 FA, indicative of decreased *de novo* FA synthesis, could be possibly attributed to isomerization of oleic acid into *trans*-monoenes such as C18:1 *trans*-10 and *trans*-11 (Dorea and Armentano, 2017) found significantly elevated in the milk of cows fed with ensiled OC, in our study. C18:1 *trans*-10 is strongly associated with MFD in cows (Gaynor et al., 1994; Griinari et al., 1998; Shingfield et al., 2009). Although we did not observe MFD, the concentration of FA with less than 16 carbons is a better indicator of inhibition of milk FA secretion compared to milk fat content due to the possible masking effect of the exogenous supply of 18-C dietary FA (Dorea and Armentano, 2017). Therefore, both a higher uptake of dietary LCFA and decreased *de novo* synthesis of MCFA in the mammary glands of cows fed with ensiled OC, without affecting milk fat percentage, are possible mechanisms.

Among SFA, a significant increase of stearic acid (C18:0) was observed in milk by OC diet inclusion, which is consistent with our (**Chapter 2**) and previous studies indicating a tendency and significantly increased levels of C18:0 (Castellani et al., 2017; He et al., 2012), respectively, in milk due to high levels of UFA 18-C FA in dairy cattle diets. Stearic acid is the final product of BH of dietary 18-C MUFA and PUFA, resulting from microbial BH saturation of double bonds in LCFA (Ferlay et al., 2017; Shingfield et al., 2010), like OA, which was abundant in the OC diet.

The reduction of SFA in the present study was accompanied by increased total MUFA content in the OC supplemented group. Similar effects and patterns have been found by researchers who tested diets supplemented with various forms of processed OC in ewes (Abbeddou et al. 2011a,b, 2015; Chiofalo et al., 2004; Vargas-Bello-Pérez et al., 2013; Symeou et al., 2019, 2021;), goats (Molina-Alcaide et al., 2010) and cows (Castellani et al., 2017). Moreover, the short-term inclusion of 10% (DM) of ensiled OC (**Chapter 2)** or the addition of olive oil (Vargas-Bello-Pérez et al., 2018) in cattle led to similar findings. It is likely that MUFA of feed, escaping BH, were transferred to milk FA content through mammary uptake from the plasma dietary FA, contributing to the higher MUFA content of milk (Shingfield et al., 2010). In addition, feeding unprotected oils rich in 18-C PUFA increased both C18:1 and C18:2 *cis* and *trans* isomers arising from ruminal metabolism and mammary desaturation of C18:0 and C18:1 *trans* (mainly 7 and 11) produced in the rumen (Chilliard et al., 2000). Indeed, milk OA can be generated either through the action of mammary Δ9-desaturase with the substrate being C18:0 or by direct transfer from feed (Collomb et al., 2006; Chilliard et al., 2007). However, since the Δ9-desaturation index in the mammary gland did not differ between groups in the current study, it can be assumed that the increase in OA is probably related more to the diet rather than to Δ9-desaturase activity.

In the present study, regarding CLA isomers, only RA and CLA *trans*-10, *cis*-12 isomers were detectable, from which the former was significantly increased in the milk fat of cows fed the OC diet, while the latter did not differ between treatments. A tendency of increased RA content has been reported in **Chapter 2** with the OC inclusion in cow diets, while previous studies in cattle observed significantly higher contents for both CLA isomers by the addition of dried olive pomace (Castellani et al., 2017) and olive oil (Vargas-Bello-Pérez et al., 2018). In line with our study, He et al., (2012) reported increased levels only for RA by adding high oleic rich diets in cows compared to the control group. It is well known that the majority of RA in the milk fat is synthesized endogenously, in the mammary gland through the action of mammary Δ9-desaturase with the substrate being VA, while a small amount of this specific CLA isomer originates from BH of UFA by rumen bacteria (Collomb et al., 2006; Buccioni et al., 2012). Thus, the elevated proportions of RA observed in the milk of the OC group could be either to the higher concentrations of VA in the mammary gland compared to the CON group or due to the incomplete BH taking place in the rumen of cows fed with the OC diet (Bauman et al., 2006).

Interestingly, the effects of OC treatment in the FA composition of Halloumi cheese were similar to those in the milk. This suggests that the improvement in nutritional quality achieved in milk because of OC supplementation is thereafter largely maintained in Halloumi cheese. Similar to the FA profile of milk, reduced levels of SFA and significantly increased levels of LCFA and MUFA were observed in Halloumi cheese when OC was supplemented in the cow diet, and consequently, the AI was diminished. Both milk and Halloumi cheese samples of the OC group had increased content of 18-C FA, including OA, the sum of C18:1 *trans*-10 and *trans*-11 and RA. Moreover, total PUFA and LA were not affected in milk nor cheese. However, in contrast to the milk FA content, linoleic acid percentages were found elevated in cheese obtained from the OC group. Those results agree with Castellani et al. (2017), who reported that proportions of total MUFA, OA, and RA increased and total SFA decreased in cheese, while the percentage of total PUFA was not modified by the inclusion of dried olive pomace in the cow diets. Additionally, studies of Vargas-Bello-Perez reported decreased SFA and AI, as well as increased total MUFA, C18:1 *cis*-9, and/or RA concentrations of cheese fat, when 10% and 25% of dried OC (2013) or olive oil (2018) were included in the diets of ewes and cows, respectively. Furthermore, these authors observed an increase in total PUFA of cheese lipids enriched in oleic acid diets. It has been reported that heating, the fermentation culture used, and the ripening time required for the type of cheese produced could modulate cheese FA composition (Collomb et al., 2006; Domagała et al., 2010; Santillo et al., 2016).

This is the first report studying the effects of OC silage on the lipid metabolism of dairy cows, and to our knowledge, no other studies are examining the potential effects of any form of olive cake on mammary or adipose gene expression in lactating cows. The current feeding trial used cows in mid-lactation, and therefore, tissue samples were obtained after the period of negative energy balance when extensive lipolysis, upregulation of lipolytic genes, and remarked downregulation of lipogenic genes take place (Sumner-Thomson et al., 2011).

During mid-lactation and according to our findings, the transcription level of *SREBF1* in adipose tissue was significantly increased in response to the increased level of C18:0 and OA in the OC diet. However, no effect in the *SREBF1* mRNA abundance of adipose tissue was reported either in nutrigenomic studies conducted in lactating cows after UFA supplementation (Thering et al., 2009; Vahmani et al., 2014) or in a recent in vitro study examining the effect of dose and type of LCFA on adipogenesis of bovine adipocytes (Yanting et al., 2018). In the study of Harvatine et al., (2009), intravenous infusion of CLA *trans*-10, *cis*-12, a well-established inhibitor of de novo milk FA synthesis, upregulated *SREBF1* in adipose tissue of dairy cows. This particular CLA isomer increases the expression of adipogenic *SREBF1* and lipid synthesis enzymes (reviewed by Bauman et al. 2011), suggesting energy partitioning towards adipose tissue fat stores (Harvatine et al., 2009). In the present study, however, milk fat content was not affected. OC increased the sum of C18:1 *trans*-10 and *trans*-11, the first of which has also been associated with reduced *de novo* synthesized FA in dairy cows (Dorea and Armentano, 2017). Likely, increased levels of C18:1 *trans*-10 or other monoenes arising from OA isomerization may have affected the expression of *SREBF1* in adipose tissue of lactating cows, but further research is needed to clarify the particular role of other CLA and *trans* monoenes on lipid metabolism when OA is fed (Dorea and Armentano, 2017).

No significant differences were observed between treatments regarding the expression of the remaining genes tested in the adipose tissue. *SREBF1* is a TF that is a master regulator of lipid synthesis (Osorio et al., 2016). This TF remains as an inactive precursor on the endoplasmic reticulum membrane and is transported to the Golgi for proteolytic cleavage prior to entering the nucleus, where it activates the expression of sterol response element (*SRE*)-containing genes (*ACACA*, *FASN*, and *SCD1*) (Bauman et al., 2011; Osorio et al., 2016). Although *SREBF1* was upregulated, we did not observe increased mRNA expression of the genes involved in FA synthesis and desaturation. Similarly, Graugnard et al. (2009) indicated that different starch levels did not affect the mRNA expression of *ACACA*, *FASN* and *SCD1* in adipose tissue of steers, despite risen mRNA expression of *SREBF1*, which could be possibly attributed to post-transcriptional regulation of *SREBF1*. In some studies testing plant oils in bovine (Thering et al., 2009; Vahmani et al., 2014) and in caprine (Bernard et al., 2009, 2012) diets, no significant effects on any of the genes expressed in the adipose tissue were reported. The differences in results regarding *SREBF1* expression could be related to OC inclusion levels or other factors that need to be further investigated. For instance, differences in plasma insulin concentrations are known to affect adipose lipogenesis (Vernon, 2005). Overall, the results of this study suggest that the adipose tissue lipogenic pathway does not play an essential role in the response of mammary lipid secretion to the OC supplement used.

In the present study, the decreased proportion of the *de novo* C < 16 FA in the cow milk of the OC group was not accompanied by changes in mammary lipogenic gene expression. Feeding cows with ensiled OC was not followed by reduced milk fat percentage, nor by changes in the expression of any of the genes involved in mammary lipid synthesis, despite the decreased proportion of the *de novo* FA in milk. Additionally, the secretion of LCFA was increased in the OC group, but this was not accompanied by variations in mammary *LPL* mRNA abundance or mRNA abundance of genes involved in the uptake, transport, and trafficking of FA in the cells (*CD36, FABP3, VLDLR, SLC2A1*). Our results concur with the studies of Bernard et al., (2005) and (2009), who observed increased secretion of 18-C FA in the milk of goats fed with plant oils, without effect on the *LPL* mRNA abundance, and with the studies of Bernard et al., (2017) and Fougère and Bernard, (2019) who reported no diet effect on the bovine and caprine mammary levels of expression of *LPL, SLC2A1, CD36,* and *FABP3* by lipid supplementation. It is likely that mammary lipid synthesis, FA uptake, and transport are regulated at the post-transcriptional or post-translational level for these genes or that short-term regulation of mRNA synthesis by nutrient supply may have taken place (Bernard et al., 2017; Fougère and Bernard, 2019).

3. 5 CONCLUSIONS

This study examined the inclusion of 10% (DM) of OC silage in the diets of lactating Holstein cows to determine whether it affects milk, corresponding Halloumi FA composition, and examine the expression of key mammary and adipose lipogenic genes. By supplementing cow diets with OC, we observed in both milk and Halloumi cheese increased levels of individual FA, which are related to positive effects on human health (i.e., RA and OA), whereas SFA were decreased. No variation in the mammary and adipose mRNA abundance due to the diets was observed, apart from *SREBF1,* which was higher in the adipose tissue of the OC group without affecting the expression of the genes *SREBF1* regulates. The results of the present study are useful for exploiting an abundant, cost-effective by-product of the olive oil industry to improve the FA profile of cow milk and derived Halloumi cheese without adverse consequences in milk fat content or milk production.

CHAPTER 4: The use of ensiled olive cake in the diets of lactating goats

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A slightly modified version of this chapter has been submitted for publication

Neofytou M. C, Hager-Theodorides A.L, Sfakianaki E., Constantinou C., Symeou S., Sparaggis D., Miltiadou D., and Tzamaloukas O. Diet inclusion of ensiled olive cake increases unsaturated lipids in milk and alters the expression of lipogenic genes in mammary and adipose tissue in goats. Journal of Dairy Science (submitted).

ABSTRACT

The present study aimed to evaluate the effect of dietary inclusion of ensiled OC as a forage substitute in dairy goats. To examine this oil rich by-product, we investigated the effects of its use on milk yield, composition, and FA profile, as well as on the expression of selected genes involved in lipid metabolism in the udder and adipose tissue of goats. Seventy-two dairy Damascus goats in mid-lactation were assigned randomly to three iso-nitrogenous and iso-energetic diets (3 pens of 8 animals each, or 24 animals, per diet treatment) for 42 days. The diets contained 0, 10, and 20% of ensiled OC on diet DM for the OC0, OC10, and OC20 treatment, respectively, as a replacement of forages, while concentrate participation in diets remained at 60 percent (DM) in all treatments. During the wk 5 and 6 of the trial, dry matter intake, milk yield, milk composition, and FA profile were recorded and analyzed using a complete randomized design with repeated measurements. At the end of the trial, mammary and perirenal adipose tissue samples were collected from 6 animals per treatment from OC0 and OC20 groups for gene expression analysis by qRT-PCR to test the effect of the highest inclusion rate on mammary and adipose tissues. The expression of 10 genes involved in FA synthesis (*ACACA*, *FASN*, *G6PDH*), FA uptake and/or translocation (*VLDLR*, *LPL*, *SLC2A1*, *CD36*, *FABP3*), FA desaturation (*SCD1*), and transcriptional regulation (*PPARγ*), was evaluated. No significant differences were observed between treatment groups concerning milk yield, 4% fat-corrected milk, fat or protein yield (kg/d). In contrast, milk fat percentage was gradually increased with increasing OC inclusion rates in the diets, while milk protein percentages were elevated in both OC groups but significantly only in the milk of OC20 group. The content of all major SFA between C4:0 to C16:0 was reduced, while LCFA and MUFA concentration was enhanced in the goat milk of OC groups. Among individual MUFA, increments of OA, C18:1 *trans*-10, and VA were

demonstrated in both OC groups compared to the OC0 group. No significant effect was reported on total PUFA levels, while the concentration of RA was increased by 11 and 21% ($P < 0.001$) with OC10 and OC20 diets, respectively, compared to the OC0 group. OC feeding treatment was associated with increased mammary expression of *SLC2A1* ($P < 0.05$), *VLDLR* ($P < 0.01$), *FABP3* ($P < 0.01$) and elevated *SLC2A1* ($P < 0.05$) and *FASN* ($P < 0.01$) gene expression in adipose tissue of goats fed the OC20 diet. Elevated LCFA and reduced *de novo* FA content in milk due to OC supplementation were accompanied by upregulation of genes involved in FA uptake and translocation in the mammary gland and increased the mRNA expression of genes involved in FA glucose transport and FA synthesis in the adipose tissue of goats. Overall, we suggest using OC silage at rates of 20% of diet DM in goats as a forage replacement since this could increase the milk protein and fat percentage and enrich its content with beneficial for human health lipids without adversely affecting milk production traits.

4.1 INTRODUCTION

The use of rich in UFA feeds in lactating animals is a feeding strategy for improving milk fat composition for human consumption (Chilliard et al., 2000, 2003; Ferlay et al., 2017). Crude OC, a by-product of olive oil extraction containing a mixture of skins, pulp, woody endocarp, and seeds, is available in appreciable quantities in the Mediterranean areas and has been tested as a ruminant feed in different forms (fresh, dried, destoned, pelleted or ensiled, reviewed by Tzamaloukas et al., (2021). Upon ensiling, OC represents an alternative feedstuff that can be used as a forage substitute, which, due to rich oleic acid content, can also improve the FA profile of milk (Symeou et al., 2019, 2021; **Chapter 2 and 3**) and Halloumi cheese produced (Symeou et al., 2021; **Chapter 3**). The use of OC in ruminant nutrition has been mainly tested in sheep diets using different types of processed OC, like dried (Abbeddou et al. 2011a,b, 2015), partly destoned fresh (Chiofalo et al., 2004), or ensiled OC (Symeou et al., 2019, 2021). In all these cases, OC significantly affected milk lipid content by decreasing SFA, increasing MUFA, and the overall unsaturation index of milk and dairy products. Additionally, studies in cows reported enhanced MUFA and CLA content of cow milk and related cheeses using dried (Castellani et al., 2017) or ensiled OC (**Chapter 3**).

Up today, there are three studies in goats that incorporated different forms of OC by-product in their experimental diets, investigating its effect on milk FA composition (Molina-Alcaide et al., 2010; Arco-Pérez et al., 2017; Marcos et al., 2020). However, in these studies, OC was examined in combination with other dietary additives, compared to control diets, and thus, their results showed contradictory effects on goat milk FA composition that cannot be attributed to the OC inclusion alone. For instance, the study of Acro-Perez et al., (2017) included ensiled OC along with olive leaves, adding up to

20% of the diet DM, and this experimental diet also supplemented with 2% (on DM) sunflower oil, which was not included in the control diet. As a result, the milk FA profile of this diet had increased SFA, decreased MUFA, unaffected PUFA but increased RA content, compared to control, which contradicts the results of OC inclusions in sheep and cows. Moreover, Molina et al., (2010) included crude OC in experimental feeds along with a row of other diet ingredients not included in the control diet, such as different quantities of fava beans, barley grains, beet molasses, and sunflower meal. They found decreased SFA and increased RA content in the goat milk of the tested feeds compared to control, while OA, total MUFA, and PUFA contents were unaffected. Similarly, Marcos et al. (2020) investigated an experimental diet that, apart from the OC (3.5%), included also 8% DDGS and 8% of dry citrus pulp (DM basis), reporting an overall increase in milk PUFA, including linoleic and CLA content, and reduced SFA, compared to control, but these effects cannot be attributed solely to OC since DDGS inclusion also affects FA profile (Anderson et al. 2006; **Chapter 5**). Thus, it remains unclear which is the effect of OC alone on goat milk quality and production and whether ensiled OC can be useful as a forage substitute in goat diets.

The underlying mechanisms of OC feeding on milk FA in ruminants have only been studied in relation to rumen microbiota alteration, fermentation, and rumen-derived FA (Mannelli et al., 2018; García-Rodríguez et al., 2020), trying to elucidate the overall effect on ovine milk composition. The investigation of the impact of dietary OC supplementation on the expression of genes involved in fat metabolism (in the mammary or other tissues) has attracted limited interest. Previous studies using different diets or supplements, rich in plant or marine lipids on FA composition examined how lipid metabolism was affected in the mammary and adipose tissues by these dietary treatments (reviewed by Shingfield et al., 2010, 2013; Bauman et al., 2011; Bernard et al., 2018). More specifically, these studies examined the effects of diet alteration on the expression of genes involved in major lipogenic pathways (*de novo* synthesis, FA uptake and transport, and FA desaturation) and their regulatory elements (e.g., transcription factors). According to the results of **Chapter 3**, we found limited effects on the expression of selected genes involved in the mammary and adipose tissue lipid metabolism in cows when partly replacing forages in cow diets with OC silage at 10% of diet DM. Species specificities of lipid metabolism have been well established (Chilliard et al., 2003; Shingfield et al., 2010; Fougère and Bernard, 2019), and so far, no studies have examined the effect of any form of OC supplementation on caprine lipogenic gene expression.

The objective of the present study was to examine the effects of the inclusion of ensiled OC, as a forage replacement, in the diet of mid-lactation Damascus goats. We tested the effects of the addition of three different levels (0, 10, and 20 % on diet DM) of ensiled OC on milk production traits and milk FA content. Furthermore, to elucidate the molecular mechanisms involved in the observed effects on milk FA composition, we performed differential expression analysis of 10 genes involved in FA synthesis (*ACACA*, *FASN*, *G6PDH*), FA uptake, and/ or translocation (*VLDLR*, *LPL*, *SLC2A1*, *CD36*, *FABP3*), FA saturation (*SCD1*) and transcriptional regulation (*PPARγ*) of lipid metabolism in the mammary and adipose tissues of goats.

4.2 MATERIALS AND METHODS

4.2.1 Animals, Management, and Experimental Diets

The experiment was conducted in the ARI farm (Athalassa, Cyprus) 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 of the Cyprus University of Technology. No animals were euthanized for the purposes of this study.

Seventy-two Damascus mid-lactating goats (98 ± 5 days in milk) were distributed randomly in 9 pens of 8 animals with average (± SEM) values of milk yield (2.81 ± 0.13 kg per head per day) and body weight (59.05 ± 1.28 kg) and assigned randomly into 3 iso-energetic and iso-nitrogenous feeding regimes (3 pens or 24 animals per treatment). Feeding treatments contained 0, 10, or 20% on DM of ensiled OC replacing forages (OC0, OC10, and OC20 treatments, respectively), with constant concentrate participation at 60% DM in all treatments **(Table 14)**. The inclusion rates of OC were chosen to be included in goats' diets based on previous studies in Cyprus (Hadjipanayiotou 1999) and unpublished pre-experimental data (Tzamaloukas' personal communication) in which suggested that up to 20% (DM) of OC can be included on ruminants' diets without adverse effects on milk traits.

The OC was prepared according to the method described previously in **Chapters 2 and 3**. Briefly, OC constituted from a mixture of skins, pulp, woody endocarp, and seeds obtained after extraction of olive oil from locally cultivated olives (Ladolia and Kalamon cv.). The OC was collected by a three-stage oil mill and ensiled according to the method developed as follows: after olive oil extraction, crude OC was obtained immediately and accumulated on the surface of a clean and hard floor of the silos. The pile of OC that did not contain any additives or other feed materials to help the fermentation process was covered with a black polyethylene film (8mm thick) and was firmly closed. The film covering the pile was stretched for expelling the air, and soil was used for covering the edges of the film. The ensiled OC was fermented in silos for 3 to 4 months before use.

All goats were fed the control diet for a 3-week adaptation period, and then the experimental diets for 6 more weeks. In each pen, the animals were group fed the diets to 1.1 times their maintenance energy and milk production requirements (NRC, 2007). All feed ingredients, apart from OC, were offered manually as a total mixed ration, two times per day after morning (0430 h) and evening (1630 h) milking. The OC supplement was offered directly only after morning milking before feeding and was consumed within 10 to 20 min of allocation. DMI and feed samples for chemical analysis were collected at weeks 5 and 6 of the trial. Water was offered ad libitum. The chemical composition of the three treatments is presented in **Table 14**. DM, ash, crude fat, and CP were determined as described by (AOAC International, 2005). Crude fiber, ADF, ADL, and aNDF were measured according to van Soest et al., (1991).

Table 14. Ingredients and chemical composition of diets contained 0 (control, OC0), 10 (OC10), and 20 (OC20) g of ensiled olive cake per 100g DM and olive cake silage used

	Treatment			
Item	OC0	OC10	OC20	Ensiled OC
Ingredient composition, %				
OC	-	9.7	19.8	
Alfalfa	11.1	10.9	10.3	
Barley hay	17.4	12.8	8.0	
Barley straw	10.8	6.2	1.7	
Concentrate mix [1]	60.7	60.5	60.2	
Chemical composition, (% DM)				
Dry matter, %	88.60	82.96	78.98	47.74
Crude protein, % DM	15.86	15.89	15.92	5.32
Crude fat, % DM	2.30	3.07	3.82	6.88
Crude fiber, % DM	18.20	18.75	19.30	49.53
Ash, % DM	7.13	6.90	6.67	2.71
Ca	1.00	0.99	0.98	-
P	0.39	0.39	0.38	-
Na	0.24	0.23	0.23	-
aNDF, % DM	37.44	37.71	38.01	71.56
ADF, % DM	22.89	23.06	23.25	54.93
Metabolized Energy (MJ/kg) [2]	10.65	10.51	10.37	-

[1]Concentrate mix = 23% barley, 21% maize, 12% soybean meal 48%, 13% sunflower cake, 10% wheat bran, 18% sugar beet pulp, 3% mineral and vitamin mix, [2]Values from NRC (2007)

4.2.2 Measurements, Sampling, and Analysis

Goats were machine milked (Fulwood, Shropshire, UK) twice daily (at 0430 h and 1630 h), and milk yields were recorded electronically (AfiMilk model Afifree 155, SAE Afikim Kibbutz, Israel). Raw milk samples for the determination of the milk composition were collected at the end of weeks 5 and 6 from each goat during two consecutive milkings (morning and evening). Measurements for total fat, protein, lactose and solids non-fat (SNF) of milk were determined using a Lactostar-milk analyzer (model 3510, Funke Gerber, Germany) previously calibrated (AOAC International, 2005).

Lipid extraction from milk was performed according to the rapid double centrifugation method described in **Chapters 2 and 3**. Briefly, 20 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 new tubes, and allowed to melt at room temperature for 20 min. The samples were then recentrifuged at 19.500g for 20 min at room temperature, and 2 mg aliquots of the resulting lipid cake were removed to fresh tubes and dispersed in 1 mL of n-hexane by shaking.

Crude fat of feeds was extracted using the Soxtec method (AOAC International, 2005), followed by the method described in **Chapters 2 and 3**. Briefly, 2 ml of hexane was added to the residue in the aluminum collection pots and transferred to a glass tube (pre-weighed). Then, the residue was dried down under a gentle stream of nitrogen gas, and the glass tube was reweighed. Subsequently, 1 ml of hexane was added for re-dissolving the residue and then, an aliquot containing 20-25 mg of crude fat was removed and put into new tubes.

FAME of milk and feed lipids were prepared according to the ISO, (2002) method and performed by using a GCMS-QP2010 Plus Gas Chromatography-Mass Spectrometer (Shimadzu, Duisburg, Germany) equipped with a 100 m x 0.25 mm x 0.2 μm column (Agilent CP-Sil 88 fused silica capillary column) with a 1:20 split ratio. The column was held for 4 min at 70°C after injection, increased at 13°C/min to 175°C, and then held at that temperature for a further 27 min. The temperature was then raised to 215°C at 4°C/min, at which it was held for a further 36 min. Helium was the carrier gas at 1 mL/min, with both injector and interface temperatures of 225°C. Chromatographic profiles were analyzed using Shimadzu GCMS Postrun Solution software. Individual peaks were identified by comparing their retention indices and mass spectra to those of commercially available standards and mass spectral libraries (NIST) quantitated by peak integration and expressed as a percentage of the total fat.

Milk AI was determined using the formula proposed by Ulbricht and Southgate, (1991): AI= (C12:0 + 4 x C14:0 + C16:0)/ (ΣMUFA + ΣPUFA) and the desaturation index (DI) was determined using the formula suggested by (Garnsworthy et al., 2010): DI = (C14:1 *cis*-9 x 100)/ (C14:0 + C14:1 *cis*-9). Fat-corrected milk yield at 4% of fat content (FCM 4%) was estimated according to Mavrogenis and Papachristoforou, (1988) for Damascus goats: FCM 4% = milk yield x (0.411 + 0.0147 x fat %).

4.2.3 Tissue Sampling for Expression Analysis

At the end of the experiment (week 6), mammary and adipose tissue biopsies were obtained from 6 animals of the control (OC0) and 6 animals of the OC20 group under anesthesia, according to the method described previously by Miltiadou et al., (2017). Briefly, perirenal adipose tissue was sampled by a veterinarian following lateral laparotomy and puncture using ultrasonography for the appropriate biopsy site selection avoiding large vessels and other organs. Udder biopsies were taken from either the left or the right gland. The biopsy site was carefully selected to prevent large subcutaneous blood vessels. The site's preparation involved shaving and washing with dilute betadine solution followed by sanitizing with ethanol (70%). Goats were given intravenous xylazine before anesthetizing the biopsy site by subcutaneous injection of lidocaine hydrochloride. An incision was made (~0.5–1.0 cm) using a scalpel blade (size 22). A Bard Magnum core biopsy instrument (Bard Peripheral Vascular Inc., Tempe, AZ) with the accompanying biopsy needle (MN1210, 12 gauge × 10 cm) was used. Mammary and perirenal adipose tissues were dissected immediately, snap-frozen in liquid nitrogen, and stored at −80 ◦C.

4.2.4 Primer Design, RNA Extraction, Synthesis of cDNA and RT-PCR

Primer design (**Table 15**) was performed using the Primer-BLAST tool at the NCBI platform using mRNA (preferably reference where available) sequences for each target or housekeeping gene. Primers were designed to avoid genomic amplification, either cross exon-exon boundaries, or each primer of a pair was designed at a different exon.

Total RNA from perirenal adipose (25 mg) and mammary gland (50 mg) tissue was isolated using NucleoZOL reagent (MACHEREY-NAGEL GmbH & Co. KG) following the manufacturer's instructions. Briefly, 25 or 50 mg of tissue were homogenized in 500 μL NucleoZOL reagent. Adipose tissue samples were centrifuged at 12000 x g for 5 min, and supernatant below the fatty layer was transferred into a new tube. 200 μL RNAse free water were added, samples were vortexed and incubated at room temperature for 15 min, then centrifuged at 12000 x g for 15 min. Supernatants were mixed with an equal volume of isopropanol, incubated at room temperature for 10 min, then centrifuged at 12000 x g for 10 min. Pellets were washed twice with 75% Ethanol and centrifuged at 8000 x g for 3 min. Finally, pellets were air-dried and diluted in 60 μL RNAse free water, and samples were stored at −80°C for further analysis. RNA purity (260/280 and 260/230) and concentration (ng/μL) were assessed using a micro-volume UV spectrophotometer (Quawell Spectrophotometer 3000). The cDNA was synthesized from 1 μg total RNA in 20 μL reactions using the PrimeScript RT-PCR Reagent Kit

(TAKARA Bio INC, Japan) according to the manufacturer's instructions, and samples were stored at − 20°C for further analysis.

The mRNA abundance of 10 candidate genes, shown in **Table 15**, was assessed via RT - qPCR. To account for variation in starting material and mRNA extraction and cDNA synthesis efficiency between samples, the mRNA abundance was normalized using the geometric mean of 3 reference genes (*UXT*, *RPS9* and *RPS15*; Bionaz and Loor, 2007).

RT - qPCR reactions were performed in an ABI 7500 Real-Time PCR system (Applied Biosystems, ThermoFisher Scientific, Waltham, Massachusetts, USA) by using FastGene IC Green 2x Universal Mix (Bioline, London, UK). Each 10 μL reaction contained 1.0 μL of cDNA (synthesized from c.a. 12.5 ng total RNA), 400 nM of forward and reverse primer and 5.0 μL of IC Green Master Mix. The RT-qPCR analyses of each studied gene were performed using cDNA from 6 biological replicates, with 3 technical replicates per biological replicate. The qPCR thermal protocol used was as follows: 1 cycle of 95°C for 2 min, 40 cycles of 95°C for 5 sec, 60°C for 30 sec, and 95°C for 30 sec. A melt curve analysis followed this to test the specificity of the amplification. Real-time PCR runs with efficiencies between 86 and 110% were considered acceptable and used for data analysis.

Data were analyzed using the 7500 Fast Software option for the quantitation-relative standard curve with SYBR green reagents (Version 2.3; Applied Biosystems). C_T values were obtained for each reaction using the automatic C_T option, in which the software calculates the baseline start and end values and the threshold in the amplification plot for a set of reactions. The software uses the baseline and threshold to calculate the C_T for each reaction. To perform relative quantitation of mRNA levels for each gene, a set of standards, consisting of 5-point serial dilutions (1:1, 1:3, 1:3^2, 1:3^3 and 1:3^4) of cDNA prepared from a pool of cDNA from adipose and mammary tissue, were used along with the samples at every qPCR run. Results from the standards reactions (triplicates for each dilution point) were used to generate the relative standard curve for each gene under study. The corresponding standard curve was identified as the best-fit regression line of C_T (dependent variable, values obtained from the reactions as described above) on the log(Qty) [independent variable with values log(1), log(1/3), log(1/3^2), log(1/3^3) and log(1/3^4)] described by the regression line formula: $C_T = m [\log (Qty)] + b$, where m is the slope, b is the y-intercept, and Qty is the relative mRNA level of the gene (quantity). Relative mRNA levels for each gene and biological sample were estimated as the mean Qty of three technical replicates, obtained using the regression formula mentioned above and applying the C_T values obtained from the reactions described above. Normalized expression was calculated as the ratio of gene mean Qty/geometric mean of reference genes mean Qty.

Table 15. Sequences (5′ to 3′) and efficiencies of the primers used in Real-Time quantification (q)PCR

Symbol	Name	Forward (F) and Reverse (R)	Access number	amplicon (bp)	R^2	Efficiency (%)
ACACA	Acetyl-Coa-Carboxylase A	F: ATCATCACCATCAGCCTGGTTA R: AGGTGTATACTTCCCTCCCGA	XM_018064168.1	154	0.992	93
FASN	Fatty Acid Synthase	F: CTCCATCCTCGCTCTCCTTC R: CATATAGTCCCGCCTTCCACC	NM_001285629.1	200	0.997	87
G6PDH	Glucose 6 Phosphate Dehydrogenase	F: CTTCCATCAGGCCGATACGC R: GGGTAGCTTTGAAGAAGGGCTC	XM_018044339.1	200	0.997	94
VLDLR	Very Low Density Lipoprotein Receptor	F: CTGCTGTGGAAATGCGATGG R: TCTCATATGGCACTGTTCTGGG	XM_018052033.1	192	0.993	89
LPL	Lipoprotein Lipase	F: CAACAAGGTCAGAGCCAAAAGA R: ACTTCAGGCAGGGTAAAAGGG	NM_001285607.1	198	0.998	88
SLC2A1	Solute Carrier Family 2 Member1	F: GTCGTGTCGCTGTTTGTGG R: GCCTGGACCCACTTCGAAAA	NM_001314223.1	189	0.991	90
FAT/CD36	Fatty Acid Translocase	F: ATTGACACATACAAAGGCAGAAAGAAT R: AGCTCCGAACACAGCATAGAT	XM_018046617.1	176	0.998	99
FABP3	Fatty Acid Binding Protein	F: CGAGTTCGATGAGACCACGG R: CATGGGTGAGTGTCAGAATGAGT	NM_001285701.1	155	0.993	99
PPARγ	Peroxisome Proliferator Activated Receptor γ	F: AAGCGTCAGGGTTCCACTATG R: CCGAACCTGATGGCGTTATGA	NM_001285658.1	199	0.997	96
SCD1	Stearoyl-Coa Desaturase	F: ACATTGATCCCCACCTGCAA R: TCAAAAACGTCATTCTGGAACGC	NM_001285619.1	185	0.998	94
Housekeeping genes						
UXT	Ubiquitously Expressed Transcript	F: CGTAAGAGCAATCTCCTCACAGA R: TGTAGCTCTCTAAGCCCCTCTA	XM_005700842.2	104	0.997	96
RPS9	Ribosomal Protein S9	F: AAGCTGATCGGCGAGTACG R: TTCATCTTGCCCTCGTCCAG	XM_018063497.1	191	0.998	98
RPS15	Ribosomal Protein S15	F: GCATTGAGACCCCGCGATAA R: TTCTACTTCCGCCATCTTGCC	XM_018050438.1	171	0.990	88

4.2.5 Statistical Analysis

DMI, milk yield, milk content, and milk FA composition data collected at week 5 and week 6 were subjected to a repeated measures analysis for a Completely Randomised Design, using SAS PROC MIXED (SAS version 9.4. SAS Institute Inc., Cary USA). The model included the fixed effects of time (T), experimental diet (D) and their interaction (T×D), and the random effect of goats and pen. Statistical significance declared at $P < 0.05$. P-values between > 0.05 and ≤ 0.10 were interpreted as trending towards significance. Data referring to FA composition of feeds were analyzed using one-way ANOVA with 3 replications, respectively. Normality of data was assessed both visually and through the normality test of Shapiro-Wilk using using the SPSS 20 software (StatSoft Inc., Tulsa, OK, USA).

The normalized relative mRNA expression data were subjected to non-parametric one-way ANOVA using the SAS (SAS version 9.4. SAS Institute Inc., Cary USA) as data were not normally distributed. For each gene, the dependent variable was the normalized mRNA expression level (12 biological replicates, 6 per treatment group), and the classification variable was the treatment group (two treatment groups, OC0 and OC20 groups). Differences between treatments' means were tested based on Wilcoxon scores followed by the Kruskal-Wallis test with DF = 1 and were declared significant when $P < 0.05$.

4.3 RESULTS

4.3.1 OC Silage and Diets

Table 16 shows the FA composition of dietary treatments and OC silage used. The inclusion of ensiled OC in the diets of goats had significant effects on all FA with oleic acid (C18:1 *cis*-9) being the major FA found at a concentration of 35 and 37% in OC10 and OC20 diets, respectively. Palmitic (C16:0) and LA (C18:2n-6) acids were also abundant, whereas stearic (C18:0) and ALA (C18:3n-3) acids were found in lesser quantities in all treatments. Both OC diets contained a significantly lower content of palmitic and linoleic acids compared to the OC0 diet. In contrast, stearic acid increased as OC proportion elevated in the goat diets ($P < 0.001$).

Table 16. Fatty acid composition (expressed as a percentage of total fatty acid methyl esters) of the dietary treatments containing 0 (control, OC0), 10 (OC10) or 20 (OC20) g of ensiled olive cake per 100 g DM, and olive cake silage (OC)

Item	Treatments			SEM	P-value[1]	Ensiled OC
	OC0	OC10	OC20			
C14:0	1.19^a	0.67^b	0.41^c	0.14	**	-
C16:0	37.29^a	30.18^b	29.61^b	0.79	**	13.45
C16:1 *cis*-9	0.35^c	0.62^b	0.97^a	0.05	**	1.48
C18:0	1.78^c	2.46^b	3.54^a	0.10	***	4.15
C18:1 *cis*-9	21.00^b	34.82^a	37.00^a	2.70	***	63.58
C18:2n-6	25.52^a	19.97^b	18.30^c	0.50	**	13.12
C18:3n-3	2.42^a	2.38^b	2.12^b	0.15	*	1.46

$^{a\text{-}b}$ Means within a row not sharing a common superscript differ, [1]Probability of significant effects; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS = Non significance

4.3.2 *Performance Variables of goats*

Table 17 shows the DMI, 4% fat-corrected milk, the daily yield of milk, fat, and protein, as well as the milk fat and protein percentages. OC substitution in the goat diets influenced neither intake, milk and protein yield nor the fat corrected milk. Similarly, the lactose and SNF percentages did not differ between treatments. However, the milk fat content increased significantly as OC rates rose in the goat diets, and milk fat yield tended to be elevated in the OC groups ($P = 0.06$). Additionally, milk protein percentage was increased in the OC20 group compared to OC0 and OC10 treatments ($P < 0.001$).

Table 17. Dry matter intake, milk production, and chemical composition of milk from goats fed diets containing 0 (control, OC0), 10 (OC10), or 20 (OC20) g of ensiled olive cake per 100 g DM

	Treatment				P-value[1]		
Item	OC0	OC10	OC20	SEM	D	T	D x T
DMI, kg/d	2.53	2.57	2.63	0.03	NS	NS	NS
Yield, kg/d	2.72	2.79	2.83	0.10	NS	NS	NS
Milk							
FCM[2]	2.35	2.43	2.50	0.07	NS	**	NS
Fat	0.082	0.085	0.091	0.004	†	*	NS
Protein	0.106	0.111	0.114	0.004	NS	†	NS
Milk composition, %							
Fat	3.08^c	3.20^b	3.30^a	0.06	*	***	NS
Protein	3.68^b	3.74^b	3.85^a	0.02	***	***	NS
Lactose	5.33	5.61	5.48	0.10	NS	NS	NS
SNF	9.56	10.07	10.05	0.08	NS	NS	NS

$^{a-c}$ Means within a row not sharing a common superscript differ due to the different diet examined ($P < 0.05$), [1]Probability of significant effects due to diet (D), time (T), and their interaction (D x T); *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS: Non significant; † $P < 0.1$: tendency, [2]4% FCM: milk yield x (0.411+0.147 x fat),

4.3.3 Milk Fatty Acid Composition

The composition of all individual FA and their aggregated groups determined in the milk of OC0, OC10, and OC20 groups, is shown in **Table 18**. The content of FA with more than 16 carbon atoms (> 16) was increased by 7 and 11% ($P < 0.001$) in the milk of goats fed with OC10 and OC20 diets, respectively, with concomitant decreased *de novo* FA (< C16) secretion ($P < 0.01$). The content of total SFA and individual SFA like C4:0, C6:0, C14:0, C16:0, and C17:0 was decreased by the addition of OC in the goat diets. No diet effect was observed in the percentage of C18:0 between treatments. In contrast, concentrations of the 18-C MUFA, like OA, as well as C18:1 *trans*-10, VA, C18:1 *trans*-12, C18:1 *cis*-11, and C18:1 *cis*-13 were significantly higher in the milk of goats that consumed OC compared to the milk of the OC0 group ($P < 0.001$). Regarding other MUFA, the content of C10:1 *cis*-9 was reduced, and the percentages of C16:1 *cis*-9 and

C16:1 *cis*-7 were increased while other monoenes were not affected in the milk of OC groups.

There were no significant differences in the concentration of total PUFA between treatments, whereas levels of RA were enhanced by 11 and 21% with OC10 and OC20 diets, respectively, compared to the OC0 group. Similar content of CLA *trans*-10, *cis*-12, and other PUFA, such as LA and ALA, was observed between treatments. Among the 20-C PUFA, the content of C20:5n-3 and C20:5n-6 were reduced by the OC inclusion in goat diets, while in contrast, no diet effect on the concentrations of other 20-C acids was observed. Milk AI diminished by 11% and 19% in OC10 and OC20 treatments, respectively, compared to the control diet, while the DI was not affected by the type of diet offered.

The differences observed in the concentrations of individual FA were reflected in changes in the SCFA, MCFA and LCFA. In particular, LCFA were significantly increased, whereas MCFA were reduced by the inclusion of 10 and 20% (DM) of OC in goat diets ($P < 0.001$), while no statistically significant diet effect in the content of SCFA among treatments was demonstrated.

Table 18. Fatty acid composition (expressed as a percentage of total fatty acid methyl esters) of milk from goats fed diets contained 0 (control, OC0), 10 (OC10), or 20 (OC20) g of ensiled olive cake per 100g DM

	Treatment				P-value[1]		
Item	OC0	OC10	OC20	SEM	D	T	D x T
C4:0	0.80^{a}	0.72^{b}	0.75^{ab}	0.01	*	**	†
C5:0	0.12	0.15	0.13	0.001	NS	NS	NS
C6:0	1.53^{a}	1.37^{b}	1.45^{ab}	0.04	**	NS	**
C7:0	0.36^{b}	0.45^{a}	0.38^{ab}	0.003	NS	NS	NS
C8:0	2.32^{a}	2.13^{b}	2.15^{b}	0.05	**	*	**
octanoic	0.039^{a}	0.037^{a}	0.028^{b}	0.002	***	NS	NS
C9:0	0.097	0.117	0.096	0.007	†	NS	NS
C10:0	5.79^{b}	6.69^{a}	5.37^{b}	0.28	**	**	*
C10:1 *cis*-9	0.21^{a}	0.16^{b}	0.17^{b}	0.008	***	***	NS
C11:0	0.15^{ab}	0.19^{a}	0.14^{b}	0.01	*	NS	†
C12:0	4.09	4.06	3.76	0.12	†	**	*
C12:1 *cis*-9	0.038	0.032	0.031	0.003	NS	***	NS
C13:0 iso	0.02	0.02	0.01	0.002	NS	NS	NS
C13:0	0.19	0.21	0.18	0.01	NS	**	NS
C14:0	9.00^{a}	8.47^{b}	8.05^{b}	0.15	***	***	***
C14:0 *iso*	0.07^{a}	0.051^{b}	0.05^{b}	0.003	***	NS	*
C14:1 *cis*-9	0.44	0.15	0.15	0.12	NS	NS	NS
C15:0	1.24^{a}	1.23^{a}	1.13^{b}	0.03	*	**	*
C15:0 *iso*	0.18^{a}	0.14^{b}	0.13^{b}	0.005	***	**	***
C15:0 antiso	0.43^{a}	0.36^{b}	0.34^{b}	0.011	***	***	***
C16:0	25.39^{a}	22.89^{b}	22.37^{b}	0.38	***	†	NS
C16:0 *iso*	0.31^{a}	0.26^{b}	0.27^{b}	0.008	***	**	***
C16:1 *cis*-9	0.13^{b}	0.15^{b}	0.18^{a}	0.008	***	NS	NS
C16:1 *cis*-7	0.28^{b}	0.27^{b}	0.31^{a}	0.009	**	**	NS
C17:0	1.07^{a}	1.01^{b}	0.94^{c}	0.02	***	***	***
C17:0 *iso*	0.54^{a}	0.46^{b}	0.41^{b}	0.01	***	**	***
C17:0 antiso	1.43	1.38	1.37	0.02	NS	NS	**
C17:1 *cis*-9	0.34	0.33	0.32	0.01	NS	***	NS
C18:0	10.40	10.12	10.35	0.46	NS	†	NS
C18:0 *iso*	0.094	0.093	0.090	0.004	NS	***	**
C18:1 *trans*-10	0.31^{b}	0.37^{ab}	0.39^{a}	0.01	***	***	NS
C18:1 *trans*-11	4.75^{b}	5.70^{a}	6.12^{a}	0.26	***	NS	†
C18:1 *trans*-12	0.33^{c}	0.41^{b}	0.68^{a}	0.05	***	†	NS
C18:1 *cis*-9	18.48^{c}	21.12^{b}	23.05^{a}	0.49	***	NS	NS
C18:1 *cis*-11	0.71^{b}	0.86^{a}	0.91^{a}	0.02	***	†	NS
C18:1 *cis*-12	0.30	0.30	0.31	0.01	NS	NS	***
C18:1 *cis*-13	0.17^{b}	0.21^{a}	0.23^{a}	0.01	***	***	NS
C18:1 *cis*-16	0.26	0.29	0.28	0.02	NS	†	NS
C18:1 *trans*-16	0.27	0.25	0.24	0.01	NS	NS	***
C18:2 *cis*-9, *trans*-13/*trans*-8, *cis*-12	0.21	0.21	0.21	0.006	NS	**	NS
C18:2 *trans-9*, *cis*-13/*trans*-8, *cis*-12	0.13	0.14	0.15	0.005	†	**	NS
C18:2 *trans*-11, *cis*-15	0.11	0.13	0.13	0.008	NS	NS	NS
C18:2n-6	4.58	4.59	4.69	0.18	NS	†	NS
C18:3n-6	0.04	0.04	0.04	0.002	NS	**	NS

C18:3n-3	0.35	0.36	0.38	0.01	NS	†	NS
C19:1 cis-9	0.043	0.043	0.042	0.003	NS	***	NS
C20:0	0.25^{b}	0.25^{b}	0.27^{a}	0.006	**	***	NS
C21:0	0.066^{a}	0.055^{b}	0.061^{ab}	0.002	***	**	**
C22:0	0.074	0.068	0.069	0.002	NS	***	*
C23:0	0.031	0.022	0.020	0.002	***	NS	NS
C24:0	0.014^{ab}	0.015^{a}	0.012^{b}	0.001	**	NS	NS
CLA *cis*-9, *trans*-11	0.41^{c}	0.46^{b}	0.52^{a}	0.01	***	†	*
CLA *trans*-10, *cis*-12	0.062	0.068	0.073	0.005	NS	NS	NS
CLA *trans, trans*	0.11	0.15	0.15	0.01	†	†	NS
C20:2n-6	0.028	0.032	0.025	0.004	NS	NS	NS
C20:3n-6	0.04	0.03	0.03	0.01	NS	†	NS
C20:3n-3	0.02	0.02	0.02	0.001	NS	***	NS
C20:4n-6	0.26	0.24	0.24	0.009	NS	***	NS
C20:5n-3	0.025^{a}	0.018^{b}	0.016^{b}	0.001	***	NS	NS
C22:4n-6	0.019	0.016	0.016	0.001	NS	***	**
C22:5n-6	0.043^{a}	0.032^{b}	0.033^{b}	0.001	***	***	†
C22:5n-3	0.055^{a}	0.046^{ab}	0.042^{b}	0.002	**	***	NS
SCFA[2]	10.93	11.09	10.22	0.30	NS	†	*
MCFA[3]	41.49^{a}	38.30^{b}	36.99^{b}	0.52	***	***	**
LCFA[4]	46.33^{c}	49.92^{b}	52.39^{a}	0.69	***	NS	NS
<C16	26.94^{a}	26.62^{a}	24.73^{b}	0.46	**	NS	**
>C16	46.55^{b}	50.19^{a}	52.40^{a}	0.69	***	NS	ND
SFA	65.51^{a}	62.46^{b}	59.68^{c}	0.69	***	NS	NS
MUFA	29.92^{b}	30.88^{ab}	32.98^{a}	0.64	**	***	***
PUFA	6.44	6.43	6.74	0.22	NS	NS	NS
AI [5]	1.16^{a}	1.03^{b}	0.92^{b}	0.03	***	†	NS
DI [6]	1.76	1.72	1.85	0.07	NS	***	NS

$^{a-c}$ Means within a row not sharing a common superscript differ due to the different diet examined ($P < 0.05$), [1]Probability of significant effects due to diet (D), time (T), and their interaction (D x T); *P < 0.05; **P < 0.01; ***P < 0.001; † P < 0.1: tendency; NS: Non significant, [2]SCFA = short-chain fatty acids (C4:0 to C8:0), [3]MCFA = medium-chain fatty acids (C10:0 to C16:1), [4]LCFA = long-chain fatty acids (C17:0 and above), [5]Atherogenic index = (C12:0+ 4 x C14:0 + C16:0) / (ΣMUFA + ΣPUFA), [6]Desaturation index = (C14:1 *cis*-9/C14:0 + C14:1 *cis*-9) x 100

4.3.4 Gene Expression

The mRNA abundance of candidate genes is presented in **Fig. 6 (A)** and **(B)** for mammary gland and adipose tissues, respectively. In mammary tissue, the mRNA abundance of *VLDLR* ($P < 0.01$) and *SLC2A1* ($P < 0.05$) was increased, while the expression of *FABP3* tended to be elevated ($P = 0.05$) in goats fed with the OC20 diet.

No diet effects were demonstrated in the expression of any of the other genes examined in mammary tissues. In the perirenal adipose tissue, the mRNA expression of *FASN* and *SLC2A1* was upregulated in goats fed 20% (DM) of ensiled OC, while no significant effects were observed in the expression of any of the other genes examined in perirenal fat.

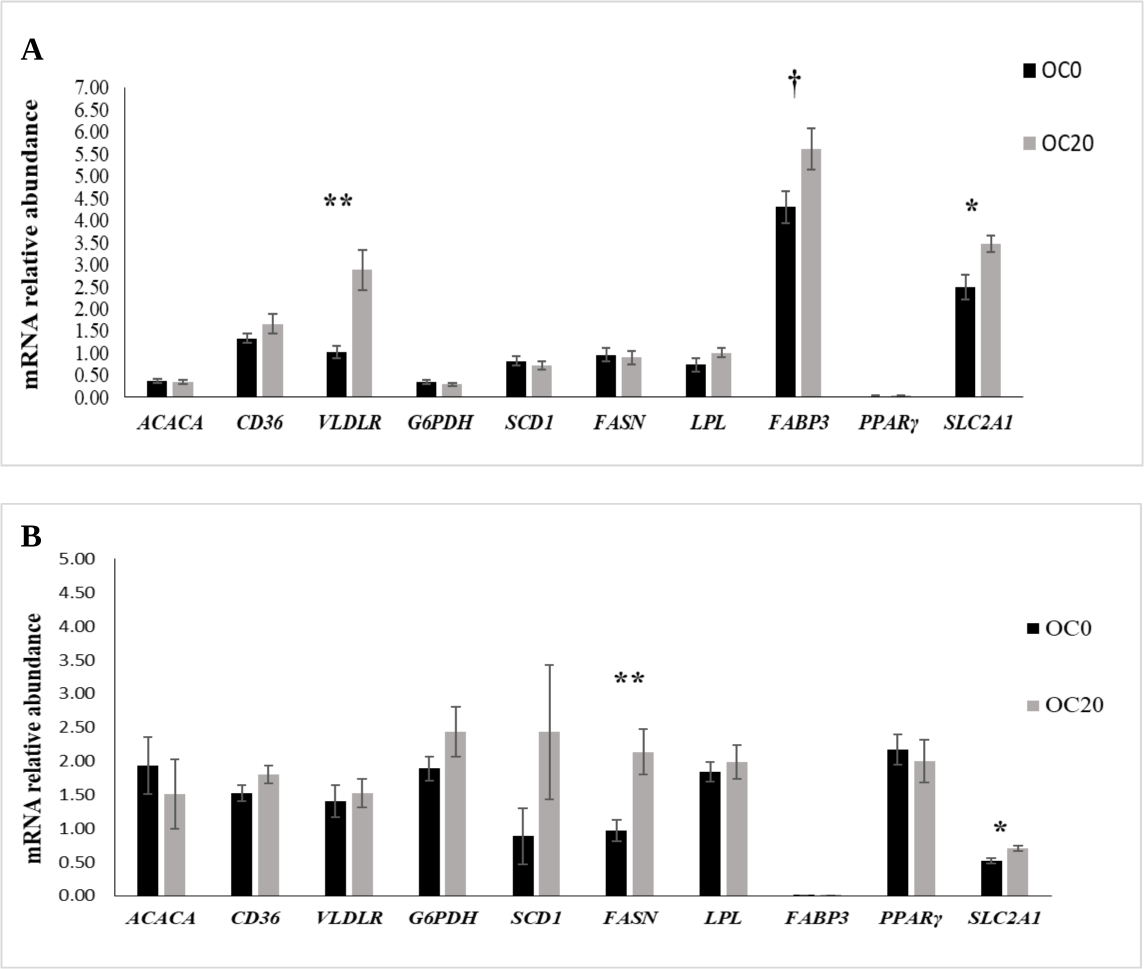

Fig. 6. mRNA relative abundance (means ± SE) of lipogenic genes and transcription factors of the mammary (A) and adipose (B) tissue in dairy goats fed a control (OC0) diet (black bar) or the conventional diet supplemented with 20% (DM) of ensiled olive cake (OC20; grey bar).

mRNA levels are expressed as abundance relative to the geometric mean of *UXT*, *RPS9* and *RPS15* mRNA. *P < 0.05; **P < 0.01; † P < 0.1: tendency. *ACACA* = acetyl-CoA-carboxylase A; *FASN* = Fatty acid synthase; *G6PDH* = glucose 6 phosphate dehydrogenase; VLDLR = very low density lipoprotein receptor; *LPL* = lipoprotein lipase; *SLC2A1* = solute carrier family 2 member 1; *CD36* = fatty acid translocase; *FABP3* = fatty acid binding protein 3; *PPARγ* = peroxisome proliferator activated receptor γ; *SCD1* = stearoyl-CoA desaturase 1.

4.4 DISCUSSION

The present study showed that milk yield was not affected by the 10 or 20 % (DM) inclusion rates of ensiled OC, and this result concurs with previous studies testing isonitrogenous and isoenergetic diets containing either 17% (DM) of ensiled OC (Hadjipanayiotou, 1999) or 20% DM of ensiled by-products of both OC and olive leaves (Acro-Perez et al., 2017) in dairy goats. Similarly, studies conducted on dairy ewes showed that milk yield was not affected by the inclusion of different forms of processed OC up to 20% diet DM (Shdaifat et al., 2013; Mannelli et al., 2018; Symeou et al., 2019, 2021) or, in cattle, when feeding ensiled or dried OC at rates up to 15% DM (Hadjipanayiotou, 1999; Meo Zilio et al., 2014; Castellani et al., 2017; Chiofalo et al., 2020; **Chapter 2 and 3**).

The substitution of forages with 10 or 20 % DM of ensiled OC in the goat diets significantly increased the milk fat content in the present study similarly to Hadjipanayiotou, (1999) and Arco-Pérez et al., (2017), while milk fat yield also tended to be elevated in the OC groups. Consistently with these findings in goats, a tendency of increased fat percentage and significantly elevated milk fat yield was reported in cows (Castellani et al., 2017; **Chapter 3**) when animals were fed diets containing 10% of dried or ensiled OC, respectively. Furthermore, significantly elevated milk fat percentage has been reported in dairy ewes by Hadjipanayiotou, (1999), who tested ensiled OC at 15 % DM inclusion rate but not in recent studies with inclusion rates up to 20 % DM (Symeou et al., 2019, 2021). However, no significant effect on the fat content and yield of cows with OC feeding was reported in **Chapter 2**, probably due to differences in the experimental duration between studies.

Milk protein percentage was numerically increased in both OC treatments but significantly only at 20% inclusion of ensiled OC in the diet DM compared to control. Increased milk protein content was observed in bovine milk following 10% (DM) of dried OC supplementation (Castellani et al., 2017) but not with 10 % (DM) of ensiled OC inclusion in cows (**Chapter 2 and 3**). It is well stated that increasing the proportion of concentrates in the ruminant diets increases milk protein content and yield, and this effect is more likely attributed to the lower energy content of high forage diets (Jenkins and McGuire, 2006). Although the forage was replaced by OC silage in the present study, the concentrate was kept at 60% for all diets, and the treatments were isoenergetic and isoproteic. Nevertheless, the *in vitro* study of Pallara et al., (2014) examining the effect of OC on rumen microbial communities showed increased rumen volatile FA production, suggesting that the OC inclusion could enhance rumen microbial activity and consequently microbial protein supply to the small intestine and content in milk, but further research is needed to establish such mechanism.

The ensiled OC inclusion positively affected the quality milk FA composition by reducing SFA and the atherogenicity index and increasing UFA in both OC10 and OC20 groups. The content of SFA with less than 16 carbons ($C < 16$), mainly MCFA, linearly decreased with increasing OC proportion in the goat diets. Those results are in line with previous studies in small ruminants (Chiofalo et al., 2004; Molina-Alcaide et al., 2010; Abbeddou et al., 2011a, b, 2015; Symeou et al., 2019, 2021; Marcos et al., 2020) and cows (Castellani et al., 2017; **Chapters 2 and 3**) reporting reduced *de novo* FA percentages and increased levels of LCFA by feeding different diets containing various forms of processed OC. In contrast, Arco-Pérez et al., (2017) reported increased SFA and decreased UFA content in milk when a mix of olive oil by-products substituted 20% (DM) of goat diet, namely ensiled OC and olive leaves, and this discrepancy could be attributed to different by-product added. Nevertheless, it is known that the milk FA of chain length C4 to C14 and approximately part of C16 are synthesized *de novo* in the mammary gland, whereas the remainder of C16 and LCFA present in milk are derived from the diet or body reserves (Chilliard et al., 2007). Therefore, the reduction of C < 16 FA observed in the present study could be due to either a higher secretion of LCFA from the blood and/or a lower *de novo* synthesis of FA in the mammary gland (Chilliard et al., 2007; Dorea and Armentano, 2017). The d*e novo* FA secretion in ruminant milk can be inhibited by FA, including trans monoenes and CLAs, with the most well-documented FA being the CLA *trans*-10, *cis*-12 in cows (Bauman et al., 2011). Since this FA did not differ between treatments in our study, other FA intermediates produced during incomplete ruminal BH of dietary LCFA, such as C18:1 *trans*-10 suggested previously to have a similar role (Bauman et al., 2011), found elevated in the milk of goats fed OC in our study. As indicated by Dorea and Armentano, (2017), this reduction of *de novo* synthesized lipids could be occurred without affecting the milk fat percentage, which was increased in OC groups in our study. Thus, summarising the above data from milk fat percentage and the FA profile, either a higher level of dietary LCFA from the blood and/or a decreased *de novo* synthesis of MCFA by this specific *trans* isomer, may be suggested as the underlying mechanisms resulting to the altered milk FA composition observed in both OC treatments. However, the latter mechanism is not supported by our results from the gene expression where none of the tested genes involved in lipid metabolism were downregulated with the inclusion of 20% OC in the diet DM.

In the present study, the content of total MUFA was higher in the milk of OC supplemented groups compared to control. Similarly to our results, previous studies in small ruminants (Chiofalo et al., 2004; Molina-Alcaide et al., 2010; Abbeddou et al., 2011b; a, 2015; Vargas-Bello-Pérez et al., 2013; Symeou et al., 2019, 2021; Marcos et al., 2020), and cows (Castellani et al., 2017; Chiofalo et al., 2020; **Chapters 2 and 3**) reported a linear decline in SFA content with concomitant increased levels of MUFA by supplemented diets with various forms of processed OC. It can be assumed that MUFA of feed, escaping rumen BH, were transferred to milk FA content through mammary uptake from the plasma, contributing to the higher MUFA content of milk (Shingfield et al., 2010). Furthermore, the inclusion of lipids rich in 18-C UFA in dairy animal nutrition could increase both C18:1 and C18:2 *cis* and *trans* isomers arising from ruminal metabolism and mammary desaturation of C18:0 and C18:1 *trans* (mainly 7 and 11) produced in the rumen (Chilliard, et al. 2000). Indeed, the linearly increased concentration of oleic acid found in milk from rising amounts of OC diet inclusion could be due to either the action of mammary Δ9-desaturase with the substrate being stearic acid or its direct transfer from feed (Chilliard et al., 2007). However, considering the results of the Δ^9-desaturation index that did not differ between groups and the unaffected expression of the *SCD1* gene in the current study, it can be assumed that the increase of oleic acid and MUFA is related more to the diet rather than to Δ9-desaturase activity.

The significantly increased levels of RA observed in the milk fat of goats fed the 10 or 20% (DM) ensiled OC diet in the present study has been reported previously in studies with goats (Molina-Alcaide et al., 2010; Arco-Pérez et al., 2017; Marcos et al., 2020) and cattle included OC at 10 % DM (Castellani et al., 2017; **Chapter 3**), while a tendency of increased RA content indicated in the milk of cows fed ensiled OC for a short- term period of 21 days (**Chapter 2**). Contradictory results observed in sheep provided different forms of processed OC (Abbeddou et al., 2011b, 2015). It is known that RA can originate from two pathways; it can be either synthesized endogenously in the mammary gland through the action of mammary Δ^9-desaturase with the substrate being vaccenic acid, which is responsible for about 60% of RA secretion in milk, or through the BH of UFA by rumen bacteria (Chilliard et al., 2007).

In order to investigate further the molecular mechanisms contributing to the observed effects due to ensiled OC supplementation, the expression of selected genes involved in lipid metabolism on mammary and adipose tissues of goats fed the OC20 and the control OC0 group was examined. Thus, the results showed that the decreased MCFA content in the OC milk was not accompanied by changes in the expression of any of the selected genes involved in mammary lipid synthesis. These results are in line with studies reporting no effect on the caprine and/ or bovine (Bernard et al., 2005, 2009, 2012, 2017; Toral et al., 2013; Fougère and Bernard, 2019), or ovine (Bichi et al., 2013a; Castro-Carrera et al., 2015) mammary levels of expression of *ACACA*, *FASN*, and *SCD1* by dietary lipid supplementation. Similarly, the results of our previous work with OC feeding in cows (**Chapter 3**) showed that decreased proportion of MCFA in OC milk could occur with no changes in the expression of genes involved in mammary lipid synthesis.

In the present study, the increased LCFA content in the milk of the OC20 group coincided with significantly elevated expression of *VLDLR* and *SLC2A1* in the mammary gland of goats. *SLC2A1* is the predominant transporter of LCFA into cells via a saturable protein-mediated mechanism, while *VLDLR,* in combination with *LPL,* take up and hydrolyze triacylglycerides (Bionaz and Loor, 2008a). In **Chapter 3**, we observed increased secretion of 18-C FA in the milk of cows fed with 10% (DM) ensiled OC, but no diet effect on the bovine mammary levels of expression of *VLDLR* and *SLC2A1* was demonstrated. Most nutrigenomic studies in goats (Bernard et al., 2012, 2017; Fougère and Bernard, 2019) reported no significant effect on the expression of any of the genes involved in mammary lipid metabolism after dietary supplementation with lipids. These differences could be attributed to different kinds of lipid supplements used or species/breed specificity. Overexpression of *SLC2A1* and *VLDLR* observed in the mammary gland of the OC20 group in the current study can be attributed to the higher amounts of LCFA, particularly oleic and stearic acid, contained in the OC diet compared to control, that were thereafter secreted in milk. Also, a tendency for higher mammary mRNA abundance of *FABP3* with OC20 diet was demonstrated in the present study. This is consistent with Invernizzi et al., (2010) that reported a significant increase of *FABP3* expression in the mammary gland of lactating cows fed marine lipids and in which MFD was induced as a result. However, MFD has not occurred in the present study, and other nutrigenomic studies in goats (Bernard et al., 2012, 2017; Fougère and Bernard, 2019) and ewes (Castro-Carrera et al., 2015) have reported no effect on the mammary levels of expression of *FABP3* by lipid supplementation. It has been noted that *FABP3* plays an essential role during lactation in cow (Bionaz and Loor, 2008b) and goat (Shi et al., 2015) lipid metabolism, channeling palmitic and stearic acids for desaturation as it provides stearoyl-CoA to SCD. This suggests that *FABP3* is a master regulator in the milk fat synthesis signaling pathway in cooperation with FA (Liang et al., 2014). Indeed, the *in vitro* study of Liang et al. (2014) reported that the addition of stearic and palmitic acids in cow mammary gland epithelial cell cultures generates an increase in *FABP3* abundance. In the present study, the increased mRNA abundance of *FABP3* could be related to the relatively higher contents of C18:0 contained in the OC20 diet compared to control.

The mRNA abundance of *FASN* was significantly increased in the adipose tissue of animals that received the OC20 diet in the present study. Previous studies testing plant oils in small ruminants (Bernard et al., 2009, 2012; Bichi et al., 2013a) or cow (Thering et al., 2009) diets reported no significant effects on the *FASN* mRNA abundance or the expression of any of the genes tested in the adipose tissue. A tendency of increased *FASN* mRNA abundance in the adipose tissue has been observed in cows after plant oil supplementation (Vahmani et al., 2014). In the study of Harvatine et al., (2009), the intravenous infusion of CLA *trans*-10, *cis*-12, which is an inhibitor of *de novo* milk FA synthesis, upregulated *FASN* in adipose tissue of dairy cows. A recent *in vitro* study examining the effect of dose and type of LCFA on adipogenesis of bovine adipocytes reported similar findings as well (Yanting et al., 2018). This particular CLA isomer has been found to increase the expression of adipogenic *FASN* and other lipid synthesis enzymes in cows (Bauman et al. 2011), but in dairy goats, adipogenesis is unresponsive or less sensitive to CLA *trans*-10, *cis*-12 (Chilliard et al., 2007), suggesting that other intermediates could have influenced the expression of *FASN* in the present study. For instance, C18:1 *trans*-10 associated with reduced *de novo* synthesized FA in dairy cows (Dorea and Armentano, 2017), could be a possible intermediate, since its concentration was higher in the goat milk of OC20 group compared to control in the present study. It is possible that increased levels of C18:1 *trans*-10, or other monoenes arising from oleic acid isomerization may be related to increased expression of *FASN* in adipose tissue of lactating goats. Nevertheless, further studies are required to discern the particular role of other CLA isomers and *trans* monoenes on lipid metabolism when feedstuffs rich in oleic acid are offered to dairy goats.

In the present study, the OC20 diet upregulated the *SLC2A1* in the adipose tissue of goats. This was not observed in our previous work in which 10% (DM) of ensiled OC was included in the diets of lactating cows (**Chapter 3**), possibly due to species-specific differences or increased OC diet content in the case of goats. The *SLC2A1* gene encoding glucose transporter-1(*GLUT1*) protein is a key transporter of glucose into the cells (Ebeling et al., 1998). The supplementation with 20% of ensiled OC led to enhanced *SLC2A1* expression that could likely increase LCFA and glycose uptake in adipose tissue of goats. Overall, our findings suggest that 20% (DM) of ensiled OC supplementation enhances FA synthesis and transport via increased expression of *FASN* and *SLC2A1* in caprine perirenal fat.

4.5 CONCLUSIONS

In the present study, the OC inclusion rates of 10 and 20% in goat diets improved milk fat quality by reducing SFA and increasing unsaturated FA percentages, including oleic, vaccenic, and rumenic, all related with positive effects to human health. Supplementation with 20% of ensiled OC led to upregulation of genes involved in FA uptake and translocation in the mammary gland consistent with increased LCFA levels. Reduced *de novo* synthesis in the mammary gland was accompanied by upregulation of genes involved in FA and glucose transport and FA synthesis in the adipose tissue. These results suggest that the inclusion of ensiled OC at rates of 10 and 20% in DM is a useful additive for dairy goat diets that can improve the fat and protein content as well as FA profile of milk and possibly other related dairy products without adverse effects on milk productivity performance.

CHAPTER 5: Evaluation of dried distillers' grains with solubles included at two rates in the diets of lactating ewes

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A slightly modified version of this chapter has been submitted for publication

Neofytou C.M., Michael C., Constantinou C., Sparaggis D., Tzamaloukas O. Feeding wheat dried distillers' grains with solubles increases conjugated linoleic acid and unsaturated lipids in ovine milk without adversely affecting milk yield. Journal of Dairy Research (accepted).

ABSTRACT

This study aimed to examine the effect of dietary supplementation with wheat-based DDGS, a by-product of bioethanol production, on yield, composition, and FA profile of ewe milk. Forty-five purebred mid-lactating Chios ewes (average milk yield 2.23 kg/d in 96 ± 5 days in lactation) were offered three iso-nitrogenous and iso-energetic diets (15 animals per diet) for a 10 days adaptation period followed by a 5-week recording and sampling period. The diets contained 0, 6, and 12% DDGS on DM basis for the DG0, DG6, and DG12 treatment, respectively, as a replacement of concentrate mix, whilst concentrate to forage ratio remained at 60:40 in all treatments. During the trial, individual milk yield, milk composition, and FA profile were recorded weekly and analyzed using a complete randomized design with repeated measurements. No significant differences were observed among groups concerning dry matter intake (overall mean of 2.59 kg/d), milk yield or 6% fat-corrected milk, and milk protein percentage or protein yield. Milk fat percentage was decreased in the DG12 (4.76%) compared to DG0 (5.69%) without, however, affecting the daily output of milk fat. The concentration of all major SFA between C4:0 to C16:0 was reduced, whereas LCFA (>16 carbons), MUFA, and PUFA were increased in the milk of DDGS groups. Among individual FA, increments of OA and C18:1 *trans*-monoenes like C18:1 *trans*-10 and VA were demonstrated in the DG12 group, whereas linoleic and RA were elevated in both DDGS groups compared to DG0. Changes in FA profile resulted in a decline in the atherogenic index of milk by 20% and 35% in DG6 and DG12 treatments, respectively, compared to DG0. In conclusion, feeding DDGS to dairy ewes increased the levels of UFA that are beneficial for human health without adversely affecting milk, protein, or fat yield.

5.1 INTRODUCTION

The rapid growth of bioethanol production from grains, like corn, wheat, and sugarcane, has generated sufficient by-products that can be used as alternative feedstuffs in dairy animals (Schingoethe et al., 2009). There are two different bioethanol production processes; the wet milling and dry grind technology, from which the latter is the main process used (Schingoethe et al., 2009). During the dry milling process, dried distillers' decoction may exist in different forms, including DDGS. DDGS represents an interesting supplement for cattle production due to its high protein, fat, and energy content (30%, 11.2 %, and 9.4 MJ NEl/kg in DM, respectively) (Schingoethe et al., 2009), and it is usually recommended to be used up to 20% of ration DM in dairy cattle (Schingoethe et al., 2009). A high concentration of DDGS in the diets may cause MFD when it coincides with reduced forage NDF content, as shown in studies implemented in cows with increasing concentration of DDGS in diet (Cyriac, 2005; Leonardi et al., 2005). In most studies, this reduction in fat percentage was not accompanied by a decrease in milk or fat yield although it has also been reported a concomitant reduction in milk yield with 25% DDGS inclusion level (Testroet et al., 2015). Nevertheless, the majority of studies in dairy cattle investigating DDGS from different grains with inclusion rates up to around 20% of DM intake showed that milk production and fat yield were increased or not adversely affected when diets contained adequate levels of forage fiber (Schingoethe et al., 2009; Chibisa et al., 2012; Gaillard et al., 2017a, b). Furthermore, the use of DDGS enriched milk lipids with FA beneficial for human health; the content of SFA was decreased, and the levels of LCFA, MUFA, and PUFA, including RA, were increased (Leonardi et al., 2005; Anderson et al., 2006; Sasikala-Appukuttan et al., 2008; Abdelqader et al., 2009; Kurokawa et al., 2013; Testroet et al., 2015; Gaillard et al., 2017a, b).

. With regards to small ruminants, up to date, only two studies investigated the use of DDGS in lactating animals (Cais-Sokolińska et al., 2015; Alshdaifat and Obeidat, 2019). In the latter one (Alshdaifat and Obeidat, 2019), the inclusion of 20 and 30% DDGS in the diet (DM basis) of dairy Awassi ewes increased milk yield without affecting the percentages of milk fat and protein, while the FA content of milk fat was not investigated. The other study (Cais-Sokolińska et al., 2015) investigated only the composition of sheep and goat milk and FA content and not milk yield. Those authors reported that the inclusion of maize DDGS at the level of 3.5% (DM) did not affect milk fat and protein percentages, while affected FA content showing increased concentrations of total PUFA and *trans* - MUFA for both ovine and caprine milk and reduced SFA, increased MUFA and CLA content only in caprine, and not in ovine milk.

The present study was designed to investigate the effects of feeding DDGS on milk production and composition, as well as on milk FA content of dairy Chios ewes during a five-week sampling period in mid-lactation. The hypothesis was that the inclusion of wheat DDGS at rates of 6% and 12% in dietary DM would positively affect yield, composition, or FA profile of milk, suggesting, therefore, its use as an alternative protein-rich supplement in sheep rations.

5.2 MATERIALS & METHODS

5.2.1 Animals, Management and Experimental Diets

The experiment was performed in the research farm of ARI (Athalassa, Nicosia) in Cyprus. All experimental procedures were carried out according to the national legislation regulations (Animal Welfare Law, 1994) and international guidelines (Directive, 2010/63/EU) and approved by the corresponding departmental committee of the Cyprus University of Technology.

Forty – five multiparous purebred, mid-lactating Chios ewes (average ± SD: 96 ± 5 days in lactation) were randomly distributed to 9 pens of 5 animals and allocated to 3 experimental feeding treatments (3 pens or 15 animals per treatment), resulting in overall means (± SEM) of 2.23 (±0.01) kg for daily milk yield and 59.3 (±0.4) kg for live weight. Animals were housed indoors and allocated to three iso-energetic and iso-nitrogenous feeding regimes for a 10 days adaptation period followed by a 5-week recording and sampling period. Feeding treatments contained 0, 6, and 12 % on DM of wheat DDGS replacing concentrates (DG0, DG6, and DG12 treatments, respectively). The inclusion rates of DDGS were chosen to be included in ewe' diets based on the review of Schingoethe et al., (2009) who has been concluded that up to 20% (DM) of DDGS can be included on ruminants' diets without adverse effects on milk traits. Animals also received barley hay of good quality as forage, obtaining a concentrate to forage ratio of 60:40 for all treatments (**Table 19**).

DMI was monitored, and feeds were offered manually, as a total mixed ration, two times per day after morning (0430 h) and evening (1630 h) milkings. In each pen, the animals were group fed the diets to 1.1 times their maintenance energy (0.401 MJ/kg of weight0.73) and milk production requirements [dietary ME (MJ/kg of milk): Y = (1.94 +

0.43X)/0.62, where X is the fat percentage and 0.62 the efficiency of utilization of dietary ME for milk production of Chios ewes (Economides, 1986). Feed samples were collected at the beginning, at the middle, and at the end of the trial, mixed per treatment, and analyzed in triplicate. Intake measurements presented were taken during the first week of the experiment and after the 10 days of the adaptation period. DM, ash, crude fat, and CP were determined as described by AOAC International., (2005). Crude fiber, ADF, ADL, and aNDF were measured according to Van-Soest et al., (1991).

Table 19. Ingredients of diets contained 0 (control, DG0) or 6 (DG6) or 12 (DG12) g of wheat - based dried distillers' grains with solubles per 100g DM of DM

Item, % DM	Diet		
	DG0	DG6	DG12
Barley hay	40	40	40
Barley grain	15.6	15.6	15.6
Corn grain	18.12	15.9	13.38
Wheat bran	4.8	4.8	4.8
Sugar beet pulp	6	6	6
Soya bean meal[1]	8.4	4.68	1.2
Sunflower meal[2]	4.8	4.8	4.8
Wheat DDGS	-	6	12
Mineral and vitamin mix[3]	2.28	2.22	2.22

[1] Containing 47% CP, [2] Containing 35% CP, [3] Containing (% DM): Magnesium oxide (5.26), Sodium Biocarbonate (21.05), Limestone (57.9), Sodium chloride (5.26), Monocalcium phosphate (5.26%), Micro - mineral and vitamin premix (5.26)

5.2.2 Measurements and Sampling

All animals were machine milked (Fulwood, Shropshire, UK) twice daily (at 0430 h and 1630 h) and milk yields were recorded electronically (AfiMilk model Afifree 155, SAE Afikim Kibbutz, Israel) at each milking. Raw milk samples for the determination of the lipid profile were collected from each ewe during the two consecutive milkings (morning and evening) at the end of each sampling week (days 17, 24, 31, 38, and 45 of the experiment), mixed and stored at -80°C for further analyses.

Measurements for total fat, protein, lactose, and SNF were performed using combined thermo-optical procedures (Lactostar 3510, Funke Gerber, Berlin, Germany) calibrated previously for protein with the Lowry protein assay and fat with the Gerber method 989.05 (AOAC International, 2005). Fat-corrected milk yield at 6% of fat content (FCM 6%) was estimated according to Mavrogenis and Papachristoforou, (1988) for Chios sheep: FCM 6% = milk yield x (0.453 + 0.0912 x fat%)

5.2.3 Lipid Analysis of Milk and Feeds

Milk fat isolated according to the rapid double centrifugation method of Feng et al., (2004). Briefly, 20 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 new tubes, and allowed to melt at room temperature for 20 min. The samples were then recentrifuged at 19.500g for 20 min at room temperature, and 2 mg aliquots of the resulting lipid cake were removed to fresh tubes and dispersed in 1 mL of n-hexane by shaking.

For the feed fat isolation, the method used was as follows: after crude fat determination (Soxtec method), 2 ml of hexane was added to the residue in the aluminum collection pots and transferred to a glass tube (pre-weighed). Then, the residue was dried down under a gentle stream of nitrogen gas, and the glass tube was reweighed.

Subsequently, 1 ml of hexane was added for re-dissolving the residue and then, an aliquot containing 20-25 mg of crude fat was removed and put into fresh tubes.

FAME of milk and feed lipids were prepared by transesterification with methanolic potassium hydroxide according to the ISO (2002) method. Essentially, either 40 µL of transesterification reagent (11.2% KOH in methanol) were added to the tubes containing extracted lipid from milk and feeds and shaken vigorously for 1 min. The tubes were then allowed to incubate for a further 5 min at room temperature before the addition of 0.1 g of sodium bisulfate. The particulate material was then removed by centrifugation at 350×g for 3 min at room temperature and aliquots of the clear organic phase transferred to amber gas chromatography vials and stored at −70°C until analyzed. Fatty acid profiles were generated by analyzing the FAME samples on a GCMS-QP2010 Plus Gas Chromatography Mass Spectrometer (Shimadzu, Duisburg, Germany) equipped with an HT280T autosampler (HTA, Brescia, Italy) following the method of Kramer et al., (2008). Typically, 1 µl aliquots of FAME were separated with a split ratio of 1:20 using an Agilent CP-Sil 88 fused silica capillary column (100m × 0.25 mm internal diameter × 0.2 µm film thickness). The column was held for 4 min at 70°C after injection, increased at 13°C/min to 175°C and then held at that temperature for a further 27 min. The temperature was then raised to 215°C at 4°C/min at which it was held for a further 36 min. Helium was the carrier gas at 1 mL/min, with both injector and interface temperatures of 225°C. Chromatographic profiles were analyzed using Shimadzu GCMS Postrun Solution software where individual peaks were identified by comparison of their retention indices to those of commercially available authentic standards (Supelco 37-FAME standard mix, CLA *cis*-9, *trans*-11 and *trans*-10, *cis*-12, C18:1 *trans*-11; 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). All FAME peaks identified were quantitated by peak integration and individual FAME expressed as a percentage of the total fat (Butler et al., 2011).

Milk AI was determined using the formula proposed by Ulbricht and Southgate, (1991) AI= (C12:0 + 4 x C14:0 + C16:0)/ (ΣMUFA + ΣPUFA) and the DI was determined using the formula suggested by Garnsworthy et al., (2010): DI = (C14:1 cis-9 x 100)/ (C14:0 + C14:1 cis-9). Fat-corrected milk yield at 6% of fat content (FCM 6%) was estimated according to Mavrogenis and Papachristoforou (1988) for Chios sheep: FCM 6% = milk yield x (0.453 + 0.0912 x fat%).

5.2.4 Statistical Analysis

Performance, milk content, and milk FA composition data were analyzed using repeated measures analysis for a Completely Randomised Design, using SAS PROC MIXED (SAS version 9.4. SAS Institute Inc., Cary, NC). The model included the fixed effects of diet (D), time (T), and their interaction (T×D) and the random effect of ewes and pen. Statistical significance declared at $P < 0.05$. P-values between > 0.05 and < 0.10 were interpreted as trends towards significance. Data referring to DMI, chemical and FA composition of feeds were analyzed using one-way ANOVA with 3 replications, respectively. Normality of data was assessed both visually and through the normality test of Shapiro-Wilk using using the SPSS 20 software (StatSoft Inc., Tulsa, OK, USA).

5.3 RESULTS

5.3.1 Chemical Composition of Experimental Diets

The chemical analysis and the FA composition of the experimental diets are shown in **Table 20**. DDGS diets contained higher fat and NDF content compared to the control diet, although in all treatments remained at common concentrations for ruminants with fat values below 2% and NDF content above 30% in diet DM. The protein and energy content were similar between experimental diets and averaged 14.46 ± 0.12 % and 11.8 ± 0.03 MJ of ME /kg DM, respectively. The FA profiles were similar between treatments, except for the content of palmitic acid (C16:0), which was slightly higher in the DGGS diets compared with the control. The major FA found in treatments were linoleic acid, around 45% (corresponding values: 44.4, 44.7, and 45.0 g/100g fat for DG0, DG6 and DG12, respectively) and oleic acid around 30% (corresponding values: 29.1, 29.4 and 28.9 g/100g fat for DG0, DG6 and DG12, accordingly) of total fat.

Table 20. Chemical composition of the DDGS feed, as well as chemical composition and fatty acid profile of diets, contained 0 (control, DG0), 6 (DG6), or 12 (DG12) g of wheat - based dried distillers' grains with solubles per 100g

Item	DDGS	Diet			SEM	P-value[1]
		DG0	DG6	DG12		
Chemical composition						
Dry matter, % as fed	90.1	89.60	89.50	90.20	0.11	NS
Crude protein, % DM	26.41	14.45	14.61	14.32	0.12	NS
Crude fiber, % DM	6.30	16.83	16.75	17.17	0.13	NS
Ether extract, % DM	6.31	1.28^{c}	1.42^{b}	1.82^{a}	0.02	***
Ash, % DM	4.23	7.38	7.22	7.37	0.05	NS
aNDF, % DM	31.68	33.37^{c}	34.09^{b}	35.07^{a}	0.40	**
ADF, % DM	16.11	20.64^{c}	21.33^{b}	22.76^{a}	0.48	***
ME (MJ/kg)[2]	10.98	11.79	11.83	11.78	0.03	NS
Fatty acid profile, % of total fatty acid						
C16:0		16.84^{b}	17.03^{b}	18.39^{a}	0.52	*
C18:0		4.12	3.52	2.79	0.46	NS
C18:1 *cis*-9		29.11	29.43	28.85	0.37	NS
C18:2n-6		44.44	44.74	45.00	0.31	NS
C18:3n-3		3.89	3.86	3.69	0.08	NS

$^{a-c}$Means within a row not sharing a common superscript differ, [1]Probability of significant effects; *P < 0.05; **P < 0.01; ***P < 0.001; NS = Non significance, [2] Calculated from NRC (2007)

5.3.2 Performance Variables of Ewes

Table 21 shows the DMI, 6% fat-corrected milk, the daily yield of milk, fat, and protein, as well as the composition of milk. DDGS inclusion in the ewe diets did not affect intake, the yield of milk, fat, or protein during the trial. Fat-corrected milk (at 6% fat content) had similar overall means among treatments during the 5-week recording period. No significant differences were observed in either total solids or protein percentage of milk. In contrast, the fat percentage was significantly reduced by 19% in the milk of ewes fed the DG12 diet, while it was not affected in DG6 milk compared to the control treatment.

Table 21. Milk production and chemical composition of milk from ewes fed diets containing 0 (control, DG0), 6 (DG6), and 12 (DG12) g of wheat-based dried distillers' grains with solubles per 100 g DM

Item	Treatment			SEM	P-value[1]		
	DG0	DG6	DG12		D	T	D x T
DMI, kg/d	2.62	2.59	2.55	0.02	NS	-	-
Yield, kg/d							
Milk	1.63	1.62	1.75	0.06	NS	***	NS
6% FCM[2]	1.55	1.56	1.53	0.06	NS	***	NS
Fat	0.098	0.097	0.088	0.003	NS	***	NS
Protein	0.081	0.080	0.084	0.002	NS	***	†
Milk composition, %							
Fat	5.69^a	5.60^a	4.76^b	0.18	**	***	NS
Protein	4.80	4.73	4.70	0.03	NS	**	*
Lactose	4.94	4.83	4.85	0.03	NS	NS	NS
SnF[3]	10.88	10.64	10.72	0.07	†	†	***

$^{a-c}$ Means within a row not sharing a common superscript differ due to the different diet examined ($P < 0.05$), [1]Probability of significant effects due to diet (D), time (T), and their interaction (D x T); *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, NS: Non significant; †$P < 0.1$: tendency, [2] 6% FCM = milk yield x (0.453 + 0.0912 x fat), [3] SnF: Solids non fat

5.3.3 Milk Fatty Acid Composition

All individual FA and their aggregated groups determined in the milk of DG0, DG6, and DG12 groups are given in **Table 22**. The content of FA with more than 16 carbon atoms was increased by 10 and 18% ($P < 0.001$) in the milk of ewes fed with DG6 and DG12 diets, respectively, at the expense of de novo FA (< C16) secretion ($P < 0.001$). Total SFA and individual SFA like C4:0, C6:0, C8:0, C10:0, C12:0, C14:0 and C16:0, were reduced by the inclusion of DDGS in the ewe diets. In contrast, the milk of DGGS groups appeared to have a tendency ($P = 0.06$) of increased concentration of C18:0 and elevated levels of 18-C MUFA, like OA ($P < 0.05$), VA and C18:1 *trans*-10 ($P < 0.01$). Regarding other MUFA, proportions of C10:1 *cis*-9, C12:1 *cis*-9, and C14:1 *cis*-9 decreased as DDGS proportion increased in the ewe diets ($P < 0.001$). On the contrary, no differences in the sum of other *cis*-C18:1 isomers and C16:1 *cis*-9 percentages were demonstrated between treatments.

The content of total PUFA was enhanced by 10 and 27% in the milk of ewes fed with DG6 and DG12 diets, respectively ($P < 0.001$). Additionally, concentrations of RA and LA were risen between 17 and 29 % with DG6 and DG12 diets, accordingly, compared to the control. The content of CLA *trans*-9, *cis*-11 isomer was increased only in the DG12 group ($P<0.05$), while no differences between treatments were displayed in the CLA *trans*-10, *cis*-12, as well as, in ALA (C18:3n-3), γ-linolenic (C18:3n-6), and AA (C20:4n-6) acids. Milk AI diminished by 19% in DG6 and by 35% in DG12 treatment compared with the control diet, while the DI was not affected by the type of diet offered.

Table 22. Fatty acid composition (expressed as a percentage of total fatty acid methyl esters) of milk from ewes fed diets contained 0 (control, DG0), 6 (DG6) and 12 (DG12) g of wheat-based dried distillers' grains with solubles per 100 g DM

	Treatment				P-value[1]		
Item	DG0	DG6	DG12	SEM[3]	D	T	D x T
C4:0	2.73^{a}	2.79^{a}	2.67^{b}	0.05	*	***	†
C6:0	2.69^{a}	2.65^{a}	2.33^{b}	0.08	***	***	NS
C7:0	0.08	0.07	0.08	0.009	NS	†	†
C8:0	3.11^{a}	3.01^{a}	2.55^{b}	0.08	**	***	NS
C10:0	7.95^{a}	7.23^{b}	6.17^{c}	0.18	**	***	NS
C10:1 *cis*-9	0.46^{a}	0.40^{b}	0.34^{c}	0.01	***	**	NS
C12:0	5.84^{a}	5.04^{b}	4.46^{c}	0.13	***	NS	NS
C12:1 *cis*-9	0.10^{a}	0.08^{b}	0.07^{b}	0.005	NS	**	**
C13:0	0.33	0.25	0.30	0.02	†	NS	*
C14:0	12.28^{a}	11.35^{b}	10.36^{c}	0.17	***	**	NS
C14:0 *iso*	0.10	0.10	0.11	0.01	NS	NS	NS
C14:1, *cis*-9	0.30^{a}	0.21^{b}	0.20^{b}	0.01	NS	***	**
C15:0	1.72^{a}	1.46^{b}	1.75^{a}	0.06	***	***	***
C15:0 *iso*	0.25^{a}	0.25^{ab}	0.23^{b}	0.008	NS	***	NS
C15:0 *antiso*	0.75^{a}	0.67^{b}	0.69^{b}	0.02	NS	NS	NS
C16:0	22.26^{a}	21.86^{a}	20.61^{b}	0.34	*	**	NS
C16:0 *iso*	0.30	0.31	0.31	0.01	NS	NS	NS
C16:1 *cis*-9	2.17	2.02	2.00	0.09	NS	NS	NS
C16:1 *trans*-9	0.60	0.57	0.62	0.02	NS	NS	NS
C17:0	0.74^{ab}	0.73^{b}	0.80^{a}	0.02	*	***	NS
C17:0 *iso*	0.46	0.45	0.44	0.02	NS	NS	NS
C17:0 *antiso*	0.71	0.67	0.70	0.04	NS	NS	NS
C17:1 *cis*-9	0.29^{b}	0.29^{b}	0.34^{a}	0.01	*	NS	NS
C18:0	5.22	6.20	5.39	0.19	†	†	NS
C18:1 *trans*-9	0.35	0.39	0.48	0.06	†	NS	NS
C18:1 *trans*-10	0.46^{b}	0.53^{b}	0.77^{a}	0.05	**	**	*
C18:1 *trans*-11	3.10^{b}	3.26^{b}	4.99^{a}	0.32	**	*	NS
C18:1 *trans*-16	0.10	0.09	0.12	0.02	NS	NS	NS
C18:1 *cis*-9	14.03^{b}	15.24^{b}	15.43^{a}	0.27	*	***	NS
Other *cis*-C18:1[2]	1.07	1.18	1.32	0.06	NS	***	NS
C18:2 *trans*-9, *cis*-13/ *trans*-8, *cis*-12	0.19	0.23	0.26	0.007	NS	**	†
C18:2 *trans*-8, *cis*-13	0.08^{b}	0.11^{a}	0.12^{a}	0.006	*	NS	**
C18:2 *trans*-11, *cis*-15	0.13^{b}	0.18^{a}	0.19^{a}	0.02	*	NS	NS
C18:2 *trans*-9, *cis*-12	0.04	0.04	0.06	0.01	NS	NS	NS
C18:2n-6	3.65^{c}	4.15^{b}	5.21^{a}	0.14	***	NS	†
C18:3n-6	0.10	0.08	0.09	0.005	†	**	NS
C18:3n-3	0.33	0.33	0.41	0.01	NS	***	NS
CLA *cis*-9, *trans*-11	0.55^{b}	0.67^{a}	0.72^{a}	0.03	*	***	**
CLA *trans*-9, *cis*-11	0.08^{b}	0.08^{b}	0.15^{a}	0.01	**	NS	NS
CLA *trans*-10, *cis*-12	0.05	0.05	0.06	0.004	NS	***	**
C20:4n-6	0.23	0.23	0.26	0.01	NS	***	NS
SCFA[3]	8.61^{a}	8.52^{a}	7.63^{b}	0.16	***	***	*
MCFA[4]	54.43^{a}	50.69^{b}	47.23^{c}	0.53	***	***	NS
LCFA[5]	34.97^{c}	39.01^{b}	43.05^{a}	0.62	***	NS	NS
<C16	36.87^{a}	33.51^{b}	30.90^{c}	0.53	***	***	NS

>C16	34.97^{c}	39.01^{b}	43.05^{a}	0.62	***	NS	NS
SFA	68.70^{a}	66.62^{b}	61.29^{c}	0.71	***	***	NS
MUFA	23.69^{b}	25.41^{b}	28.12^{a}	0.41	***	***	NS
PUFA	5.66^{c}	6.35^{b}	7.80^{a}	0.21	***	***	NS
AI[6]	2.82^{a}	2.27^{b}	1.82^{c}	0.06	***	NS	*
DI[7]	2.84	2.41	2.50	0.11	NS	***	NS

$^{a-c}$ Means within a row not sharing a common superscript differ due to the different diet examined ($p < 0.05$), [1]Probability of significant effects due to diet (D), time (T), and their interaction (D x T); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS: Non significant; †$P < 0.1$: tendency, [2] Other C18:1 *cis* = C18:1 *cis*-11; C18:1 *cis*-12; C18:1 *cis*-13; C18:1 *cis*-14; C18:1 *cis*-15, [3]SCFA = short-chain fatty acids (C4:0 to C8:0), [4]MCFA = medium-chain fatty acids (C10:0 to C16:1), [5]LCFA = long-chain fatty acids (C17:0 and above), [6]Atherogenic index = (C12:0+ 4 x C14:0 + C16:0) / (ΣMUFA + ΣPUFA), [7]Desaturation index = (C14:1 *cis*-9 / C14:0 + C14:1 *cis*-9) x 100, Unidentified picks were less than 2% in all groups

5. 4 DISCUSSION

In the present study, milk production and the 6 % fat-corrected milk yield were not affected by the inclusion of 6 or 12% of wheat-based DDGS in sheep diets which was expected due to similar DM and nutrient intake of the compared groups. There is only one study (Alshdaifat and Obeidat, 2019) testing corn DDGS in dairy sheep production and reported increased milk yield for 20 and 30% DM inclusion rates compared to control (daily milk production of 0.95, 1.17, and 1.19 kg/d for inclusion rates of 0, 20 and 30%, respectively). The discrepancy of the results in milk production in that study compared to the present may be attributed to (a) the different inclusion rate of DDGS followed, (b) the origin of DDGS (corn as opposed to wheat-based) as has been previously shown to affect the animal response (Kleinschmit et al., 2006; Chibisa et al., 2012; Kalscheur et al., 2012) or (c) the different basal diet and nutrient intake. Nevertheless, most cow studies with isoenergetic and isonitrogenous experimental diets and similar nutrient intake reported no effect on milk yield, although DDGS used up to 30% DM inclusion (Abdelqader et al., 2009; Gaillard et al., 2017a; b; Testroet et al., 2018). The increased, or a tendency of increased, milk yield with DDGS inclusion in the diets of lactating cows reported in some studies (Janicek et al., 2008; Chibisa et al., 2012; Benchaar et al., 2013) has been attributed to either the slightly increased energy density, due to higher fat content in DDGS diets, or to greater DM intake and, therefore, higher energy acquisition by the animals offered DDGS. In contrast to those, Testroet et al., (2015) reported a reduction of milk yield in cows with 25% DM inclusion of corn DDGS but no effect when the level of inclusion was 10% in diet DM.

DDGS inclusion did not affect milk protein levels in the present study, which was also reported in a previous related study in sheep (Alshdaifat and Obeidat, 2019). Nonetheless, the results in earlier studies with cows on the effect of DDGS on milk protein content are controversial, reporting either an increase (Testroet et al., 2015) or a decrease (Kleinschmit et al., 2006; Benchaar et al., 2013; Kurokawa et al., 2013), or no significant effect (Anderson et al., 2006; Janicek et al., 2008; Sasikala-Appukuttan et al., 2008; Chibisa et al., 2012). Reviewing the studies in cows, Kalscheur et al., (2012) concluded that milk protein content is rarely reduced by feeding DDGS unless protein is limiting in the diet or DDGS levels are higher than 30% DM of the diet reflecting the high rumen undegradable protein of the diet, which may result in a reduction of provided lysine for milk protein synthesis in the mammary gland. However, none of these prerequisites occurred in our study where adequate feed protein levels and lower DDGS inclusion rates were used.

Milk fat percentage, in the current study, was reduced with the inclusion rate of 12%, but not with 6% of DDGS in the diet (on DM basis) compared to control (corresponding mean values were 5.7, 5.6, and 4.7 for DG0, DG6, and DG12 milk), without however affecting the fat yield. Reduction of milk fat was not observed in a previous study in sheep with either 20 or 30% DM inclusion rates of corn DDGS (Alshdaifat and Obeidat, 2019). Nevertheless, it is well established in lactating cows that the addition of high levels of DDGS in diets could result in a decrease of milk fat percentages when NDF content and forage participation in the diets are low, a fact that has also been observed in practice (Schingoethe et al., 2009). A meta-analysis by Kalscheur et al., (2012) demonstrated that milk fat depression in cows fed with DDGS occurred only when diets contained less than 22% forage NDF and 50% total forage. Furthermore, studies of Cyriac (2005) and Testroet et al., (2015) observed a reduction in milk fat percentage when cows were fed increasing DM concentrations from 7 to 25% DDGS whilst the dietary NDF was more than 32% and forage participated in the rations with 40% corn silage and 15% alfalfa hay in the diet DM. This finding led these authors to suggest that the key to maintaining milk fat percentage is to provide not only adequate NDF and a specific percentage of forages in the diet but also sufficient amounts of effective fibre (i.e., hay as opposed to corn silage). Although our study was designed to provide adequate dietary NDF content (34 and 35% DM in DG6 and DG12, respectively) and the barley hay participation was at 40% of the diet DM (usual practice in Cyprus), it is not known whether these practices were adequate to prevent milk fat depression in sheep with the 12% DDGS inclusion in the diet. Further studies in dairy sheep and goats are needed to elucidate the role of different NDF levels and particular forage fibre in minimizing the effect of oil containing DDGS on milk fat content in order to make DDGS widely usable.

Apart from the reduction of milk fat percentage observed in the DG12 group, the DDGS inclusion had a marked effect on milk FA composition by reducing SFA and increasing UFA in both DG6 and DG12 treatments. Indeed, levels of LCFA and particular FA with 18 carbon atoms such as OA, RA and LA were increased, while total SFA and major individual SFA like C4:0, C6:0, C8:0, C10:0, C12:0, C14:0, and C16:0 as well as the AI were decreased with DDGS inclusion in the diets of ewes.

The content of SFA with less than 16 carbons ($C < 16$) was significantly reduced with increased DDGS proportion in the ewes’ diets. Those results are in line with that of a previous study in ewes (Cais-Sokolińska et al., 2015) and with many studies conducted on cows (Leonardi et al., 2005; Anderson et al., 2006; Sasikala-Appukuttan et al., 2008; Abdelqader et al., 2009; Hippen et al., 2010; Kurokawa et al., 2013; Gaillard et al., 2017a,b) showing diminished *de novo* FA percentages and increased levels of LCFA as a result of DDGS dietary inclusion. Milk FA of chain length C4 to C14 and approximately part of C16 are synthesized *de novo* in the mammary gland, whereas the remainder of C16 and longer chain FA present in milk are derived from the diet or body reserves (Chilliard et al., 2000). It was reported previously that the inclusion of LCFA in ruminant diets is associated with lower levels of FA with less than 16 carbon atoms, particularly with decreased concentration of SCFA and MCFA (Chilliard et al., 2000; Shingfield et al., 2010; Dorea and Armentano, 2017). This is due to either a higher secretion of LCFA from the blood or/and a lower *de novo* synthesis of FA in the mammary gland (Chilliard et al., 2000; Dorea and Armentano, 2017). Although in the present study, the content of LCFA of feeds did not differ in FA profile between dietary treatments, DDGS diets contained slightly higher levels of ether extract than the control diet, indicating that ewes fed with DDGS consumed greater amounts of PUFA than the control group and therefore an increased quantity was transferred to milk. Furthermore, *de novo* FA secretion can be inhibited by FA, including *trans* monoenes and CLAs (Bauman et al., 2011). The most well-documented FA is CLA *trans*-10, *cis*-12 (Bauman et al., 2006, 2011) that did not, however, significantly differ between treatments. Therefore, the decreased C < 16 FA, indicative of decreased *de novo* FA synthesis, could be possibly attributed to the production of other FA intermediates of the *trans*-10 pathway produced during incomplete ruminal BH of dietary PUFA, such as C18:1 *trans*-10 (Bauman and Griinari, 2003; Shingfield et al., 2010; Dorea and Armentano, 2017) found elevated in the milk of ewes fed with 6% and 12% of DDGS, although significantly in DG12 treatment. Thus, both/or a higher level of dietary LCFA from the blood and a decreased *de novo* synthesis of SCFA and MCFA by this specific *trans* isomer may have been the underline mechanisms resulting in the milk FA composition reported here from ewes fed DDGS diets.

In the present study, the tendency of greater concertation of C18:0 observed in the milk of DDGS groups has been reported as a statistical increment previously in studies conducted on cows after inclusion of DDGS up to 20% (DM) into their diets (Leonardi et al., 2005; Anderson et al., 2006; Hippen et al., 2010; Kurokawa et al., 2013; Testroet et al., 2015). Increased levels of C18:0 have been reported, also, by researchers who tested diets supplemented with either high proportions of linoleic (Capper et al., 2007; Gómez-Cortés et al., 2009; Bodas et al., 2010; Castro-Carrera et al., 2015) or oleic (Bodas et al., 2010; Symeou et al., 2019) acids in ewes' diets. Stearic acid is the final product of BH of dietary mono-unsaturated and poly-unsaturated C18-FA, resulting from microbial BH saturation of double bonds in LCFA (Shingfield et al., 2010; Ferlay et al., 2017), like linoleic and oleic acids, which were rich in the DDGS diets.

The reduction of SFA in the present study was accompanied by increased total PUFA and MUFA for the DDGS groups. Similar effects and patterns have been displayed in studies that examined diets supplemented up to 20% of DDGS (DM) in cows (Anderson et al., 2006; Sasikala-Appukuttan et al., 2008; Hippen et al., 2010; Kurokawa et al., 2013; Gaillard et al., 2017b) in goats and ewes (Cais-Sokolińska et al., 2015). It is likely that MUFA and PUFA of feed, escaping the rumen without BH or with incomplete BH, were transferred to milk FA content through mammary uptake from the plasma dietary FA, contributing to the higher MUFA and PUFA content of ewes' milk (Shingfield et al., 2010). In particular, the inclusion of DDGS in ewes' diets positively affected the proportion of C18:1, like OA (C18:1 *cis*-9) and C18:2 acids in milk, results that have been verified by other researchers who assessed similar proportions of DDGS, as in the present study, in bovine milk (Anderson et al., 2006; Sasikala-Appukuttan et al., 2008; Abdelqader et al., 2009; Hippen et al., 2010; Testroet et al., 2015). Indeed, milk C18:1 *cis*-9 can be generated either through the action of mammary Δ9-desaturase with the substrate being stearic acid or by direct transfer from feed (Collomb et al., 2006; Chilliard et al., 2007). However, since the Δ9-desaturation index in the mammary gland did not differ between groups, it can be assumed that the increase in oleic acid is possibly related more to the diet rather than to Δ9-desaturase activity.

Milk fat concentration of the predominant CLA isomer, RA, was increased in response to feeding the DDGS diets compared to the control diet. This observation is consistent with previous studies after the inclusion of DDGS up to 20% in cows' diets (Leonardi et al., 2005; Anderson et al., 2006; Sasikala-Appukuttan et al., 2008; Hippen et al., 2010; Kurokawa et al., 2013; Gaillard et al., 2017b) and in ewes with either 3.5% of DDGS (DM) supplementation (Cais-Sokolińska et al., 2015) or high levels of linoleic acid (Gómez-Cortés et al., 2009; Bodas et al., 2010; Castro-Carrera et al., 2015; Carreño et al., 2016) into their diets. It is well known that the majority of RA in the milk fat is synthesized endogenously, in the mammary gland through the action of mammary Δ^9-desaturase with the substrate being VA, while a small amount of this specific CLA originates from BH of UFA by rumen bacteria (Lock and Bauman, 2004; Collomb et al., 2006; Buccioni et al., 2012). Thus, the elevated proportions of RA observed in the milk of DDGS groups are either due to the higher concentrations of VA those groups contained compared to the DG0 group or due to the incomplete BH that took place in the rumen of ewes that fed with the DDGS diets.

Regarding other CLA isomers, *trans*-10, *cis*-12 was not affected by the type of diet. Inconsistent with our results, Leonardi et al., (2005) demonstrated no effect on CLA *trans*-10, *cis*-12 content after 5 - 15 % (DM) of DDGS supplementation in cow diets. Nevertheless, other studies reported significantly higher CLA *trans*-10, *cis*-12 proportions by the addition of DDGS up to 20% (DM) in diets of cows (Anderson et al., 2006; Sasikala-Appukuttan et al., 2008; Hippen et al., 2010) and ewes (Cais-Sokolińska et al., 2015), although in the latter study, RA and CLA *trans*-10, *cis*-12 were not separately identified. Although, in cows, the low - milk fat syndrome is strongly associated with increases in CLA *trans*-10, *cis*-12, in dairy ewes, the causative factors, and mechanisms involved in this syndrome are still uncertain (Bauman et al., 2011), indicating that other CLA isomers or *trans*-monoenes may inhibit milk fat production in ewes. In this regard, we observed increased concentrations of CLA *trans*-9, *cis*-11, which is also a FA with confirmed or putative antilipogenic properties in lactating cows (Perfield et al., 2007; Shingfield et al., 2010), but these increments were lower than those reported to affect milk fat synthesis, suggesting that further research is required to clarify the role of other less - known rumen intermediates on lipid metabolism.

Concerning non-conjugated C18:2 FA, LA (C18:2n-6:) concentration was modified and increased with DDGS in the ewes' diets. This is in line with previous studies in cows that indicated elevated levels of LA after inclusion of DDGS up to 20% into their diets (Leonardi et al., 2005; Anderson et al., 2006; Hippen et al., 2010) and in dairy ewes fed with diets enriched in LA (Mele et al., 2006; Bodas et al., 2010; Bichi et al., 2013b) like DDGS in the present study. Although the content of LA did not statistically differ in the fat of dietary treatments, increments of this FA in the milk of ewes fed the DDGS may be attributed to the higher ether extract the DDGS diets contained compared to the control.

5. 5 CONCLUSIONS

This study examined the inclusion of 6% and 12% on ration DM of wheat-based DDGS in lactating Chios ewes diets. Although the 12% of DDGS inclusion decreased the fat content of milk by 1 percent (from 5.7 to 4.7% in control and DG12 milk, respectively), DDGS in both treatments did not affect milk production or milk protein. DDGS use in the diets in both inclusion rates improved milk fat quality with reduced SFA and increased MUFA, PUFA, and individual FA, such as oleic, vaccenic, rumenic, and linoleic acids, all related to positive effects on human health. These results are useful for exploiting a cost-effective proteinaceous by-product in dairy sheep and concurrently improve the FA profile of ewe milk and possibly other related dairy products without adverse effects on milk traits.

CHAPTER 6: General Discussion

Milk and dairy products are rich in saturated and *trans* FA, which are related to hypercholesterolemic and thrombogenic effects, thereby increasing the risk for cardiovascular diseases (Shingfield et al., 2008, 2013). The World Health Organization (WHO, 2003) suggested a reduction in the intake of SFA and *trans* FA and an increase in the consumption of the LCFA as an attempt to lower the economic and social burden of chronic diseases (Shingfield et al., 2013). In this framework, the scientific community has focused on strategies to reduce the SFA content and improve the nutritional properties of ruminant milk through nutrition, which is the most crucial factor affecting the variability of FA composition of milk (Chilliard et al., 2000; Ferlay et al., 2017). With that scope, several diets have been tested in ruminants, including different types of forage, lipid supplements, the forage to concentrate ratio, and the associated starch level, or their combination, to evaluate the changes in duodenal flow and the proportion of each FA in milk and derived dairy products (Chilliard et al., 2000; Bernard et al., 2018).

Among different feedstuffs, the use of industrial by-products rich in UFA represents an interesting supplement for ruminants and could be a promising way for enhancing the quality of milk and its products (Schingoethe et al., 2009; Tzamaloukas et al., 2021). Moreover, the replacement of traditional feeds with alternative low-priced industrial by-products could have a profit for farmers, and subsequently**,** environmental issues associated with by-products accumulation and waste management could be assisted (Correddu et al., 2020).

Based on the aforementioned aspects, the main objective of the present thesis was to evaluate the effects of different by-products derived from olive oil processing, like ensiled OC, and the biofuel industry, like DDGS, on the milk yield and composition, on the FA profile of milk and related Halloumi cheese. However, it is well-known that oil rich diets may confer adverse effects on milk fat percentage through milk fat syndrome, often referred to as MFD (Shingfield and Griinari, 2007). In particular, diets causing MFD alter ruminal lipid metabolism, resulting in the formation of specific biohydrogenation intermediates in the rumen that directly inhibit milk fat synthesis (Bauman and Griinari, 2001) and thereby act to down-regulate the expression of key lipogenic genes involved in milk fat metabolism (Bauman et al., 2011). In this sense, in the present thesis, the effect of ensiled OC on the expression of specific genes involved in lipid metabolism of mammary and adipose tissue in ruminants was investigated to elucidate likely mechanisms of action when an oil rich by-product is included in their diets.

Effect of OC Feeding on Milk Yield and Composition in Cows and Goats

In **Chapters 2 and 3**, the effects of dietary inclusion of 10% (DM) of ensiled OC, for a short and long term, respectively, on milk yield, composition, and FA profile of cow milk and derived Halloumi cheese (**Chapter 3**), were evaluated. Furthermore, to assess the use of this by-product on other ruminant species, an additional study was implemented in lactating goats testing the effects of 10 and 20% inclusion rates (DM) of ensiled OC supplementation, for a six-week period, on milk yield, composition and FA profile (**Chapter 4**).

As reported in **Chapters 2 and 3**, the inclusion of 10% of OC in the diets of lactating cows did not affect milk yield, a result that is in accordance with previous studies in cattle (Hadjipanayiotou, 1999; Meo Zilio et al., 2014; Castellani et al., 2017), neither affected protein content or yield, similarly to Hadjipanayiotou, (1999) and Meo Zilio et al., (2014). Furthermore, the milk fat content was not significantly affected, which is in line with previous works (Hadjipanayiotou, 1999; Meo Zilio et al., 2014; Castellani et al., 2017), while, in contrast, an increase in fat yield in the milk of cows fed with OC for a longer period (**Chapter 3**) compared to the short-term feeding (**Chapter 2**), was demonstrated. Other cattle studies showed that by feeding OC up to 15% (DM), milk fat yield was not significantly affected (Hadjipanayiotou, 1999; Meo Zilio et al., 2014).

Equivalent results were observed in caprine milk by feeding 10 or 20% (DM) of ensiled OC (**Chapter 4**). Indeed, there was no diet effect on milk yield, which is in line with other studies in goats (Hadjipanayiotou, 1999; Arco-Pérez et al., 2017) and ewes (Cabiddu et al., 2004; Shdaifat et al., 2013; Mannelli et al., 2018) showing no significant effect on milk yield when different forms of processed OC (i.e., dried, pelleted or destoned) up to 20% (DM) included in the diet. Moreover, the milk fat content of goats increased significantly in the present study, similarly to Hadjipanayiotou, (1999) and Arco-Pérez et al., (2017), while milk fat yield tended, also, to be elevated in the OC groups.

In contrast to cows (**Chapters 2 and 3**), the milk protein percentage of goats was numerically increased in both OC treatments, significantly only at the 20% inclusion, compared to control. Increased milk protein content has been observed in bovine milk after 10% (DM) of dried OC supplementation (Castellani et al., 2017). It is well-known that by increasing the concentrate content in ruminant diets, milk protein percentage and yield can be increased due to the high energy contained in high concentrate diets (Jenkins and McGuire, 2006). Although the forage was replaced by OC silage in the present study, the concentrate ratio was kept at 60% and the NDF content was similar in all diets that were also isoenergetic and isoproteic. Nevertheless, the *in vitro* study of Pallara et al., (2014) examining the effect of OC on rumen microbial communities showed increased rumen volatile FA production, suggesting that the OC inclusion could enhance rumen microbial activity and consequently microbial protein supply in small intestine and therefore protein content in milk, but further research is needed to establish such mechanism.

Effect of Ensiled OC on FA Profile of Bovine and Caprine Milk

The levels of total SFA, particularly the medium-chain FA (MCFA), were reduced, while LCFA increased significantly in the milk of cows fed with ensiled OC (**Chapters 2 and 3**). Those results are in line with previous studies implemented in cows and examined the addition of either a high level of UFA (reviewed in Dorea and Armentano, 2017) or feeds rich in oleic acid, as in our studies (He et al. 2012; Castellani et al. 2017; Weld and Armentano, 2018). The inclusion of either 10 or 20% (DM) of ensiled OC in the diets of dairy goats led to similar results (**Chapter 4)** and are in line with previous studies in sheep fed with various forms of processed OC (Chiofalo et al., 2004; Abbeddou et al., 2011a, b, 2015; Symeou et al., 2019, 2021). In contrast to the findings of our study (**Chapter 4)** and those in ewe studies, Arco-Pérez et al. (2017) reported reduced MUFA and increased SFA content in caprine milk after feeding animals with 20% (DM) of a mixture of olive oil by-products, namely silage from both OC and olive leaves, and this discrepancy could be attributed to different by-products added. The reduction of MCFA concentration and the increment of LCFA content reported in **Chapters 2, 3 and 4** could be attributed to either a higher uptake of long-chain FA from the blood and/ or a lower *de novo* synthesis of FA in the mammary gland (Chilliard et al., 2000; Dorea and Armentano, 2017). The *de novo* FA synthesis could be inhibited by FA, including CLAs and *trans*-monoenes such as CLA *trans*-10, *cis*-12 and C18:1 *trans*-10, respectively (Bauman et al., 2011; Dorea and Armentano, 2017), from which the latter was significantly elevated in the milk of cows and goats fed with ensiled OC in our studies. As indicated by Dorea and Armentano, (2017), this reduction of *de novo* synthesized lipids could be occurred without affecting or even with an increase in the total milk fat percentage. This was observed in the present thesis since MCFA was reduced in all trials, whereas milk fat content found increased in OC groups in the trials presented in **Chapters 3 and 4** and was unaffected in the trial of **Chapter 2**. Thus, the results from milk FA profile and milk fat content suggest that it is likely that both a higher uptake of dietary LCFA and decreased *de novo* synthesis of MCFA by specific *trans* isomer in the mammary glands of cows and goats fed with ensiled OC are possible mechanisms of action, without however reducing the total milk fat content.

Moreover, significantly increased total MUFA content in the OC supplemented groups was reported in all trials of the present thesis (**Chapters 2, 3 and 4)**. Previous studies tested diets containing various forms of processed OC in ewes (Chiofalo et al., 2004; Abbeddou et al., 2011a, b, 2015; Vargas-Bello-Pérez et al., 2013; Symeou et al., 2019, 2021;), goats (Molina-Alcaide et al., 2010) and cows (Castellani et al., 2017) reported similar results, as well. Since the OC diets contained higher amounts of MUFA compared to the control diet, it is likely that MUFA escaping BH were transferred to milk FA content through mammary uptake from the plasma dietary FA, contributing to the higher MUFA content of milk of OC groups. Furthermore, the inclusion of lipids rich in 18-C UFA in dairy animal nutrition could increase both C18:1 and C18:2 *cis* and *trans* isomers arising from ruminal metabolism and mammary desaturation of C18:0 and C18:1 *trans* (mainly 7 and 11) produced in the rumen (Chilliard, et al. 2000). Based on this framework, the higher milk content of C18:1 *cis*-9 could be resulted either through the action of mammary Δ9-desaturase, with the substrate being stearic acid, or by direct transfer from feed (Collomb et al., 2006; Chilliard et al., 2007), and therefore found elevated in the bovine **(Chapters 2 and 3)** and caprine **(Chapter 4)** milk of OC groups. However, since there was no significant effect in the Δ9-desaturation index in the mammary gland of either cows or goats of the present thesis, the increment of OA in the milk of OC groups was attributed to the OC diet rather than to Δ9-desaturation activity.

Additionally, there was an increase in the RA content of bovine **(Chapters 2 and 3)** and caprine **(Chapter 4)** milk due to OC feeding, a fact which is consistent with previous studies in cows, in which, either dried olive pomace (Castellani et al., 2017) or olive oil (Vargas-Bello-Pérez et al., 2018) or feeds rich in oleic acid (He et al., 2012) included in their diets. Studies implemented in goats (Molina-Alcaide et al., 2010; Arco-Pérez et al., 2017; Marcos et al., 2020) led to similar results as well, while contradictory findings were observed in sheep fed different forms of processed OC (Tzamaloukas et al., 2021). This specific CLA isomer can be synthesized either endogenously in the mammary gland through the action of mammary Δ^9-desaturase with the substrate being VA, or through the BH of UFA by rumen bacteria (Collomb et al., 2006). Therefore, the elevated proportions of RA observed in the milk of OC groups in the present thesis could be either due to the higher concentrations of vaccenic acid in the mammary gland, compared to the control group or due to the incomplete BH taking place in the rumen of cows and goats fed with OC.

Effect of Ensiled OC on the FA Profile of Halloumi Cheese

As shown in **Chapter 2**, the effects of OC treatment in the FA composition of Halloumi cheese were similar to those reported for milk. More specifically, reduced levels of SFA with concomitant increased levels of LCFA and MUFA, including individual FA like OA, the sum of C18:1 *trans*-10 and *trans*-11 and RA were observed in Halloumi cheese when OC was supplemented in cow diet, and consequently, the AI was diminished. Those results are in line with Castellani et al., (2017) and Chiofalo et al., (2020) in cows, as well as with the studies of Vargas-Bello-Pérez et al., (2013, 2018), who reported decreased SFA and AI, as well as increased total MUFA, C18:1 *cis*-9 and/or RA concentrations of cheese fat, when 10% and 25% of dried OC (2013) or olive oil (2018) were included in the diets of ewes and cows, respectively. The similarity in the results of milk and cheese due to OC feeding reported in **Chapter 3** suggests that the improvement in nutritional quality achieved in milk because of OC supplementation is thereafter largely maintained in Halloumi cheese. In a recent study of our group, similar results were observed in Halloumi cheese produced from the milk of ewes fed 10% and 20% of ensiled OC (Symeou et al., 2021). This could be very useful for the dairy industry in Cyprus, where Halloumi cheese is among the most well-known and the top exported commodity of Cyprus and may improve its FA profile since about 70% of total FA presented in Halloumi cheese are saturated (Tzamaloukas et al., 2015b).

Effect of OC Feeding on the Expression of Selected Genes in Cows and Goats

Mammary Tissue

To study the underline mechanisms and understand the effects of dietary inclusion of feedstuffs rich in UFA on ruminant milk quality, the determination of the molecular aspects related to the overall lipid digestion, absorption, and metabolism in dairy animals, is of paramount importance (Bionaz et al., 2020). The onset of RT-qPCR was an essential step toward a better understanding of the transcriptional control of milk fat synthesis and helped the scientific community to characterize several genes that were thought to be involved in all steps of milk fat synthesis in mammary tissue of dairy cows (Bionaz and Loor, 2008a).

Thus, in the present thesis, for examining any possible impact of dietary inclusion of 10% and 20% (DM) of ensiled OC on bovine (**Chapter 3)** and caprine **(Chapter 4)** lipogenic gene expression, respectively, changes in mRNA abundance of selected genes involved in FA synthesis (*ACACA*, *FASN*, *G6PDH*), FA uptake and/ or translocation (*VLDLR*, *LPL*, *SLC2A1*, *CD36*, *FABP3*), FA saturation (*SCD1*) and transcriptional regulation (*SREBF1*, *PPARγ*) in mammary and adipose lipid metabolism, were evaluated. The knowledge gap regarding this field exists since no other nutrigenomic trials have examined the potential effects of any form of OC on mammary or adipose gene expression in lactating cows and goats.

The gene expression results of **Chapter 3** in cows showed that the increased LCFA and the reduction in *de novo* FA reported in the milk of cows by 10% (DM) of OC feeding were not associated with alterations in the mammary expression of tested genes involved in FA synthesis, uptake, translocation, and regulation of lipogenesis. In contrast to those results, when goats fed 20% of ensiled OC (**Chapter 4**), the results showed that the increased secretion of LCFA in the milk accompanied by significantly elevated expression of *VLDLR* and *SLC2A1* genes in the caprine mammary gland. Previous nutrigenomic studies in cows and/or goats (Bernard et al., 2012, 2017; Fougère and Bernard, 2019) reported no significant effect on the expression of any of the genes tested and involved in mammary lipid metabolism after lipid supplementation, including *SLC2A1*. The differences could be attributed to different kinds of lipid supplements, the quantity of the lipid supplementation, and species specificity or breed effects. Both of these genes play an essential role in the lipid metabolism of ruminants; *SLC2A1* is the predominant transporter of LCFA into cells via a saturable protein-mediated mechanism, while *VLDLR*, in combination with *LPL*, take up and hydrolyze triacylglycerides (Bionaz and Loor, 2008). The overexpression of *SLC2A1* and *VLDLR* observed in the mammary gland of goats fed 20% and not in the cows fed 10% (DM) of ensiled OC could be possibly attributed to the higher content of LCFA and particularly, oleic and stearic acids contained in the 20% OC diet or to species specificity reported by other researchers to play a role (Bernard et al., 2017; Fougère and Bernard, 2019).

Moreover, a tendency for higher mammary mRNA abundance of *FABP3* with 20% (DM) of OC feeding was demonstrated in the study implemented in goats (**Chapter 4)** but not in cows fed 10% OC **(Chapter 3)**. Increased mRNA abundance of *FABP3* has been reported by Invernizzi et al. (2010) in the mammary gland of lactating cows fed marine lipids that induced MFD. However, MFD has not occurred in our studies (**Chapters 3 and 4**), and other nutrigenomic studies in goats (Bernard et al., 2012, 2017; Fougère and Bernard, 2019) and ewes (Castro-Carrera et al., 2015) have indicated no effect on the mammary levels of expression of *FABP3* by lipid supplementation. It has been noted that *FABP3* plays an important role during lactation in cow (Bionaz and Loor, 2008b) and goat (Shi et al., 2015) lipid metabolism, channeling palmitic and stearic acids for desaturation as it provides stearoyl-CoA to SCD. This suggests that *FABP3* is a master regulator in the milk fat synthesis signaling pathway in cooperation with FA (Liang et al., 2014). Indeed, the *in vitro* study of Liang et al. (2014) reported that the addition of stearic and palmitic acids in cow mammary gland epithelial cell cultures generates an increase in *FABP3* abundance. In **Chapter 4**, the increased mRNA abundance of *FABP3* could be related to the relatively higher contents of C18:0 contained in the caprine OC20 diet compared to control, while the quantity of OC offered in cows (10%) was not enough to affect the expression of this gene. Moreover, species differentiation may be involved

(Bernard et al., 2017; Fougère and Bernard, 2019). Further studies in cows and goats examining the differences in the responsiveness of the two species or species-specific lipogenic genes pathways would be of great interest.

Adipose Tissue

According to the findings of **Chapter 3**, the expression of the studied genes was unaffected apart from an increase in the mRNA abundance of *SREBF1* in perirenal fat from cows fed the OC diet. Previous nutrigenomic studies conducted in lactating cows reported no effect in the *SREBF1* mRNA abundance of adipose tissue after UFA supplementation (Thering et al., 2009; Vahmani et al., 2014). Upregulation of adipogenic *SREBF1* has been observed in dairy cows by intravenous infusion of CLA *trans*-10, *cis*-12 (Harvatine et al., 2009), which, as mentioned above, is a well-established inhibitor of de *novo* milk FA synthesis (Bauman et al., 2011).

In the caprine adipose tissue **(Chapter 4)**, significant upregulation was also observed in the mRNA abundance of *FASN* in the animal fed 20% OC. This upregulation has not been detected by previous studies testing plant oils in small ruminants (Bernard et al., 2009, 2012; Bichi et al., 2013), which reported no significant effects in the mRNA abundance of any of the genes tested, including *FASN*, in the adipose tissue. However, our results agree with one nutrigenomic study that found a tendency of increased *FASN* mRNA abundance in the adipose tissue after plant oil supplementation (Vahmani et al., 2014).

According to the review of Bauman et al., (2011), upregulation of genes involved in FA synthesis, FA uptake and desaturation in the adipose tissue could be occurred in cows by the action of CLA *trans*-10, *cis*-12 isomer. However, since we did not observe MFD and this specific CLA isomer differed in neither **Chapter 3** nor **Chapter 4**, it was hypothesized that the increment of the sum of C18:1 *trans*-10 and *trans*-11 in the study with cows and VA in the case of goats or other isomers arising from oleic acid isomerization may have affected the expression of *SREBF1* and *FASN* in adipose tissue of lactating cows and goats, respectively. Further research is needed to clarify the particular role of other CLA and *trans* monoenes on lipid metabolism when oleic acid is fed.

Concerning the overexpression of the *SLC2A1* gene observed in the adipose tissue of goats fed with 20% of ensiled OC, we suggested that this specific gene could likely increase LCFA and glucose uptake in adipose tissue of goats because its main role is to transport glucose into the cells, as well as FA in several tissues including adipose (Ebeling et al., 1998).

Effect of DDGS Feeding in Sheep

In **Chapter 5** another alternative feed that was also studied was the wheat based-DDGS, a bioethanol by-product, included at rates of 6% and 12% (DM) in diets of dairy Chios ewes, highlighting its potential effects on milk production and content, as well as on milk FA composition. According to the literature, most studies tested DDGS were implemented in dairy cattle, while, up to date, only two studies have investigated the use of DDGS in dairy goats and ewes (Cais-Sokolińska et al., 2015; Alshdaifat and Obeidat, 2019).

The results referring to intake and milk yield obtained after DDGS supplementation in ewe diets of our study showed that neither the intake nor the yield of milk was affected by diet, a fact that is in contrast with the previous study of sheep (Alshdaifat and Obeidat, 2019), in which increased milk yield was reported by feeding ewes with 20 and 30% (DM) of corn based-DDGS. The discrepancy of the results may be attributed to differences in the (a) inclusion rate of DDGS, followed by (b) the origin of DDGS, as has been shown to affect the animal response to DDGS supplementation (Kalscheur et al., 2012) or (c) differences in basal unbalanced diet and nutrient intake. Nevertheless, the majority of the studies implemented in cows and tested diets that were isoenergetic and isonitrogenous, as in our case, reported no significant effect on milk yield, by up to 30% (DM) of DDGS inclusion in diets (Abdelqader et al., 2009; Gaillard et al., 2017a, b; Testroet et al., 2018).

Regarding milk composition, DDGS inclusion in our study did not affect milk protein percentage (**Chapter 5**), and this is in line with the previously referred study in sheep (Alshdaifat and Obeidat, 2019), in which higher inclusion rates of DDGS examined (20 and 30% of DDGS on DM basis). Furthermore, a reduction in milk fat percentage was reported only in the case of ewes fed with 12% and not with 6% (DM) of wheat based DDGS in the diets (**Chapter 5**). Reviewing the studies in cows, Schingoethe et al., (2009) reported that a reduction in milk fat content can be occurred by the addition of corn DDGS in diets when this is accompanied by low dietary NDF and forage content, particularly less than 22% of forage NDF and with less than 50% of total forage in the diet (Kalscheur et al., 2012). Since none of these circumstances took place in the present study, it is unknown whether the 12% (DM) of DDGS inclusion in the ration could result in MFD in sheep.

Regarding the FA results, reduced content of saturated lipids and increased unsaturated ones in ewe milk was achieved with DDGS feeding. Indeed, results indicated the significant contribution of this by-product in improving the quality of ewe milk by minimizing all major saturated FA between C4:0 to C16:0 and rising, the content of total MUFA and PUFA in both DDGS groups. Those results are in line with those of a previous study in ewes (Cais-Sokolińska et al., 2015) and with many other studies conducted in cows (Leonardi et al., 2005; Anderson et al., 2006; Sasikala-Appukuttan et al., 2008; Abdelqader et al., 2009; Hippen et al., 2010; Kurokawa et al., 2013; Gaillard et al., 2017a, b).

Increments of C-18 carbons FA like OA, C18:1 *trans*-10, VA, RA and LA with the addition of 6% and 12% (DM) of DDGS in ewe diets were observed in the present study. Similar effects have been reported in studies that examined diets from 5 up to 20% DM inclusion levels of corn DDGS in cows (Kalscheur et al., 2012; Kurokawa et al., 2013; Gaillard et al., 2017a, b) and also observed in ewes fed lower DDGS inclusion compared to ours (3.5% in diet DM, Cais-Sokolińska et al., 2015). Therefore, the use of DDGS in sheep diets, apart from an alternative cost-effective protein feed, can be suggested to the farmers as a means of increasing beneficial for human health FA and improving overall milk quality.

Conclusions and Future Research Plans

The substitution of conventional feeds with waste biomasses like ensiled OC and wheat-based DDGS produced by agro-industrial processes in ruminant diets is a good practice for enhancing the quality of dairy products, as well as the sustainability of ruminant production, in regions where animal feedstuffs are inadequate and mostly imported, like Cyprus. Moreover, using such by-products as feed ingredients could represent an important component of the global strategy to reduce the environmental impact of agro-industrial wastes and enhance nutrient recycling with their use in livestock production.

Overall, in the experiments with olive oil by-product **(Chapters 2, 3 and 4)**, the inclusion of up to 20% (DM) of ensiled OC in cow and goat diets had a marked effect on milk and/or Halloumi cheese FA composition by reducing saturated lipids and increasing at the same time the unsaturated ones, suggesting an improvement in fat quality of milk and related products. Our hypothesis of using this amount of OC in ruminant diets is further supported by a recent review that summarizes the existing literature about the use of olive oil by-products in the diets of ruminants, suggesting that OC can safely be included up to 15–20% on DM basis in the ration of ruminants without negative effects on milk traits (Tzamaloukas et al., 2021). However, further studies examining the potential side effects of ensiled OC on animal performance and product quality (e.g oxidative stress), would be of great interest.

Additionally, the milk FA differences observed in studies presented in **Chapters 3 and 4** were not associated with adverse changes in the expression of mammary and adipose lipogenic genes due to OC feeding, reducing, for instance, the milk fat content. In this sense, our studies give evidence for using the ensiled OC as a forage replacement. Furthermore, our study showed that milk protein content was increased in goats fed OC at 20 % of their diet DM, and this finding merit further investigation to identify potential factors or mechanisms that may influence the protein content when OC is fed to ruminants. Recent development in “omics” technologies could be a useful tool for a thorough and deeper understanding of the genes and biological networks that regulate bovine and caprine milk composition.

Moreover, concluding the results of **Chapter 5**, although the inclusion of wheat DDGS up to 12% (DM) in ewe diets gave promising results regarding the FA profile of ovine milk, the decrease in the milk fat content observed in ewes fed with the 12% (DM) of DDGS would cause a negative effect on cheese industry, since ewe milk is mainly transformed into cheese, and the farmers have to evaluate the implementation of this feeding strategy in accordance to forage percentage in the diets. Therefore, we recommend that the wheat based DDGS inclusion up to 12% on a DM basis would be tolerable in sheep rations for improving the FA profile of milk, partly replacing protein feeds such as the combination of corn grains and soybean meal, without adverse effects on milk yield and production traits. It has been recommended that DDGS could be incorporated at 20% of the diet DM to achieve optimum performance of lactating dairy cows if diets contain at least 50% forage (Schingoethe et al., 2009). This could be economically profitable when commodity prices for common feeds such as soybean-based protein supplements and grains are expensive. However, since the literature referring to the use of DDGS in the diets of small ruminants is scant, further studies are required to address the possible effects of feeding DDGS in small ruminants and with advances in bioethanol production, more by-products could become available.

The findings of this dissertation point out the challenge and complexity in increasing the milk fat content with beneficial FA for human health by using alternative feedstuffs in ruminants. We have shown that, despite public opinion and farmers perception, low-quality olive oil by-products, such as OC, or the non-common bioethanol by-product, such as DDGS, can be included in ruminant diets and improve milk quality by increasing several classes of FA, which are beneficial for human health. Yet, enriching these FA in milk and dairy products with the use of industrial by-products in dairy ruminants with the final scope to develop a functional food product is a challenging research endeavor. Towards this end, a next step would be the evaluation of feed supplements like rumen-protected lipids or other by-products on ruminants' diet, as well as assessing the underline molecular mechanisms related to lipid metabolism.

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Thesis Deliverables

- **Neofytou M.C.,** Miltiadou D., Sfakianaki E., Constantinou C., Symeou S., Sparaggis D., Hager-Theodorides, A.L., Tzamaloukas, O. (2020). *The use of ensiled olive cake in the diets of Friesian cows increases beneficial fatty acids in milk and Halloumi cheese and alters the expression of SREBF1 in adipose tissue.* Journal of Dairy Science, 103 (10), 8998-9011.
- **Neofytou M.C.,** Miltiadou D., Symeou S., Sparaggis D., Tzamaloukas· O. (2021) *Short-term forage substitution with ensiled olive cake increases beneficial milk fatty acids in lactating cows.* Tropical Animal Health and Production 53: 257
- **Neofytou M. C.,** Michael C., Constantinou C, Sparaggis D., Tzamaloukas O. (2021) *Feeding wheat dried distillers' grains with solubles increases conjugated linoleic acid and unsaturated lipids in ovine milk without adversely affecting milk yield.* Journal of Dairy Research (in press)
- **Neofytou M.C**, Miltiadou D., Sfakianaki E., Constantinou C., Symeou S., Sparaggis D., Hager-Theodorides A.L, Tzamaloukas O. *Diet inclusion of ensiled olive cake increases unsaturated lipids in milk and alters the expression of lipogenic genes in mammary and adipose tissue in goats*. Journal of Dairy Science (submitted)

Other Publications during Ph.D

- Tzamaloukas, O., **Neofytou M.C**., Simitzis P.E. 2021. Application of Olive By-Products in Livestock with Emphasis on Small Ruminants: Implications on Rumen Function, Growth Performance, Milk and Meat Quality. *Animals*. 11: 531. https://doi.org/10.3390/ani11020531

Abstracts in International Conference Proceedings

- **Neofytou, M.C**., Sparaggis, D., Constantinou, C., Symeou, S., Miltiadou, D., Tzamaloukas, O. (2018) *Feeding ensiled olive cake affected fat and fatty acid composition of cow milk.* Proceedings of the European Federation for Animal Science, 69th conference, EAAP 2018, Dubrovnik, Croatia. vol. 24, page 182
- Tzamaloukas, O., **Neofytou, M.C.,** Simitzis, P.E., Sparaggis, D., Miltiadou, D. (2021) *Invited talk: The use of olive cake by-product in ruminant diets.* Accepted for the proceedings of the European Federation for Animal Science, 71st conference, EAAP 2021, Davos, Switzerland