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DOCTORAL THESIS IN VETERINARY SCIENCE

"Medicinal plant supplementation in the diet of lactating ruminants: investigations on milk productive traits, quality and nutrigenomics"

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“Everything you can imagine nature has already created”

(*Albert Einstein*)

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Glossary

AA: arachidonic acid

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

ACACA: acetyl-coenzyme A carboxylase

ADF: acid detergent fiber

ADL: acid detergent lignin

ALA: alpha linolenic acid

ANOVA: analysis of variance

ATP: adenosine triphosphate

BW: body weight

cDNA: complementary DNA

CAT: catalase

CLA: conjugated linoleic acid

CF: crude fiber

COPs: cholesterol oxidation products

CP: crude protein

Ct: cycle threshold

DHA: docosahexaenoic acid

DIM: days in milk

DM: dry matter

DMY: daily milk yield

DNA: deoxyriboNucleic Acid

DPPH: 2,2-diphenyl-1-picrylhydrazyl

EE: ether extract

EPA: eicosapentaenoic acid

ER: endoplasmic reticulum

FA: fatty acids

FAME: fatty acid methyl esters

FASN: fatty acid synthase

FCM: fat-corrected milk

FFA: free fatty acids

FSP: fennel seeds powder

GAE: gallic acid equivalents

GC-MS: gas chromatography/mass spectrometry

GLUT1: glucose transporter member 1

GPx: glutathione peroxidase

GTF: general TF

HATs: histone acetyltransferases

HDL: high density lipoprotein

HMTs: histone methyltransferases

HNF4: hepatic nuclear factor 4

IGF1: insulin-like growth factor I

IL: interleukin

LA: linoleic acid

LCFAs: long-chain fatty acids

LDL: low-density lipoproteins

LdNRs: ligand-dependent nuclear receptors

LEP: leptin

LEPR: leptin receptor

LPL: lipoprotein lipase

LXRs: liver X receptors

MECs: mammary epithelial cells

MFG: milk fat globules

miRNA: microRNA

mRNA: messenger RNA

MUFA: monounsaturated fatty acid

MW: metabolic weight

NADPH: nicotinamide adenine dinucleotide phosphate

ND: not detected

NDF: neutral detergent fiber

NR: nuclear receptors

NRF2: nuclear factor erythroid 2-related factor 2

PDO: Protected Designation of Origin

PIC: pre-initiation complex

Pol II: polymerase II

PPARs: proliferator-activated receptors

PRL: prolactin

PRLR: prolactin receptor

PSCs: plant secondary compounds

PUFA: polyunsaturated fatty acids

RIN: RNA integrity

RNA: riboNucleic Acid

ROMs: reactive oxygen metabolites

ROS: reactive oxygen species

RT-qPCR: real-time quantitative polymerase chain reaction

SCD: stearoyl-CoA desaturase

SCFAs: short chain fatty acid

SEM: standard error of the mean

SFA: saturated fatty acids

SOD: superoxide dismutase

SPME: solid-phase microextraction

TAC: total antioxidant capacity

TAG: triacylglycerol

TC: total cholesterol

TE: trolox equivalent

TF: transcription factors

TG: triacylglycerides

TL: total lipids

TLR4: toll-like receptor 4

TLRs: toll-like receptors

TPC: total phenolic content

UFA: unsaturated fatty acids

UFL: feed unit for lactation

VFAs: volatile fatty acids

VLDL: very low-density lipoproteins

VOCs: volatile organic compounds

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This work was made possible by a fruitful collaboration with other Italian universities and national research institutes, whose analyses are incorporated into this project.

The study of this fascinating topic, along with the extensive range of effects that this type of integration may produce, has led to a multifaceted investigation into various aspects related to product quality and the biological mechanisms underlying the quantitative and qualitative production of milk and cheese. It is worth noting that this research has benefited from the contributions of numerous working groups.

The INRA research centre (Clermont-Ferrand) provided invaluable assistance with gene expression analyses, facilitating the study and application of methods for mRNA extraction and quantification of gene expression, which enabled me to conduct my analyses. My seven-month tenure at INRA also afforded me the opportunity to interact with leading experts in the field, including Dr. Laurence Bernard, Dr. Christelle Cebo, and Dr. Sébastien Bes. In addition to forging a strong professional relationship, I had the privilege of conducting gene expression analyses and contributing to the French LIPOMEC project, which studies lipolysis in the milk of diverse ruminant species. I am indebted to them for their guidance and support in my development as a researcher, and for the invaluable friendship they have bestowed upon me.

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Chapter I

Introduction

The objective of this doctoral research project was to evaluate the effects of incorporating medicinal plants into the diet of lactating ruminants on their productive characteristics in terms of both quantity and quality.

The decision to use medicinal plants was driven by the necessity to identify more natural supplements capable of enhancing animal performance and wellbeing. In light of the aforementioned considerations, medicinal plants, given their plethora of properties, present themselves as an optimal supplement. Three plants were selected for this purpose: fennel seeds, green aniseeds and milk thistle, specifically chosen for their galactagogue and antioxidant properties in order to test their benefits on production in both quantitative and qualitative terms. Over the course of the three-year doctoral programme, four trials were conducted: one in 2022, one in 2023, and two in 2024. The goat milk trials were carried out at the Amato livestock farm, while the buffalo milk trial was performed at Prete Carolina livestock farm of Dr. Massimo Campagna.

For this purpose, specific plant parts were selected that had undergone only thermal treatments, such as drying or grinding. The plants were provided by the company BIOKYMA. Although products derived from extraction techniques are more concentrated and sometimes more effective in achieving specific results, we opted to refrain from using extracts in order to minimise the economic impact of this supplementation, which already represents an additional expense for the animals' diet. Moreover, the objective of extraction methods is frequently the extrapolation of individual active ingredients, which often represent a minor proportion of the bioactive compounds present in the whole plant. Accordingly, the decision to use the entire plant was also driven by the knowledge that the diverse bioactive compounds within a plant, which collectively constitute its phytocomplex, often work in a synergistic manner, exerting a multitude of effects on the organism. Therefore, the amount of product was chosen for the trials according to previous

Figure 1. Grazing goats. Picture by the author.

works which tested the effects of the whole plant in ruminants' nutrition (Kholif et al., 2017; EI-Shereef 2019; Singh et al., 2023).

Similarly, the selection of plants indigenous to the Mediterranean region was made with the objective of reducing the environmental and economic costs associated with the importation of exotic plants, while simultaneously enhancing the value of resources available in this area.

These plants have not been the subject of extensive investigation in the field of animal feeding, albeit their widespread use in human nutrition. The advantages of using natural supplements in the nutrition of ruminants have been the subject of considerable investigation in recent years and have been well received by consumers, who are increasingly aware of and concerned about the impact of the use of synthetic supplements on product quality and the environment.

The nutritional characteristics of the products obtained from the trials were evaluated through chemical composition analysis, fatty acid analysis, total polyphenol content and antioxidant activity.

Moreover, much of the work was dedicated to the examination of the expression of candidate genes associated with lipid metabolism, lactation and the antioxidant system. In each trial, with the exception of the first, the RNA was extracted, and the selected gene panel was evaluated. The objective of these analyses is to gain insight into the underlying mechanisms of the galactagogue and antioxidant effects associated with the selected herbs.

Figure 2. Italian Mediterranean dairy buffalo. Picture by the author.

This thesis is structured into eight chapters: I) introduction; II) an overview of the current state of knowledge regarding milk production and the nutritional significance of dairy products with particular reference to oxidative potential; presentation of the main characteristics of the herbs chosen for the trials; III) a review of the current state of knowledge regarding the use of gene expression analyses and the presentation of the candidate genes in this PhD project; IV) trial on dairy grazing goats: "Dietary fennel seeds supplementation affects yield, fatty acid composition and

flavour profile of milk and cheese in grazing goats"; V) trial on dairy grazing goats with two medicinal herbs: "Effects of milk thistle and green anise supplementation in grazing goats' diet on milk and cheese nutritional characteristics and on the expression of a panel of genes"; VI) trial on dairy buffaloes housed in stall: "Fennel seed powder in the diet of dairy buffalo affects milk vocs profile, antioxidant activity and SCD mRNA level"; VII) trial on dairy goats housed in stall "Fennel seed powder supplementation in dairy goats: investigation on milk productive traits, fa profile and gene expression"; VIII) conclusions and general considerations.

Chapter II

The role of dairy products in human nutrition

Milk plays an indispensable role in the diets of approximately 6 billion people worldwide (Visioli and Strata, 2014). World milk production in 2023 reeached 944 million tonnes, an increase of 0.9 percent from 2022 (FAO, 2023). While milk is primarily produced by mammals for the nourishment of their young, humans in many parts of the world continue to consume milk throughout their lives. In addition to milk, a variety of dairy products, including cream, butter, yoghurt, kefir and cheese, have been produced and consumed globally for millennia. The influence of milk and dairy products on human health is therefore considerable and has been the subject of extensive investigation, into both the products as a whole and their individual components. Of particular interesest are the fat content of milk (which is primarily composed of saturated fatty acids) and certain minor constituents, such as calcium and oligosaccharides, which are being actively researched with regard to their potential health benefits.

In recent years, the growing demand for foods with potential health benefits has led to an increased focus on the development of strategies to improve the nutritional qualities of food products. Currently, consumers tend to view nutrition as a fundamental aspect of well-being, which is driving a market for foods that demonstrate health benefits (Infascelli et al., 2021). In accordance with the European Commission Concerted Action on Functional Food Science in Europe (FUFOSE), a functional food is defined as a food that positively influences one or more specific functions of the body, beyond the effects of its nutritional value. This improves health and well-being and/or reduces the risk of disease. It is imperative that functional foods retain their fundamental nature as foodstuffs, exhibiting their effects at the doses consumed in a normal diet. They are not to be considered as pills or capsules, but rather as an integral part of the regular diet (Bellisle et al., 1999).

In this new context, it is of the utmost importance that the dairy industry offers products that are not only free from any potential health risks but also possess organoleptic, nutritional, and nutraceutical characteristics that encourage consumers to choose them.

The factors that can cause both quantitative and qualitative variations in milk fat are numerous and can be classified as endogenous and exogenous. Among the endogenous factors, breed, the individual animal within the breed, the animal's health and well-being status and especially the animal's lactation stage play a fundamental role. Among the exogenous factors, climate, farming techniques, milking methods and diet are of particular significance. Nevertheless,

dietary treatment represents the most influential factor and is also the one where farmers have the greatest ability to intervene (Lestingi and De Palo, 2006).

The practice of grazing and the forage to concentrate ratio in the diet are of significant importance in enhancing the nutritional value of ruminant milk. The complete feeding of dairy ruminants on pasture has been demonstrated to significantly enhance the quality of the milk fat. In such circumstances, the fat exhibits reduced levels of lauric and palmitic acids, which are regarded as atherogenic and thrombogenic when consumed in excess, while the proportion of unsaturated fatty acids with 18 carbon atoms, including CLA, is elevated. These fatty acids have been linked to beneficial effects on human health. The nature of the forage is also a significant factor, with mountain grazing resulting in higher C18:1 content and double the CLA compared to low-altitude pastures (Lestingi and De Palo, 2006). Furthermore, milk from pasture-fed animals exhibits a natural enrichment in n-3 fatty acids in comparison to milk from animals fed hay or silage. The maturity stage of the forage is of particular importance, as grass is known to be particularly rich in lipid precursors, especially during the early vegetative stage. A number of studies have reported a more favourable n-6/n-3 fatty acid ratio in animals raised on pasture, with levels significantly lower than those observed in animals raised in stalls (Santos-Silva, 2002; D'Urso et al., 2008; Tudisco et al., 2014).

Milk production

Lactation is a comprehensive process that encompasses the synthesis and secretion of milk, as well as the development and restoration of the mammary gland. The process can be divided into four stages: mammogenesis (development of the mammary gland), lactogenesis (initiation of milk synthesis and secretion), galactopoiesis (maintenance of milk secretion), and involution (restoration of the mammary gland).

Mammogenesis starts around day 32 of embryonic development, however, structural and functional alterations, such as differentiation and involution, occur throughout the various physiological stages. Lactogenesis denotes the beginning of milk production, encompassing the enzymatic and cytological differentiation of mammary alveolar cells from the early stages of pregnancy to the attainment of full lactation following childbirth. During this stage, the mammary gland uses blood-derived precursors to synthesise the various components of milk.

The maintenance of milk production, known as galactopoiesis, follows a dynamic pattern, beginning with a rapid increase, reaching a peak, and gradually declining until the end of lactation. The neuroendocrine reflex serves as the stimulus for milk ejection in response to suckling. Mammary involution is the process by which the mammary gland returns to its pre-

lactation state through tissue remodelling following the cessation of milk production. The interactions of various hormones and growth factors are essential for the proper regulation of each stage of lactation.

Lactation is a period of high energy demand, requiring enhanced mitochondrial function. If nutrient sensing is compromised, these energy needs cannot be met. Energy production is closely linked to the generation of reactive oxygen species (ROS) and without sufficient antioxidant activity to balance this, oxidative stress can increase, potentially impairing lactation (Kelleher et al., 2024). Likewise, increased ROS can trigger inflammation, which, whether systemic or localized in the mammary gland, may reduce milk production and result in a low milk supply.

Consequently, proper nutrition and health management during this period are crucial for ensuring adequate milk production.

Feeding treatment and the role of phenolic compounds

The practices employed in livestock farming are inextricably linked to the health, productivity and overall welfare of the animals. To guarantee optimal animal health and to produce safe, high-quality animal products, the management of their diet is frequently regarded as the most straightforward strategy that can be implemented at the farm level. Significant advancements have been made in various aspects of beef and dairy production, including animal genetics, care, management, health, and nutrition.

The quality of animal well-being is dependant on a number of factors including the quality of the nutrition provided, animal's reproductive health, overall health, and farm management practices (Bach, 2012).

In addition, there is a growing interest in the production of healthier animal products through the enhancement of the ratio of polyunsaturated (PUFA) to saturated fatty acids through dietary modulation. Nevertheless, this strategy is associated with an increase in lipid peroxidation. It is therefore, important to mitigate oxidative deterioration in order to maintain the quality and safety of meat and dairy products (Serra et al., 2021). The addition of antioxidants to animal feed can assist in the control and reduction of the onset of oxidation.

Indeed, there is a substantial body of scientific research and practical experience which has demonstrated the efficacy of cost-effective methods for enhancing the profitability of ruminants by optimising their feed with an appropriate balance of nutrients and additives (McGrath et al., 2018). The strategic incorporation of feed additives offers a significant opportunity to enhance feed efficiency and overall animal production. Feed additives are defined as substances added

to an animal's diet that produce a beneficial effect, often without providing direct nutritional value (Hutjens, 1991). Their primary function is not to meet the animal's nutritional needs directly but to modify digestive or metabolic processes, thereby enhancing nutrient absorption and boosting productivity.

A diverse range of feed additives is available, encompassing minerals and probiotics, among other substances. In recent years, there has been a growing interest in plants and their extracts due to their nutraceutical properties. Furthermore, herb-based feed additives are preferred to synthetic ones, due to their reduced environmental impact and cost-effectiveness (Jiang et al., 2016) and are perceived by consumers as safe. In extensive farming systems, ruminants naturally consume a variety of plants when allowed to graze freely, selecting different types to strengthen their immune systems, defend against parasites, alleviate gastrointestinal issues, and more (Villalba et al., 2024). This behaviour may be regarded as a natural means of enhancing the nutritional value of their diet, due to the presence of plant secondary compounds (PSCs). These PSCs constitute a large group of molecules produced by plants as secondary metabolites, often containing the active ingredients responsible for the plant's observed effects (van Vliet et al., 2021).

Among the various classes of PSCs, those of the phenolic group are of particular interest due to their extensive range of applications and effects. A substantial body of research has investigated the potential benefits of using plant extracts or by-products, with a particular focus on those rich in polyphenols. Such products can be employed to enhance feed efficiency (Fahim et al., 2022), improve animal welfare and elevate the quality of animal products (Villalba et al., 2024).

Phenolic compounds represent a vast and diverse group of chemical substances, encompassing over 8,000 different compounds with varied chemical structures and biological activities (Serra et al., 2021). They constitute a significant component of both human and animal diets. These compounds play a pivotal role in plant signalling and defence mechanisms, enabling plants to withstand stress caused by pathogens and predators. Additionally, they neutralise free radicals by transferring a hydrogen atom from their hydroxyl group. The mechanism through which phenolic compounds (ROO-) react with peroxyl radicals involves the concerted transfer of a hydrogen ion from the phenol to the radical, forming a transition state with an H-O bond sharing one electron.

Effect of dietary treatment with natural antioxidants on milk production and animal health

As has been previously indicated, there is a growing interest in the use of natural antioxidants in livestock feed due to the numerous potential benefits that these compounds offer in relation to animal welfare and the quality of animals' products. This has been corroborated by various authors (Bešlo et al., 2023; Ponnampalam et al., 2022; Tsiplakou et al., 2021).

The incorporation of natural antioxidants into the diets of livestock animals has been shown to result in notable improvements in both the quantity and quality of milk production. These natural compounds, derived from sources such as herbs, plant extracts, or by-products of the food industry, exert a beneficial influence on the oxidative stability and bioactive content of milk while supporting overall animal health.

The inclusion of natural antioxidants like phenolic compounds, flavonoids, and vitamins (A and E) has the effect of enhancing the nutritional profile of milk. For instance, the supplementation of dairy cattle diets with herbal mixtures (e.g., oregano, thyme, and cinnamon) has been demonstrated to increase the content of essential proteins such as β-lactoglobulin and lactoferrin, and fat-soluble vitamins. This is accompanied by an improved antioxidant capacity in milk (Stobiecka et al., 2013). This boost in antioxidant properties is crucial for preventing oxidative stress in both animals and consumers of dairy products (Tsiplakou et al., 2021). The supplementation of garlic essential oil and powder to cows and ewes has been demonstrated to increase the content of beneficial FA in milk. These include CLA isomers, as evidenced by studies conducted by Yang et al. (2007) and El-Shereef (2019), as well as rosemary extract in goats' milk, as documented by Kholif et al. (2017).

A variety of dietary interventions are indeed designed to enhance the quality of animal-derived products by increasing the PUFA content, thereby producing healthier end products. It is therefore of great importance to maintain the quality of meat and dairy products by mitigating oxidative decay. It has been demonstrated in several studies that the supplementation of naturally derived antioxidant molecules can effectively control and reduce the occurrence of oxidative stress in the final product (Ponnampalam et al., 2022). These mechanisms contribute to the extension of product shelf life, the enrichment of the product with antioxidant compounds that may have beneficial effects on consumers, and the protection of certain macromolecules, such as unsaturated fatty acids, from lipid peroxidation processes, which can occur during the maturation of cheeses or meat.

Lipid oxidation is regarded as one of the most deleterious processes affecting animal tissues and animal-derived products, such as milk and meat. These reactions result in the formation of

oxidised lipids and fatty acids, which subsequently generate free radicals. The oxidation by-products, including (2E)-4-hydroxyalk-2-enals and aldehydes (e.g., malondialdehyde), alkanes, lipid epoxides and alcohols, have the potential to interact with proteins and nucleic acids, resulting in molecular damage (Ponnampalam et al., 2022). The cumulative effects of lipid oxidation can impair various cellular functions, including reduced membrane fluidity, increased permeability of cell membranes and damage to membrane proteins. This results in the inactivation of receptors, enzymes, and ion channels.

Studies on buffaloes and cows show that natural antioxidant supplementation can modestly improve milk yield by enhancing metabolic efficiency and reducing stress-related production drops. For example, buffaloes fed diets enriched with antioxidants displayed better milk clotting properties and sustained production levels even under challenging conditions (Evangelista et al., 2022). However, while antioxidant supplementation has a stabilizing effect on milk production, the extent of the increase depends on the specific additive used and the baseline health of the animals.

In addition, the inclusion of natural antioxidants in the diet of livestock has been shown to significantly influence their endogenous antioxidant systems, offering multiple benefits for health and productivity. Natural antioxidants, such as flavonoids, polyphenols, and essential oils derived from plant sources, act by neutralizing reactive oxygen species (ROS) and improving the oxidative stability of animal cells. This enhancement reduces oxidative stress caused by environmental factors, high metabolic demands, and diseases, ultimately promoting cellular health.

Several studies have reported that natural antioxidants can increase the activity of key antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) (Manuelian et al., 2021). These enzymes are essential for neutralizing harmful free radicals, protecting cell membranes, and maintaining redox balance in tissues. For example, research has demonstrated that supplementing ruminant diets with plant-based antioxidants enhances their antioxidant capacity, particularly under conditions of stress such as weaning or high-density housing. This results in improved immune responses and overall resilience (Wang et al., 2022).

Additionally, dietary antioxidants help mitigate oxidative damage to lipids, proteins, and DNA, which can impair reproductive and metabolic functions. In ruminants, antioxidant-enriched diets have been associated with lower cortisol levels, enhanced growth performance, and improved feed efficiency (Wang et al., 2022).

The mode of action and efficacy of these supplements can vary depending on the antioxidant type, dosage, and administration method.

Natural antioxidants present a promising alternative to synthetic additives for the production of high quality dairy products aligning with consumer demand for such products. Their role in improving milk's oxidative stability, nutrient content, and production efficiency highlights their potential for broader application in livestock farming.

Medicinal plants

In order to enhance production efficiency, optimise animal health and address concerns pertaining to sustainability, researchers and farmers are increasingly turning to nutraceuticals. These bioactive compounds, derived from natural sources, have the potential to confer health benefits (Pandey et al., 2023). They function as alternative feed additives, exerting a beneficial influence on the immune system through their antioxidant, anti-inflammatory, and antimicrobial properties. This, in turn, has the effect of improving animal health and productivity. Specifically, their antioxidant activity helps to regulate oxidative stress, which can have a severely detrimental impact on immune system functions, increasing disease incidence. An expansion of the knowledge base regarding these plants and their benefits offers an opportunity to use local resources to enhance animal performance and reduce the environmental impact of livestock production, while also minimising the use of synthetic drugs.

A review of the historical record reveals that plants have been employed as remedies in a variety of medical traditions (Balick and Cox, 2020). Ethnobotanical studies demonstrate the pervasive utilisation of a multitude of plant species, reflecting the substantial potential of these substances, which have been employed for centuries. A substantial body of evidence from human medicine and nutrition demonstrates the preventive and therapeutic value of plants, thereby supporting their regular use to promote health (Gurib-Fakim, 2006). Nevertheless, research in the field of animal husbandry that is focused on enhancing production through commercial application remains limited. These plants are increasingly being recognised for their potential in controlling parasites in ruminants, with their consumption being linked to enhanced performance and anthelmintic effects.

Medicinal plants can be defined as the plants that possess therapeutic properties or exert beneficial pharmacological effects on human or animal body (Namdeo, 2018). Among them, the galactagogue group appears to be of particular interest in dairy ruminant since these plants are characterized by different bioactive compounds able to enhance milk production.

Medicinal plants have been demonstrated to be effective in ruminants for the treatment of parasitic diseases (González et al., 2020), mastitis (Nair and Punniamurthy, 2016), and other conditions (Abdalla and McGaw, 2020). These plants contain a diverse array of bioactive molecules, including flavonoids, tannins, glucosides, alkaloids, saponins, terpenoids, amines, non-protein amino acids, and organosulfur compounds (Irchhaiya et al., 2015).

A considerable number of plant species have been demonstrated to possess anti-inflammatory properties, making them a viable option for the treatment of a multitude of conditions marked by inflammatory processes, including infections, trauma, and infestations. These plants have

been shown to provide symptomatic relief in such cases. Extensive in vitro and in vivo research has elucidated the composition and effects of these plants, demonstrating their mechanisms of action and the conditions under which they operate. In general, the majority of herbs demonstrate anti-inflammatory and antioxidant properties to varying degrees, being suitable as natural remedies. However, certain plants are employed in the treatment of specific diseases, and when intended for multiple purposes, they may be prepared in a manner that differs from the standard method. This versatility is attributable to the diverse range of bioactive compounds present within the plant, which exert multiple therapeutic properties upon a single organism.

The effective utilisation of plant-based remedies necessitates the utilisation of specific pharmaceutical formulations in order to achieve the desired preventive or therapeutic effects. A thorough examination of the chemical composition and properties of bioactive compounds allows for the selection of the most effective extraction and administration methods, which can then be used to exhibit specific effects. It is common practice to combine multiple herbs or their extracts in order to achieve a synergistic effect, thereby enhancing the benefits of individual herbs and, in some cases, mitigating the side effects of specific drugs.

Plant-based remedies can be administered in a variety of forms, including powdered (in either loose form or pills) or in extracts prepared through different processes (such as infusions, decoctions, dry extractions, or liquid extractions) (Gurib-Fakim, 2006). Furthermore, less common formulations, including mother tinctures, gemmotherapy extracts and essential oils, are also employed. Selecting the optimal mode of administration is a pivotal aspect of ensuring the efficacy of a given treatment or supplement. This step must be carefully tailored to a specific objective, as different formulations of the same remedy can yield varied pharmacological results (Abubakar and Haque, 2020).

In the last years the use of synthetic chemicals has become prevalent and in response, public awareness of the environmental and health risk has increased the drive to find more natural alternatives (Rochfort et al., 2008). As a matter of fact, recently, the interest in ethnoveterinary medicine has grown leading to more than thousand papers published in European countries between 1990 and 2013 which considered the use of medicinal plants in ruminants (Mayer et al., 2014).

Fennel (Foeniculum vulgare Mill.)

Fennel (*Foeniculum vulgare Mill*) is an aromatic plant belonging to the family of *Apiaceae* traditionally used as a medicinal plant in human healthcare (Badgujar et al., 2014), employed also for its carminative, digestive and diuretic properties (Rather et al. 2016). *Foeniculum Vulgare Mill.* is a perennial, aromatic plant with many subspecies and varieties. *F. vulgare* subsp. *vulgare* var. dulce is called sweet fennel, while *F. vulgare* mill. Subsp. *vulgare* var. vulgare is referred to as bitter fennel. The plant has grooved stems and intermittent leaves that are combined with dark green, fluffy leaves and sheathed petioles. The flowers are typically bisexual, irregular, or regular, and have yellow umbrellas that resemble oval beads. The seeds of fennel are characterised by a fragrant scent and a pleasant flavour. The seeds are approximately 8 mm in length and 3 mm in width. The seeds of fennel exhibit a long, thin, cylindrical morphology, with dimensions that fluctuate in accordance with the growth of the plant (Noreen et al., 2023). Fennel is still largely used in popular medicine, especially in Mediterranean countries and in Iran (Akbar, 2020a); it is indeed one of the most commonly consumed herbs by more than a quarter of Italian pregnant women every day for at least 3 months during pregnancy (Facchinetti et al., 2012).

Figure 1. Fennel flower. Source: https://www.piante.it

Hippocrates and Dioscorides mentioned fennel as diuretic and emmenagogue, and the juice was supposed to sharpen the eyesight. As one of the ancient Saxon people's nine sacred herbs, fennel was credited with the power to cure, and was valued as a magic herb (Akbar, 2018). In the Middle Ages it was draped over doorways on Midsummer's Eve to protect the household from evil spirits. It is one of the most frequently quoted plants in The Chilandar Medical Codex,

the best preserved medieval Serbian manuscript on European medical science from the 12th to 15th centuries (Akbar, 2018).

Figure 2. Fennel seeds. Source: https://www.misya.it

From a nutritional standpoint, fennel seeds are notable for their protein content, comprising 15.85% of the seed, along with 8.29% crude oil and an essential oil content of approximately 2.60% (Unal et al., 2013). In fennel seed oil, an average of 93% of total oil consists of UFA. The C18:1 c6, C18:2, C18:1 c12 and C16:0 acids corresponding to approximate 97% of total oil are recorded as the principal fatty acids, and the values of C18:0, C14:0, C18:3 and C20:0 are lower than 2% (Coşge et al., 2008).

Its main phytocomponents are phenolic compounds, glycosides and other volatile organic compounds (VOCs) among which the trans-anethole, estragole, limonene and fenchone are the most abundant and of particular importance for their medical properties (Rather et al., 2016).

In particular, fennel's galactagogue effects are related to the great content in trans-anethole, a monoterpene able to inhibit the link of dopamine with its own receptors acting as antagonist (Hassanzadeh et al., 2022) hence reducing the effects of dopamine on prolactin (PRL) (Benker et al., 1990). Through this process, PRL works for a longer time enhancing the production of milk by a direct effect on mammary gland (Lacasse et al., 2012).

Several studies demonstrated that supplementing breastfeeding women diet with fennel seeds increases both milk production and the weight of the newborn (Akbar, 2020b; Alachkar et al., 2011; Facchinetti et al., 2012) whereas in animal field the studies are much fewer, especially on dairy ruminants. El-Hendawy et al. (2018) tested fennel seeds as supplement for Damascus goats showing an improvement in reproductive parameters, milk yield and chemical composition, while Moosavi-Zadeh et al. (2023) investigated fennel effects on dairy cows'

feeding by evaluating milk productive characteristics. Both in cows and in goats, fennel supplementation increased milk yield, while different results were observed for the content of lactose, protein and fat. As far as we know from the literature, few studies have been made on the characteristics of cheese obtained by animals supplemented with fennel seeds, while the effects of adding fennel (as it is or as aqueous extract or as essential oil) during cheesemaking are more investigated and consistent. Previous studies have shown that incorporating antioxidant additives into the diet of animals can enhance the nutritional properties of their food (Esposito et al., 2023; Morittu et al., 2021). The addition of aqueous extracts of fennel during cheesemaking, indeed, improved stability and shelf-life of the products (Mansha Rafiq and Ghosh, 2021) by increasing its antioxidant properties (Caleja et al., 2015). Similar results were observed in cheese obtained by fennel supplemented goats (Ismail, 2004) where the chemical, microbiological and organoleptic properties of Domiati cheese were evaluated. A significant decrease in lipolysis and proteolytic phenomena as in the viable bacterial count of coliform and spore-forming bacteria was observed.

Milk thistle (Silybum marianum L.)

Silybum marianum L., more commonly known as milk thistle, is a biennial plant belonging to the *Asteraceae* family. The species is native to the Mediterranean region, but it is now cultivated globally (Bijak, 2017). For centuries, it has been employed in traditional medicine, primarily for the treatment of conditions affecting the kidneys, spleen, liver, and gallbladder (Flora et al., 1998; Schadewaldt, 1969). In accordance with an ancient Christian tradition, the epithet 'marianum' is thought to have its origins in the white streaks that adorn the plant's leaves. These are believed to symbolise the milk of the Virgin Mary. According to the Christian tradition, these markings were left in the aftermath of Mary spilling some of her milk while nursing the infant Jesus during their run to Egypt with Joseph.

Figure 2. "Madonna litta". Leonardo Da Vinci.

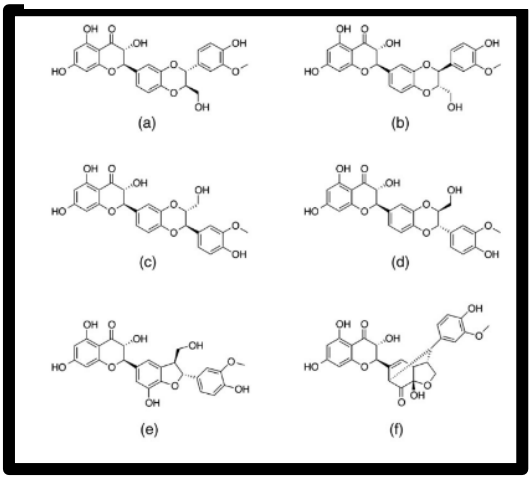
As early as the sixteenth century, Pietro Andrea Mattioli, a renowned Italian humanist and physician, described the qualities of milk thistle:

«*La radice scalda, monda, apre e assotiglia. La cui decottione dà utilmente nelle oppilationi del fegato e delle uene, per prouocar l'orina ritenuta...Prouoca la medesima i menstrui non solamente beuta, ma anchora sedendouisi dentro...*»

"*The root warms, cleanses, opens, and thins. Its decoction is useful for liver and vein obstructions, to stimulate retained urine... It also induces menstruation, not only when ingested, but also when sitting in it...*"

Figure 3. Milk thistle leaves. Photo by © Giancarlo Dessì

Figure 4. Chemical structures of main flavolignans contained in silymarin, namely (a) silybin A, (b) silybin B, (c) isosilybin A, (d) isosilybin B, (e) silycristin and (f) solydianin. (Abenavoli et al., 2018)



The leaves of the milk thistle are notable for their high protein content, which can reach up to 25%, whereas crude oil, which is primarily concentrated in the seeds and flowers can reach up to 20% (Marceddu et al., 2022). The lipid fraction is primarily constituted of UFA, particularly C18:3n6 and C18:2n6, while other less prevalent fatty acids are C16:0 and C18:0 (Malekzadeh et al., 2011).

Its main phytocostituent is silymarin, which is a well-established milk thistle seeds standardized dry extract containing mainly flavonolignans (about 70%–80%) as well as polymeric and oxidized polyphenolic compounds consisting of a mixture of flavonoids. Flavonolignans, which have been first discovered in the seeds of *Silybum marianum L.*, are a relatively small subclass of compounds, where the flavonoid part of the molecule is fused with a lignan. The main silymarin flavonolignans are silybin, isosilybin (A and B), silydianin, and silychristin (Abenavoli et al., 2018).

The main part of the literature available studies has been dedicated to flavonolignans giving less attention to minor components. This has led to problems in determining the exact composition of silymarin, which can vary depending on the processing, variety of the plant, soil composition, and climatic conditions during the plant growth. Silymarin contains also a mixture of undefined polyphenolic compounds, often referred to as "polymeric fraction" (Bijak, 2017). Nonetheless, the oil fraction, which contains linoleic, oleic, and palmitic acids, sterols, tocopherol (vitamin E), and phospholipids, has been not fully explored (Chambers et al., 2017). Despite being widely used as a galactagogue in breastfeeding women, few studies have been conducted in dairy animals, and one study conducted in rodent models, where a 14-day treatment with Silymarin BIO-C (25–200 mg kg−1 BW) demonstrated increased serum PRL levels in lactating rats (Capasso et al., 2009).

Figure 6. Milk thistle flower. Source: https://ciclamino.it

The primary mechanism of action of milk thistle on the liver is through silymarin. The hepatoprotective effects of silymarin are attributed to a number of mechanisms, including its

antioxidant, anti-lipid peroxidation, and anti-inflammatory properties. These compounds facilitate liver detoxification by inhibiting phase I detoxification processes while enhancing glucuronidation and preventing the depletion of glutathione. Furthermore, silymarin has been demonstrated to reduce inflammation by inhibiting the synthesis of leukotrienes and prostaglandins. Additionally, it has been shown to promote liver cell protein production, thereby facilitating liver tissue regeneration (Porwal et al., 2019).

Silymarin is frequently employed for its hepatoprotective effects in animals during the peripartum period, with the objective of reducing the risk of conditions such as negative energy balance, ketosis, and liver fat accumulation (Tedesco et al., 2004). While the use of a liver-protective agent is beneficial during this phase, it is crucial to maintain liver health throughout lactation. The liver plays a role in milk production by synthesising plasma proteins and processing fats for conversion into milk. Furthermore, it is responsible for the production of hormones and other molecules that are essential for lactation.

Green anise (Pimpinella anisum L.)

Anise (*Pimpinella anisum L.*) is an aromatic annual herb belonging to the *Apiaceae* family which origins from eastern Mediterranean and western Asian areas. It is now cultivated in a number of regions, including southern Europe, northern Africa, South America, China, and Japan. The principal exporters of anise are Turkey, Egypt, and Spain, whereas Russia, Spain, and Poland are the predominant suppliers of anise oil (Singletary, 2022). The plant's small fruit, frequently erroneously designated a seed is usually identified as "aniseed." Once the fruit has ripened and undergone the drying process, it is used as a popular spice with a sweet, liquorice-like flavour. The fruit and its essential oil are employed as flavourings in a variety of foodstuffs, including baked goods, sweet treats, puddings, curries and frozen desserts.

Aniseeds are characterised by a protein content of approximately 17.40% and a crude oil content of 16.23% (Jordanovska et al., 2023). The lipid fraction of the seeds is composed of a considerable quantity of C18:2n6 and C18:1n9 (Topčagić et al., 2022).

The essential oil derived from aniseed comprises a number of principal compounds, which vary depending on the geographical region in which the plant is cultivated and the method employed for its extraction. These typically include trans-anethole (85%–95%), γ-himachalene (0.4%–8.2%), methyl chavicol (0.5%–5.0%), p-anisaldehyde (0%–5%), and pseudoisoeugenol-2-methylbutyrates (1.3%–3.0%) (Singletary, 2022). Historically, aniseed was employed to facilitate childbirth and stimulate milk production. It remains a component of folk medicine, utilized for the treatment of a range of ailments. Due to its high trans-anethole content, aniseed is employed in the treatment of coughs, respiratory issues, migraines, digestive problems, colic and skin infections. Additionally, it has been demonstrated to possess sedative, aphrodisiac and galactagogue properties. Current research is exploring the potential of aniseed to assist with conditions such as diabetes, dysmenorrhea, and menopausal symptoms, as

Figure 5. Green anise. Source: https://www.armoniedinatura.it

Figure 8. Green anise flower. Source: https://www.gaiagarden.com

well as its antioxidant, anti-inflammatory and antimicrobial properties.

In ancient times, Dioscorides observed that anise could enhance milk production, while Pliny the Elder noted that its effects seemed to be linked to estrogenic properties (Albert-Puleo, 1980). Dalion, a prominent herbalist, recommended anise for the management of labour pain and uterine discomfort, often in conjunction with other herbs such as dill. Furthermore, it was postulated that inhaling or ingesting the substance in the form of a drink could facilitate the process of childbirth. Pliny even described it as an aphrodisiac. In addition, medieval herbals highlighted the estrogenic properties of anise and fennel, citing their capacity to stimulate lactation and enhance libido (Albert-Puleo, 1980).

Aniseed has been employed in veterinary field as a growth promoter, immune booster and to increase dry matter intake in animals. It has been demonstrated to stimulate digestive enzymes and to possess antiparasitic, antibacterial, antifungal and fever-reducing properties. The effects of aniseed are largely attributed to its anethole content, which has a chemical structure similar to that of catecholamines, thereby producing sympathomimetic effects (Iftikhar et al., 2017).

Chapter III

Epigenetics and nutrigenomics

The interaction between genetics and the environment, nature and nurture, is a fundamental determinant of health and disease across all domains (Simopoulos and Childs, 1975). Nutrition represents one of the most significant environmental factors influencing this interaction. Advances in molecular biology and genetics have facilitated the study of inherited diseases at the DNA level and the investigation of nutrients at a molecular level. This has resulted in the emergence of research focusing on the evolutionary aspects of diet and how nutrients influence gene expression (for example, PUFA have been observed to suppress the expression of the fatty acid synthase gene, a concept known as nutrigenomics) (Simopoulos, 2020).

Nutrigenomics may provide a framework for the development of novel foods designed to promote health and manage chronic diseases in accordance with specific genotypes.

The term "nutrigenomics" is used to describe the interactions between nutrients and the genome. It is important to note that the term "nutrigenomics" does not refer to the impact of nutrients on the DNA sequence itself. Rather, it encompasses the influence of nutrients on gene expression through the involvement of transcription factors (TF) in the short to medium term and epigenetic mechanisms over the medium to long term. Bioactive compounds with the potential to exert nutrigenomic effects are present in the typical diets of dairy ruminants. These compounds can either directly or indirectly influence the activity of TF.

As a discipline that has emerged from the post-genome era, nutrigenomics has attracted increasing attention due to its potential implications for humans, companion animals and livestock species. In dairy cattle and ruminants more broadly, this field is still in its infancy. Nevertheless, it offers considerable potential for improving both health and productivity (Coffey, 2007).

The field of epigenetics introduces an additional layer of regulation at the genomic level, influencing the transition from genotype to phenotype by regulating the timing and location of gene expression. The field of functional genomics provides researchers with the tools necessary to generate new hypotheses regarding the molecular mechanisms through which nutrients exert their effects. Nutrients act as dietary signals, which are detected by cellular sensor systems. This influences gene and protein expression, which in turn affects metabolic outcomes.

In ruminants, these effects may play a pivotal role in physiological adaptations during the onset of lactation, such as the increase of specific gene sequences through alterations in DNA methylation (Osorio and Moisa, 2019). This process enables the transcription of essential proteins, such as caseins, in the mammary glands of dairy ruminants. This nascent field of

research, designated "nutriepigenomics," promises to yield invaluable insights into the molecular-level impact of nutrients on ruminant biology (Osorio and Moisa, 2019). The advent of nutriepigenomics introduces an additional layer of complexity to this field, necessitating a profound comprehension of these interactions and, eventually, the capacity to manipulate them through dietary strategies.

The term "epigenetics" is currently used to describe modifications that occur on top of the genetic code, while the word 'epigenesis', first coined by the Greek philosopher Aristotle, was used to describe the process by which any organism develop (Hyde et al., 2020). This concept was introduced as a contrast to the preformation theories that were prevalent at the time. In the 1930s, systems biologist Conrad Hal Waddington further developed the concept during his studies on the embryological development of Drosophila melanogaster. The current definition of epigenetics is that it is a heritable and reversible phenomenon that alters gene expression without changing the underlying DNA sequence. Each somatic cell in an organism possesses the same genome, yet only a subset of genes is expressed in each cell type, thereby determining its identity and function within the tissue environment (Hyde et al., 2020). Epigenetics is the mechanism that determines which genes are expressed in different cell types. As embryonic stem cells differentiate from a pluripotent state, their epigenetic patterns undergo continuous modification, resulting in the formation of distinct cell types that constitute tissues and organs. Epigenetic alterations entail the attachment of chemical tags to DNA or its associated proteins, without modifying the nucleotide sequence itself.

In order to fit within the cell, the DNA is wound around proteins called histones, forming a structure known as a nucleosome. These nucleosomes are subsequently compacted into chromatin fibres and ultimately condensed into chromosomes. For a gene to be actively transcribed, the chromatin structure surrounding it, must undergo a relaxation process, thereby allowing the transcriptional machinery to gain access to the DNA. Epigenetic marks play a key role in regulating chromatin compaction and serve as docking sites for transcriptional regulators, thereby coordinating gene expression.

Epigenetic "readers" are proteins that bind to these marks and can either modify chromatin structure or recruit other proteins to regulate gene expression. Together with epigenetic "writers" and "erasers", these readers play a role in the overall orchestration of gene expression profiles within a cell (Hyde et al., 2020). During the process of cell division, the epigenetic patterns of the parent cell are replicated in the newly formed DNA of the daughter cells, thereby ensuring the preservation of these patterns. However, epigenetic patterns can also be influenced

by environmental factors, thereby enabling cells and organisms to adapt to changes in their environment, including variations in nutrition, stress, or toxin exposure.

In recent years, the application of concepts from molecular biology has been extended to encompass the study of food components and essential nutrients as factors that influence gene expression. In the context of chronic diseases, the impact of dietary cholesterol and fatty acids on gene expression is of particular significance. For example, dietary cholesterol has been demonstrated to exert a pronounced inhibitory effect on the transcription of the gene encoding β-hydroxy-β-methylglutaryl-CoA reductase (Simopoulos, 2020). PUFA have been demonstrated to reduce the hepatic messenger RNA (mRNA) production of fatty acid synthase, thereby influencing serum lipid levels. The extent of this suppression is contingent upon the degree of fatty acid unsaturation, with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) exhibiting greater efficacy than arachidonic acid (AA). Furthermore, omega-3 fatty acids have been observed to reduce mRNA levels for platelet-derived growth factor and interleukin (IL)-1β, indicating that this process may be subject to transcriptional regulation (Simopoulos, 2020).

Gene expression is the process by which the information encoded in DNA is converted into functional proteins or RNA within a cell or the body. This encompasses genes that code for mRNA, which are translated into proteins, as well as genes for noncoding but functional RNA molecules, including transfer RNA, ribosomal RNA, small nuclear RNA, small nucleolar RNA, microRNA (miRNA) and others. The regulation of gene expression is a highly controlled process. Despite the fact that all cells in an animal's body originate from a single cell, namely the fertilised egg or zygote, and thus essentially share the same genome, the portion of expressed information differs from one cell type to another. The number of cellular proteins present varies considerably, with some being abundant, while others are present in only a few copies per cell. Some genes are expressed at relatively constant levels (constitutive genes), whereas others exhibit variable expression in response to internal and/or external signals (inducible genes) (Bonet and Palou, 2020).

Modifications in gene expression are of great importance for the regulation of cell fate and differentiation, as well as for the adaptation to stress and the control of metabolism. The regulation of protein-coding gene expression can occur at various stages, from the synthesis of mRNA to the protein's lifespan, and is often coordinated at multiple stages. Post-transcriptional regulatory mechanisms, including alternative mRNA splicing, polyadenylation, RNA editing, alternative translation initiation, and gene silencing via miRNA, also exert a considerable influence (Bonet and Palou, 2020).

The transcription of protein-coding genes starts with the formation of a pre-initiation complex (PIC) on the core promoter, which is the minimal DNA sequence indispensable for accurate transcription initiation. The PIC comprises RNA polymerase II (Pol II) and general TF (GTF), which are proteins that are indispensable for the initiation of transcription of all protein-coding genes. In a chromatin environment, the assembly of the PIC can be inefficient due to the wrapping of eukaryotic DNA around nucleosomes and the formation of higher-order chromatin structures, which restrict access to the transcription machinery. Consequently, in eukaryotes, the default state of DNA expression is largely constrained. In order to achieve optimal transcription levels within a nucleosomal context, the assembly of the PIC frequently necessitates the assistance of sequence-specific DNA-binding TF (Bonet and Palou, 2020).

Transcription factors play an important role in nutrigenomics, working as intermediaries between dietary nutrients and alterations in gene expression. Nutrient-responsive TF can be activated either directly or indirectly by nutrients. Upon activation, these TF migrate from the cytoplasm to the nucleus, where they modify the transcription of specific target genes. TF bind to short DNA sequences, typically 6–12 nucleotides in length, known as response elements. These are found in enhancer regions located upstream of the gene sequence (Osorio and Moisa, 2019). The capacity of TF to regulate gene expression in response to nutrients renders them a pivotal element in the field of nutrigenomics.

Although estimates suggest that there are between 2,000 and 3,000 TF with sequence-specific DNA-binding domains in the human genome, only approximately 100 have been experimentally confirmed for their DNA-binding and regulatory functions (Vaquerizas et al., 2009). The Animal Transcription Factor Database (Animal TFDB) has collated data on approximately 1,300 transcription factors and around 400 transcription cofactors for *Bos taurus* (Zhang et al., 2015). Rather than functioning in isolation, TF are believed to operate within a regulatory network, which is essential for coordinating responses to external stimuli and translating them into changes in gene expression.

Among the various TF, the nuclear receptor (NR) superfamily is of particular importance as nutrient sensors. A subset of transcription factors within this superfamily has been identified as ligand-dependent nuclear receptors (LdNRs), which are capable of binding to and being activated by both macronutrients and micronutrients. Recent reviews have concentrated on the principal LdNRs with potential involvement in nutrigenomics, particularly in large and small ruminants (Osorio and Moisa, 2019). The key LdNRs associated with macronutrients, such as fatty acids, include peroxisome proliferator-activated receptors (PPARs), liver X receptors (LXRs), and hepatic nuclear factor 4 (HNF4).

PPARs play a pivotal role in regulating genes involved in lipid metabolism, inflammation, glucose uptake in mammary tissue and in milk fat synthesis. A noteworthy attribute of PPARs is their capacity to bind to and be activated by long-chain fatty acids (LCFAs) in both monogastric and ruminant species.

Transcription factors regulate target gene transcription through a variety of mechanisms, frequently involving direct protein-protein interactions with GTFs and/or RNA Pol II. These interactions facilitate (activators) or impede (repressors) the assembly of PICs, the initiation of transcription, or the reinitiation of transcription. Additionally, numerous transcription factors possess enzymatic activity that can remodel chromatin at the core promoter or other cis-regulatory DNA elements. This remodelling affects the accessibility of these elements to trans-acting binding factors (GTFs or TFs) and may also result in the recruitment of other protein complexes with such activity (Bonet and Palou 2020).

Furthermore, TF may facilitate chromatin looping or post-initiation functions by regulating the transition of RNA Pol II from a paused state to productive elongation.

The interplay between chromatin structure, encompassing its dynamic organisation in nucleosomes and higher-order structures, and TF interactions with DNA is of particular importance for regulating gene expression at the transcriptional level. The majority of transcription regulation is achieved by modulating the regulators themselves, namely by altering the abundance and/or activity of TF, coregulators, chromatin re-modellers and other involved proteins. Signals that impact gene expression include those derived from nutrition and metabolism, hormones, second messengers, and various forms of stress (thermal stress, oxidative stress, organelle stress, hypoxia, and DNA damage, among others). Some signals act directly, for example, by binding to a TF molecule, while others act indirectly by influencing metabolic pathways and signalling cascades that, in turn, impact gene regulatory mechanisms, often through covalent modifications of TF or TF-interacting proteins (Bonet and Palou 2020).

Control of gene expression by dietary factors

Dietary factors that influence gene regulation encompass a range of specific food components, including macronutrients, micronutrients, phytochemicals and other bioactive compounds, as well as xenobiotics. Additionally, different dietary patterns, such as those with varying macronutrient compositions, and nutritional conditions, such as calorie restriction, overfeeding and frequent feeding, also have an impact.

Dietary factors exert an influence on gene expression, whether directly or indirectly, at various stages of the process, from transcription to translation and protein activity. At the level of transcription, they exert an influence on both the activity of TF and chromatin regulation, both in the short and long term. The impact of dietary factors on gene expression can facilitate the utilisation and storage of dietary nutrients, enable adaptation to changes in nutrient availability, influence cell fate (e.g., cell growth and proliferation), and may be associated with the maintenance of health or the development of diet-related diseases (Bonet and Palou, 2020).

The impact of dietary factors on gene expression can be tissue-specific and dependent on a number of factors, including genetic polymorphisms, DNA and histone modifications (epigenetics), other individual factors (e.g. health status) or other dietary components. These sources of variability contribute to the complexity of the control of gene expression by dietary factors and to the research in this area.

An increase in nutrient abundance results in a corresponding elevation of the nuclear acetyl-CoA pool, which in turn facilitates histone acetylation and gene transcription. It is noteworthy that this increased histone acetylation is predominantly observed in genes that facilitate cell growth and those involved in nutrient utilisation and storage, resulting in their upregulation.

Nutritional factors also exert an influence on methylation processes, as specific nutrients (such as methionine, choline, folate, vitamin B12, vitamin B6, zinc, and selenium) serve as substrates or essential cofactors in the one-carbon metabolism pathway, which generates S-adenosylmethionine, the universal cellular methyl donor. It has been demonstrated that alterations in the consumption of these nutrients can impact DNA methylation in cells, which can subsequently affect gene expression, typically resulting in transcriptional silencing (Bonet and Palou, 2020). Furthermore, specific food compounds have the capacity to modulate chromatin re-modellers within cells via an allosteric mechanism. For example, dietary phytochemicals such as polyphenols derived from black raspberries, apples, and green tea have been demonstrated to act as DNA methyltransferase inhibitors, while other compounds including curcumin, soy isoflavones, isothiocyanates derived from cruciferous vegetables, and sulfur compounds derived from garlic have been shown to inhibit type I and II histone

deacetylases. Butyrate and other short-chain fatty acids, produced by the fermentation of specific dietary fibres by the colonic flora, also inhibit histone deacetylases. The inhibition of DNA methyltransferases and/or histone deacetylases by such dietary compounds has been observed to reactivate silenced tumour suppressor genes in cancer cell lines and animal models, suggesting a potential role in cancer prevention.

Histone modification

Histones are essential for the organisation of DNA, facilitating its compacting in an orderly manner and enabling cells to store it within the limited space of the nucleus. In humans, the genome is compacted by a factor of approximately 10,000, with approximately 2 metres of DNA being packed into a nucleus that is only around 2 micrometres (2 μm) in diameter (Strachan and Read, 2021).

The N-terminal tails of histones can undergo a variety of modifications, including acetylation, methylation, ubiquitination, and phosphorylation, which are catalysed by specific enzymes. These modifications exert an influence on the chromatin structure, which may facilitate or inhibit gene transcription, or affect other critical cellular processes. There is substantial evidence to suggest that a considerable number of transcription activators possess histone acetyltransferase activity, while a similar number of repressors exhibit histone deacetylase activity.

Histones, and consequently nucleosomes, should not be viewed as merely passive, structural components. On the opposite, they are in fact dynamic structures that are susceptible to a plethora of post-translational modifications. It is now evident that an intricate "histone code" exists, which is capable of regulating gene expression in an epigenetic manner. In the following paragraphs only two, among the multiple possible histone modification, will be discussed.

Histone acetylation

This type of modification is fundamental to the process of gene expression. This process is facilitated by a class of enzymes known as histone acetyltransferases (HATs), which transfer an acetyl group (donated by acetyl-coenzyme A) to the nitrogen on the side chain of histones. This modification results in a reduction in the ability of histones to bind tightly to the negatively charged phosphate groups of DNA. Consequently, the DNA at that site becomes more relaxed, thereby facilitating interaction with other proteins or enzymes. It can be seen, therefore, that acetylation plays a pivotal role in the activation of transcription. The principal classes of HATs include *p300* and its homologue, CREB-binding protein. These enzymes are widely expressed

and acetylate histones in response to a variety of cellular signals. Their activity is particularly elevated in nerve cells, bone marrow cells, and specific endocrine structures, particularly in cell types that are actively replicating or exhibiting a high metabolic rate, including cancer cells. Recently, HATs have emerged as a potential target for cancer therapy (Strachan and Read, 2021).

Histone methylation

The process of histone methylation is mediated by a group of enzymes known as histone methyltransferases (HMTs). This reaction entails the transfer of a methyl group to either lysine or arginine residues situated at the N-terminal ends of histones H3 or H4. The methyl group donor in this process is S-adenosylmethionine.

The acetylation of lysine on histone H3 is typically associated with transcriptionally active chromatin. However, when methylation occurs, it recruits both histone deacetylases, which remove acetyl groups, and histone methyltransferases, which methylate histones. This results in chromatin condensation and transcriptional repression. The effects of histone methylation are variable. For example, the methylation of lysine 9 on histone H3 results in the formation of a binding site for heterochromatin protein-1, which in turn induces chromatin packaging and gene silencing. Conversely, methylation of lysine 4 on histone H3 has the opposite effect, promoting chromatin relaxation and increased gene expression.

By leveraging this mechanism, researchers have developed chemical compounds that can interfere with the cellular functions of certain HMTs (Strachan and Read, 2021).

Gene regulation in ruminants

The increasing utilisation of molecular biology techniques and bioinformatics in ruminant nutrition and physiology research has considerably enhanced our comprehension of the regulatory mechanisms that regulate pivotal biological processes, such as milk production. This knowledge has highlighted the necessity for a shift in the approach to ruminant nutrition. It suggests that nutrients in the diet should be viewed as bioactive molecules with the capacity to alter molecular mechanisms, depending on the animal's physiological state. These alterations occur via gene regulatory mechanisms. As research in this field continues to uncover further insights into the interactions between nutrients and genes in ruminants, it is anticipated that this knowledge will be applied in practical ways to enhance performance and efficiency in milk production and muscle growth.

The incorporation of sophisticated molecular techniques in ruminant nutrition research has facilitated a more profound comprehension of the manner by which nutrients influence animals at the cellular level. This has led to a shift in perspective regarding the role of nutrients, which are now viewed not merely as building blocks for cells, tissues, and organs, or as sources of energy for cellular metabolism, but also as bioactive molecules that can regulate fundamental molecular mechanisms based on the animal's physiological stage.

The molecular effects of nutrients vary considerably due to the inherent differences in gastrointestinal physiology between ruminants and monogastric animals, with the susceptibility of a nutrient to rumen fermentation being a key factor. It is therefore evident that rumen fermentation and kinetics play a key role in the interactions between nutrients and genes in ruminants. From a nutrigenomic perspective, a nutrient present in a ruminant diet will either undergo fermentation in the rumen or be bypassed by this process. In the event of fermentation, the nutrient may contribute to microbial biomass or be converted into intermediate metabolites, such as volatile fatty acids (VFAs), which can be absorbed through the rumen wall and enter the animal's metabolism. Nutrients that are not fermented in the rumen may undergo conversion into intermediate metabolites, trigger signal transduction cascades or directly bind to and activate TF or NR. Subsequently, these transcription factors bind to specific DNA regions located upstream of target genes, thereby initiating alterations in gene expression.

It is possible that some transcription factors may trigger a secondary wave of gene expression by upregulating other TF, thereby creating a network of coordinated responses to dietary inputs. Furthermore, intermediate metabolites can result in modifications to the DNA or histones, thereby altering the genetic information through epigenetic effects. A potential epigenetic mechanism involves transcription factors increasing the transcription of noncoding RNAs, such

as miRNAs, which then target coding RNAs before they are translated into proteins (Osorio and Moisa, 2019).

Lactogenesis and the mammary gland gene expression

Lactogenesis is a defining characteristic of mammals and plays a crucial role in gene regulation, driving the mammary gland to produce milk. This process, which coincides with the formation of colostrum and the timing of parturition, has been shown to have profound effects at the cellular level on numerous occasions, as evidenced by transcriptomic analyses (such as microarray and RNA sequencing) across a diverse range of mammals (Osorio and Moisa, 2019). The substantial alterations in the mammary gland transcriptome from pregnancy to lactation underscore the pivotal function of these modifications in initiating and sustaining milk production. In cattle, for instance, comprehensive studies have elucidated the transcriptional alterations that occur at the onset and throughout lactation, particularly in relation to fat (Bionaz and Loor, 2008) and protein synthesis (Bionaz and Loor, 2011). During the lactation period, there is a notable increase in the expression of genes involved in the uptake of fatty acids from the blood, intracellular transport, and key transcription factors associated with lipogenesis. With regard to milk protein synthesis, the regulation of amino acid and glucose transport across cell membranes is of particular importance. These processes are fundamental in controlling protein production within the mammary gland. On a broader scale, the impact of lactogenesis on the mammary gland transcriptome has been linked to epigenetic changes that alter the structure of DNA and, consequently, the genetic information available for transcription. For example, research conducted by Bionaz et al. (2012) have identified chromatin alterations associated with lactogenesis. This research observed a reduction in euchromatin (actively transcribed chromatin) at the onset of lactation, followed by an increase in euchromatin status as milk production declines in late lactation. This shift may be indicative of the mammary gland intrinsic capacity to curtail further epigenetic alterations at the outset of lactation, while concurrently sustaining a stable transcriptome until the decline in milk synthesis in the subsequent stages.

Transcriptomic analysis in milk

As stated by Wang et al. (2009), the utilisation of gene expression analysis in livestock is becoming increasingly prevalent due to its capacity to facilitate a deeper comprehension of intricate biological phenomena such as lactation physiology. By analysing the expression profiles of genes associated with milk production, researchers can gain a more accurate understanding of the molecular processes involved in lactation.

The traditional method for studying gene expression in the mammary glands of dairy animals has been through the use of mammary biopsies. However, these biopsies are invasive, painful, costly, and disrupt the normal lactation process (Shaban et al., 2024).

The milk of mammals contains somatic cells, which include a diverse population of cells, such as those of the epithelial type shed from the mammary epithelium. During the lactation period, mammary epithelial cells (MECs) undergo a significant degree of turnover, with the existing cells being replaced by new cells (Capuco et al., 2001). The shed cells are released into the milk, constituting a minor proportion of the somatic cells present. The utilisation of RNA extracted from this subset of somatic cells has the potential to significantly enhance our capacity to assess gene activity in the mammary secretory epithelium, facilitating straightforward and repeated sampling without causing damage to the mammary tissue (Boutinaud et al., 2002). However, the majority of epithelial cells present in milk are no longer viable and have reached the conclusion of their secretory lifespan. Consequently, the genetic information they contain may not accurately reflect the true metabolic state of MECs.

An alternative approach was proposed by Maningat et al. (2007) who put forth the use of milk fat globules (MFG) as a source of mammary RNA. Cytoplasmic crescents can be incorporated into MFG, as first described by Wooding et al. (1970) in goats and humans. This secretion process, designated the apocrine mechanism, entails the exfoliation of secretory cell material. Lipid droplets, which are initially formed in the endoplasmic reticulum, exert pressure against the apical plasma membrane. This eventually results in their envelopment and subsequent pinching off from the cell, whereby they become MFG. This process results in the entrapment of intracellular components, including membranes and organelles, within the MFG. These MFG also contain cytoplasmic material other than the nucleus.

Therefore, the utilisation of MFG provides a non-invasive alternative, as MEC are responsible for milk fat synthesis during lactation (Brenaut et al., 2012). It has been demonstrated that RNA extracted from MFG exhibits a high degree of similarity to RNA extracted from MEC during the production of milk. This makes MFG a reliable and accessible source for investigating gene expression related to lactation. The gene expression patterns derived from MFG RNA are more

representative than those derived from MEC RNA. This is due to the fact that MFG can be collected at any point during lactation and the RNA extraction process is simpler.

Candidate genes selection

The utilisation of gene expression analysis in livestock research is becoming increasingly prevalent, as it facilitates a deeper comprehension of intricate biological phenomena such as lactation physiology. Lactation is typically characterised by a substantial increase in the expression of lipogenic genes. The expression of genes involved in mammary lipid metabolism reaches its peak around 60 days postpartum, which aligns with the lactation curve. The majority of genes associated with fat metabolism demonstrate elevated expression levels during the transition and peak lactation phases.

In the present project, a number of candidate genes were selected for the evaluation of differences in lipogenic (*ACACA, LPL, FASN* and *SCD1*), metabolic (*GLUT1, LEP, LEPR* and *PRLR*), anti-inflammatory (*TLR4*) and antioxidant-related (*SOD, NRF2*) gene expression according to dietary treatments in dairy goats and buffaloes. The following subparagraphs present the selected candidate genes.

ACACA (Acetyl-coenzyme A carboxylase)

Location on chromosome 19 in goat (NCBI, ID: 100861224) and on chromosome 3 in buffalo (NCBI, ID: 102415834).

Acetyl-coenzyme A carboxylase α (ACACA) plays a key role in the synthesis of fatty acids, facilitating the conversion of acetyl-CoA into malonyl-CoA. In mammals, this enzyme is located in the cytosol and requires ATP to perform the carboxylation of acetyl-CoA, resulting in the formation of malonyl-CoA. This represents a pivotal stage in the synthesis of long-chain fatty acids (Cronan and Waldrop, 2002). Given its role in milk fat metabolism, *ACACA* represents a gene of interest for the study of the impact of genetic differences on milk traits in goats.

During lactation, the body increases lipid metabolism to meet the elevated demand for milk fat, which enhances both the activity and quantity of ACACA in the mammary gland (Badaoui et al., 2007).

The correlation between the *ACACA* genotype and lactose content is a particularly fascinating area of study, given the close connection between the processes of lactose synthesis and fat production. Glucose, which is required for the synthesis of lactose and glycerol-3-phosphate, is also a vital component in triglyceride synthesis. Furthermore, the entry of glucose into the

pentose phosphate pathway results in the production of NADPH, a molecule that is essential for the biosynthesis of fatty acids, as it provides the necessary reducing power.

LPL (Lipoprotein lipase)

Location on chromosome 8 in goat (NCBI, ID: 100860750) and on chromosome 3 in buffalo (NCBI, ID: 102412415).

Lipoprotein lipase (LPL) is one of three enzymes in the lipase gene family, which is responsible for the breakdown of triacylglycerol found in chylomicrons and very low-density lipoproteins (VLDL). The process results in the release of two free fatty acids and a monoacylglycerol, which can then be utilised by tissues. LPL is produced in the epithelial cells of the mammary gland, and its activity is particularly high during lactation, rendering these cells indispensable for the release of fatty acids in the gland (Zhao et al., 2014). LCFA are taken up from the plasma following their release from circulating triglycerides in chylomicrons or VLDL by LPL, or they originate from plasma free fatty acids.

The activity of this enzyme in the mammary tissue increases significantly at the onset of lactation, which is likely to be a critical factor in the initiation of milk fat secretion.

The *LPL* gene, which encodes the enzyme, plays a pivotal role in the synthesis of milk components. The hydrolysis of triacylglycerols and the uptake of free fatty acids from the plasma are processes that are driven by LPL and are vital for the production of milk.

FASN (Fatty acid synthase)

Location on chromosome 19 in goat (NCBI, ID: 100861286) and in chromosome 3 in buffalo (NCBI, ID: 102409414).

Fatty acid synthase (FASN) is a versatile enzyme that is responsible for the production of fatty acids. In ruminants, it plays an important role in the synthesis of all short- and medium-chain fatty acids, as well as approximately half of the palmitic acid (C16:0) (Zhu et al., 2014). The primary building block for fatty acid synthesis in ruminants is acetate, with beta-hydroxybutyrate and propionate contributing to a lesser extent. The initial conversion of acetate to acetyl-CoA is carried out by the acetyl-CoA short-chain gene family. Subsequently, ACACA facilitates the two-step process of converting acetyl-CoA into malonyl-CoA. Malonyl-CoA, which is produced by FASN, is used to add two carbon atoms from acetyl-CoA to the growing fatty acid chain, ultimately resulting in the synthesis of palmitate (Haile et al., 2016). The thioesterase catalytic domain of FASN (thioesterase I) is responsible for terminating the fatty

acid chain, resulting in the production of palmitic acid (C16:0), which is the main product of FASN activity in both ruminants and monogastric animals (Barber et al., 1997).

Fatty acids with 16 or more carbon atoms are unable to undergo further elongation by the mammary gland and are instead hydrolysed by thioesterase I. This results in the synthesis of short- and medium-chain fatty acids directly in the mammary epithelial cells. Consequently, the proportion of these fatty acids in milk is indicative of the contribution of de novo fatty acid synthesis to the overall milk fat content. The majority of the short- and medium-chain fatty acids (C4:0–C14:0) and approximately half of the C16:0 fatty acids present in milk are derived from this de novo synthesis process (Haile et al., 2016).

SCD (Stearoyl Co-A desaturase)

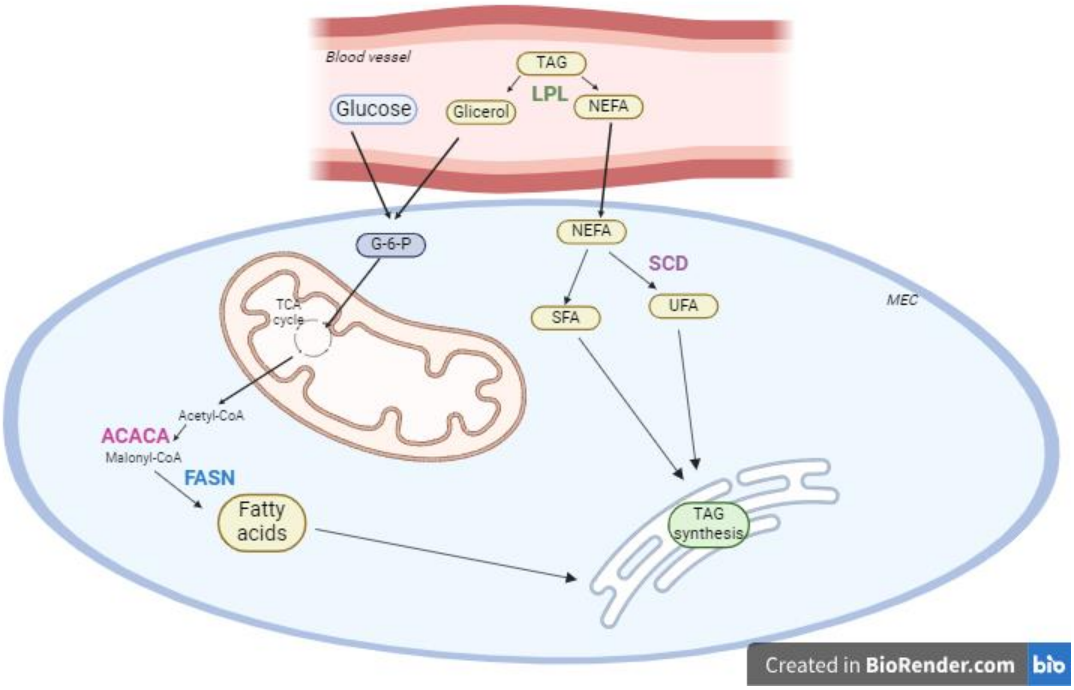
Location on chromosome in goat 26 (NCBI, ID: 100860763) on chromosome 23 in buffalo (NCBI, ID: 102401664).

Stearoyl-CoA desaturase (SCD) is a pivotal enzyme that regulates the rate of monounsaturated fatty acid (MUFA) synthesis by catalyzing the desaturation of fatty acids. The expression of the *SCD* gene in the mammary glands of lactating ruminants has been the subject of considerable research interest, given its impact on both the technological and nutritional qualities of milk consumed by humans (Bernard et al., 2013). Also known as Δ9-desaturase, SCD plays a crucial role in the production of monounsaturated fatty acids and specific conjugated linoleic acid (CLA) isomers, which are unique to ruminant products. One of the primary functions of SCD is to introduce a cis double bond between the 9th and 10th carbon atoms of fatty acids, a process that is essential for the synthesis of essential milk components such as oleic acid, palmitoleic acid, myristoleic acid, and rumenic acid (a CLA isomer).

The presence of these fatty acids has a significant impact on milk fat content and composition, thereby affecting its nutritional value. The synthesis of beneficial fatty acids, such as cis-9, trans-11-CLA and, to a lesser extent, cis-9-18:1, by SCD serves to enhance the nutritional quality of milk fat. Furthermore, the introduction of a cis-9 double bond by SCD reduces the melting point of milk fat, which is crucial for its physical properties (Parodi, 1982).

The role of SCD is of particular importance in ruminants, given the extensive biohydrogenation of dietary PUFA that occurs in the rumen. This process generates saturated fatty acids (mainly 18:0) and various intermediates, including trans fatty acids, which can be further desaturated by SCD in the mammary gland.

Figure 1. Mechanism of action of lipogenic genes. Created with Biorender.com.



GLUT1 (Glucose transporter member 1) (Known also as SLC2A1)

Located on chromosome 3 in goat (NCBI, ID: 102174800) and on chromosome 6 in buffalo (NCBI, ID: 102392377).

In order to supply glucose to the mammary gland during the period of peak lactation, the body reduces insulin-independent glucose uptake in the majority of tissues, with the exception of the mammary gland. Two principal types of transporters are of paramount importance for the transportation of milk components in dairy animals: the ATP-binding cassette (ABC) superfamily and the solute carriers/glucose transporters (SLC/GLUT). The SLC/GLUT transporters facilitate the uptake of glucose across the plasma membrane via a process known as diffusion (Pradeep et al., 2014). The distinct biological roles of each GLUT transporter are indicated by their unique transport efficiency, substrate affinity, and tissue distribution. A number of solute carrier genes have been identified, including SLC2A1 (GLUT1), SLC2A3 (GLUT3), SLC2A4 (GLUT4), SLC2A5 (GLUT5), SLC2A8 (GLUT8) and SLC2A12 (GLUT12).

At the onset of lactation, the mammary glands undergo a substantial increase in glucose uptake, driven by the upregulation of glucose transporters (*GLUTs*). It has been demonstrated that the uptake of glucose by mammary epithelial cells (MECs) represents a pivotal stage in the

synthesis of milk during lactation, with the yield of milk being directly correlated with the rate of glucose uptake by MECs (Threadgold et al., 1982).

LEP (Leptin)

Location on chromosome 4 in goat (NCBI, ID: 100860794) and on chromosome 8 in buffalo (NCBI, ID: 102401642).

Leptin (LEP), which is primarily synthesised by adipose tissue, is a determinant in regulating and controlling the productive performance of animals (El-Shorbagy et al., 2022).

As a protein hormone secreted from white adipose tissue, leptin exerts a profound influence on food intake, body energy balance, and the distribution of nutrients between tissues, thereby playing a pivotal role in energy metabolism. Furthermore, leptin plays a regulatory role in lactogenesis. It has been demonstrated that polymorphisms in the leptin gene can influence circulating leptin levels, as well as the yield and quality of meat and milk in a range of farm animals (Avondo et al., 2019). In cattle, polymorphisms in the leptin or leptin receptor genes have been demonstrated to exert an influence on milk yield, milk fat, milk protein and milk fatty acid composition (Avondo et al., 2019). Furthermore, Glantz et al. (2011) identified correlations between leptin gene polymorphisms and milk traits that are crucial for cheese production, including gel strength and fat globule size.

LEPR (Leptin receptor)

Location on chromosome 3 in goat (NCBI, ID: 102180987) and on chromosome 6 in buffalo (NCBI, ID: 102402159).

The leptin receptor (LEPR) mediates the effects of leptin, including its influence on feeding behaviour, nutrition, obesity, and reproduction (Wang et al., 2011).

It is noteworthy that the expression of *LEPR* mRNA exhibits considerable alterations during the course of pregnancy in cattle, indicating the potential existence of a critical threshold that must be surpassed in order to stimulate the reproductive axis (Wójcik-Gładysz et al., 2006).

The *LEPR* gene appears to be a strong candidate for influencing reproductive traits. Further research on the *LEPR* gene may yield valuable insights into gene polymorphisms in livestock, potentially offering farmers a molecular marker for the early selection of traits such as hyperprolificacy.

TLR4 (Toll-like receptor 4)

Location on chromosome 8 in goat (NCBI, ID: 100860955) and on chromosome 3 in buffalo (NCBI, ID: 102407022).

TLR4 is one of the 13 well-characterised mammalian toll-like receptors (TLRs) and activates the nuclear factor (NF)-κB signalling pathway. This pathway is of critical importance, as NF-κB functions as a principal regulator of a vast number of genes associated with inflammatory processes (Chang et al., 2015). This factor complex functions as a pivotal regulator, orchestrating the expression of a multitude of immune response genes. Among these genes are those encoding cytokines, which show a significant increase in expression in the livers of cows suffering from mastitis (Chang et al., 2015).

Toll-like receptors (TLRs) are essential for the recognition of microbial components, including proteins, carbohydrates, lipids, and nucleic acids. This initiates a complex signalling cascade that activates various transcription factors and inflammatory cytokines (Takeda and Akira, 2004).

PRLR (Prolactin receptor)

Location on chromosome 20 in goat (NCBI, ID: 100861318) and on chromosome 19 in buffalo (NCBI, ID: 102415743).

The prolactin receptor (PRLR) is a transmembrane receptor that is expressed in a wide range of mammalian cells. It plays a pivotal role in numerous physiological processes, including the growth of muscle, bone, and cartilage, as well as lactation (Shi et al., 2016). During lactation, PRLR signalling is essential for the production of milk components, including milk proteins, lactose, and triglycerides, and also regulates their secretion.

In ruminants, two forms of PRLR have been identified: a short form and a long form (Shi et al., 2016). The long form of PRLR (lPRLR) is more prevalent than the short form (sPRLR) during lactation, indicating that lPRLR plays a more critical role in regulating lactation in mammary epithelial cells. It has been demonstrated that PRLR signalling is essential for milk synthesis and mammary cell survival in mice and that the inhibition of PRLR can result in a reduction in milk production in dairy cows (Lacasse et al., 2012). Although previous studies have established a link between PRLR and milk content and yield in dairy cattle (Picazo et al., 2004; Brym et al., 2005), the role of PRLR in regulating milk fat metabolism during lactation in dairy goats remains to be elucidated.

SOD (Superoxide dismutase)

Location on chromosome 1 in goat (NCBI, ID: 100861196) and on chromosome 1 in buffalo (NCBI, ID: 102414313).

Superoxide dismutase (SOD) plays a crucial role in cellular protection by converting superoxide radicals into hydrogen peroxide (H_2O_2), thereby acting as a primary defence against oxidative damage. The activity of some antioxidant enzymes has been shown to be influenced by a number of factors, including soil type, pasture conditions and season, as evidenced by research conducted on grazing sheep (Celi, 2011). In dairy goats, there is a tendency for SOD activity to decrease during the postpartum period. This is likely due to a reduction in peroxide production, as evidenced by a decrease in the levels of reactive oxygen metabolites (ROMs).

Levels of ROMs declined significantly by the fourth week postpartum, although they were notably higher in the second week, indicating that oxidative stress was more pronounced at the outset of the postpartum period.

NRF2 (Nuclear factor erythroid 2-related factor 2) known as NFE2L2

Location on chromosome 2 in goat (NCBI, ID: 102176737) and on chromosome 2 in buffalo (NCBI, ID: 102405811).

Reactive oxidants are endogenously generated in healthy cells under controlled conditions and perform vital functions as signalling molecules. They are indispensable for processes such as cell division, immune function, and responses to inflammation and stress (Finkel, 2011). However, stress has the potential to disrupt this equilibrium, resulting in an overproduction of oxidants. Such damage can impair cellular functions, impede immune responses, and increase the risk of inflammatory diseases.

The transcription nuclear erythroid 2-related factor 2 (NRF2) plays a key role in regulating the body's antioxidant defences. It plays a role in the protection of cells from oxidative damage and it has been demonstrated that mice lacking the Nrf2 gene exhibit heightened susceptibility to oxidative stress, chemical toxicity, and a heightened propensity for severe health complications (Grewal et al., 2022). Conversely, the administration of pharmacological agents that enhance Nrf2 activity has been demonstrated to markedly diminish oxidative damage in animal models (Talalay et al., 2003).

Chapter IV

Dietary fennel seeds supplementation affects yield, fatty acid composition and flavour profile of milk and cheese in grazing goats

Experimental design

The trial was performed at Azienda Zootecnica Antonio Amato located in Casaletto Spartano (SA, Italy; 832 m a.s.l. (40°09' N; 15°37'E)) on 20 multiparous lactating Murciana goats from April to September 2022.

Sixty days after kidding (February), the goats, homogeneous for parity (3^{rd}), body weight (BW = 50 kg ± 2.0 kg) and DMY (daily milk yield) (1950 ± 120 g/day), were randomly allocated into two groups of ten goats each (C: control and F: fennel). All the animals were fed on a permanent pasture from 9.00 to 16.00 and received 400 g/head/day of corn meal in individual pen; in addition, group F received 15 g/head/day of organic dry fennel seeds (*Foeniculum vulgare Mill.*) which was mixed into the corn meal before been offered to the animals. All the animals had free access to fresh water.

Feeds samples and analysis

Feed antioxidant activity, phenolic content and VOCs

Fennel seeds and pasture samples were characterized for antioxidant activity (TPC, ABTS and DPPH) and VOCs. Total phenolic content in samples was determined by the Folin-Ciocalteu colorimetric method described by Taga et al. (1984). The concentration was calculated using gallic acid as standard (Sigma-Aldrich), and the results were expressed as mg gallic acid equivalents (GAE)/g.

To evaluate the antioxidant activity of the samples, the ABTS method was applied according to the procedure described by Re et al. (1999), with minor modifications. In particular, a 7 mM solution of ABTS in 2.45 mM aqueous potassium persulfate was prepared, and after 16 h of incubation in the dark at room temperature, the solution was diluted with ethanol to obtain an absorbance of 1.000 ± 0.020 at 734 nm. Then, 100 µL of the extract was added to 1000 µL of this solution. Measurements of absorbance were taken at 734 nm after 2.5 min of incubation. In addition, Trolox was used as the reference and activities were expressed as Trolox equivalents (mg/g TE).

The antiradical activity of feed was measured using DPPH method (Sanchez-Moreno et al., 1998), and expressed as EC50 (µg/ml), the concentration necessary for 50% reduction of DPPH.

The volatile compounds (VOCs) extraction from feed samples was performed by solid-phase microextraction (SPME), whereas the GC-MS analysis was carried out with a gas chromatograph (Perkin Elmer, Waltham, MA, USA) coupled with a mass spectrometer (SQ8S; Perkin Elmer, USA). The gas chromatograph was equipped with an Elite-5MS column (Perkin Elmer, USA). A total of 5 g of sample were mixed with 10 ml of saturated sodium chloride solution (360 g/l), and then 10 µl of internal standard solution (4-methyl-2-heptanone; 10 mg/kg in ethanol) was added. The vials were sealed with a polytetrafluoroethylene-silicone septum (Supelco, Bellefonte, PA, USA) and stirred at 60°C; VOCs were extracted from the headspace with a divinylbenzene-carboxen-polydimethylsiloxane SPME fiber (Supelco, USA) with an exposition time of 60 min. After adsorption time, the extracted VOC were thermally desorbed into the gas chromatograph injector splitless mode for 1 min at 250°C. The oven temperature was held at 50°C for 1 min, increased at a rate of 3°C/min up to 200°C and held for 1 min, and then increased from 200°C to 250°C at 100.15°C/min and held for 15 min. Helium was used as a carrier gas at a flow rate of 1 ml/min. The mass spectrometer operated in electronic impact ionization mode at 70 eV, and data were collected in full scan mode. Source and interface temperature were held at 250°C. Compounds were identified by comparing their mass spectra with those contained in the National Institute of Standards and Technology (NIST) 14 library (Gaithersburg, MD, USA). The compounds were considered as correctly identified when their spectra presented a library match factor > 85. The VOCs were expressed on percentage of total volatile compounds.

Feed chemical composition

Corn meal and fennel seeds were collected monthly whereas pasture samples were collected weekly as described in Musco et al. (2022); briefly, every week pasture samples (1 kg each) were collected from four different areas of 2.5 m2 each, cutting the plants at 3 cm from the ground and then air oven dried at 65 °C. Corn meal, fennel seeds and the representative weekly samples, which were weighted and pooled to obtain monthly samples and trimmed at 1 mm, were analysed for chemical composition, according to AOAC (2012) procedures, and fibre content as suggested by Van Soest et al. (1991). Feed nutritional value was calculated according to INRA (2018).

Feed fatty acid analysis

Lipid fraction of feed samples was extracted from 15 g of sample using a mixture of chloroform and methanol (2/1 vol/vol) according to Gray et al. (1967). Lipid extract was methylated adding

1 mL of hexane and 0.05 ml of 2 N methanolic KOH. Separation of the methyl esters in forage and cheese samples was performed according to Di Trana et al. (2015). Fatty acid methyl esters (FAME) were identified with reference to the retention time of FA standard mixture of Supelco 37 Component FAME Mix (Supelco, Bellafonte, PA). The content of FA was quantified using internal standards (Supelco, Bellafonte, PA) added during the methylation step. Briefly, 100 mg of lipid extract was mixed with 50 µl of 2 N methanolic KOH and 1 ml of hexane containing the internal standards (20 mg/mL) according to Giorgio et al. (2019).

Milks samples and analysis

Starting from 60 days after kidding, milk yield was daily registered and individual milk samples were collected monthly by proportionally mixing the milk from the two daily milkings.

One-hundred ml of milk were analysed for fat, lactose and protein by Milko Scan 133B (Foss Matic, Hillerod, Denmark) standardized for goat milk. The FA profile (50 ml of milk) has been determined as described by Tudisco et al. (2014). Briefly, milk fat was extracted by using hexane and isopropane (3/2 v/v) (Hara and Radin, 1978). The fatty acid methyl esters were prepared by direct transesterification with sulfuric acid and methanol (1:9, v/v) of the lipids (Christie, 1993) and then analysed in a gas chromatograph (Agilent technologies, model 5890) fitted with an SP-2560 fused silica capillary column (100 m × 0.25 mm i.d. × 0.2 µm film thickness, Supelco, Inc., Bellefonte, PA, USA). Helium was used as carrier gas and set at a constant pressure of 180 kPa, splitting flow of 50 mL/min, and injection volume of 1 µL. The column parameters were: initial temperature of the column maintained at 170 ◦C for 15 min; then, with an increase of 5 ◦C/min, it was brought up to 240 ◦C. The total execution time was 64 min. Chromatogram peak areas were acquired and calculated by Chemstation software (Agilent, technologies) and expressed as g/100g, considering 100g as the summation of the areas of all FAME identified.

Cheese samples and analysis

Cheeses were sampled after 1 month of treatment and at the end of the trial. They were obtained separately with the milk of the two groups, made from raw milk heated at 37 °C with the addition of rennet. The curd was broken and then reassembled in the ‘fuscelle’, typical wicker baskets which allow the whey to drain for 24 h. Then it was salted (around 2 g of NaCl/100 g of curd) and ripened for 20 days at 10°C. Four small cheeses (around 500g) for each sampling period were obtained for each group and stored in vacuum packages at -20 °C until analysis.

Cheese yield was calculated dividing the sum of the weight of all the cheeses obtained from the milk of one group by the initial weight of milk.

Cheese samples were thawed over night at 4°C and then they were grinded homogenized in a stainless-steel blender. The moisture content was determined using oven drying, while the fat and protein contents were analysed using the Gerber and Kjeldahl methods, respectively (AOAC 2012).

Cheese samples fatty acids composition was determined by gas chromatography (GC), according to Bligh and Dyer method (1959) and International Organization for Standardization (ISO) as has been reported previously by Papaloukas et al. (2016).

Briefly, aliquots (3 g) of grinded cheese were added with 4.50 ml of chloroform and 9,0 ml of methanol and 3,6 ml of deionized water. This mixture was stirred for 1 minute. Subsequently, 4.5 ml of chloroform and of deionized water were added. The tube was shaken for 1 minute and then centrifuged for 10 minutes at 2500 rpm at 4 °C, and the upper organic layer was transferred to a glass test tube. The lower aqueous layer was extracted twice more, with 1.5 mL of n-hexane each time after suspension centrifugation. The pooled hexane layer was evaporated under a stream of nitrogen. The previously dried solution was derivatized according to Moltò-Puigmartì et al. (2007).

The extracted fat was stored at −20 °C until further analysis. One microliter of each sample was injected into a Thermo GC 6890 gas chromatography system equipped with a flame-ionization detector (Thermo Fisher Scientific., Waltham, Massachusetts, US). Fatty acid methyl esters from all samples were separated using a 100-m length, 0.25-mm i.d., 0.25-µm capillary column (CP-Sil 88; Chrompack, Middelburg, the Netherlands).

Individual FAME were identified, based both on retention time and on the comparison with a standard mixture of 37 pure components (Supelco 37 Component FAME Mix, Merck Life Science, Milano, S.r.l, Italy) and literature data. The injector temperature was kept at 250°C and the detector temperature was kept at 250°C, with an H2 flow of 40 mL/min, air flow of 400 mL/min, and a constant He flow of 45 mL/min. The initial oven temperature was held at 80°C for 1 min, increased at 5°C/min to 100°C, held for 2 min, increased at 10°C/min to 175°C, held for 40 min, and then finally increased at 5°C/min to a final temperature of 240°C and held for 55 min. Helium was used as the carrier gas at a flow rate of 0,70 ml/min.

The identification of the isomers of the conjugated linoleic acid (CLA) was accomplished by comparing the retention time of each chromatographic peak and those of a mixture of chromatographic standards (Conjugated (9Z,11E)-Linoleic acid; Conjugated (10E,12Z)-Linoleic acid solution, Merck Life Science, Milano, S.r.l, Italy). The internal standard used was

the Nonadecanoic Acid Methyl Ester (Merck Life Science, Milano, S.r.l, Italy). The concentrations of fatty acids were expressed as mg/100 mg total fatty acids. All determinations were carried out in triplicate.

The volatile compounds (VOCs) extraction from samples of cheese was performed with the same method used for analysing fennel seed VOCs content. Total antioxidant capacity (TAC) of cheese extract samples was assessed using the ferric ion reducing antioxidant power assay as following the methods reported Benzie and Strain (1997). The standard curve was constructed using iron sulphate heptahydrate ($FeSO4·7H2O$) and data were expressed as mol $FeSO4$ equivalents per kg of cheese.

Statistical analysis

Data from pasture were analysed by the one-way ANOVA with SPSS IBM software (V29.0.1.0) using as factor the period of sampling; the means were compared using Tukey's test. Differences were considered statistically significant at $P < 0.05$.

Milk and cheese data were analysed using the two-way ANOVA for repeated measures on SPSS IMB software (V29.0.1.0). In the statistical model, group (G), period (P) and their interaction (G x P) were considered as fixed effects.

Results

Diet composition

VOCs and antioxidant capacity of fennel seeds and pasture are reported in table 1. In pasture samples tetradecanoic acid, 1-butanol-3-methyl and 2-nonanone resulted the most abundant compounds, whereas some differences occurred during the months of trial regarding pasture antioxidant activity, as reported in figure 1.

Feeds chemical composition (g/kg DM) and nutritive value are reported in table 2. Pasture had the highest content of protein and fat in July and the highest NDF and the lowest ADF and ADL content in May. The fennel seeds had high fat and crude fibre contents. The ash content of pasture was significantly higher during the sampling of September compared to the other months.

Table 1. Fennel seeds antioxidant activity and Volatile organic compounds (VOCs).

Item %	Fennel seeds	Pasture
Butanoic acid	0.10	nd
Hexanoic acid	0.10	3.61
Nonanoic acid	0.10	2.34
Decanoic acid	0.10	0.09
Dodecanoic acid	0.10	3.50
Tetradecanoic acid	0.10	20.58
2,3-Butanediol	0.10	0.98
1-butanol-3-methyl	0.10	31.26
Phenylethyl alcohol	0.10	12.14
Ethyl hexanoate	0.80	0.42
Ethyl octanoate	0.40	nd
Ethyl decanoate	0.10	0.21
2-Heptanone	0.60	0.90
2-Nonanone	0.10	23.42
Limonene	14.50	0.09
Fenchone	2.50	nd
Estragole	4.30	nd
trans-Anethole	75.50	nd
2-Butanol	0.20	nd
Acetic acid	0.10	0.37
Antioxidant activity		
TPC, mg GAE/g	4.06	56.018
ABTS, mg/g TE	6.74	23.56
DPPH, EC50 µg/ml	12.04	36.162

TPC: total phenolic content; ABTS: 2,20-azino-bis(3-ethylbenzothiazoline-60-sulfonic acid) diammonium salt; DPPH: 2,2-diphenyl-1-picrylhydrazyl. SEM: standard error of the mean. Values within a row with unlike superscripts differ at P <0.01 when uppercase and P<0.05 when lowercase.

Figure 1. Pasture antioxidant activity along the trial.

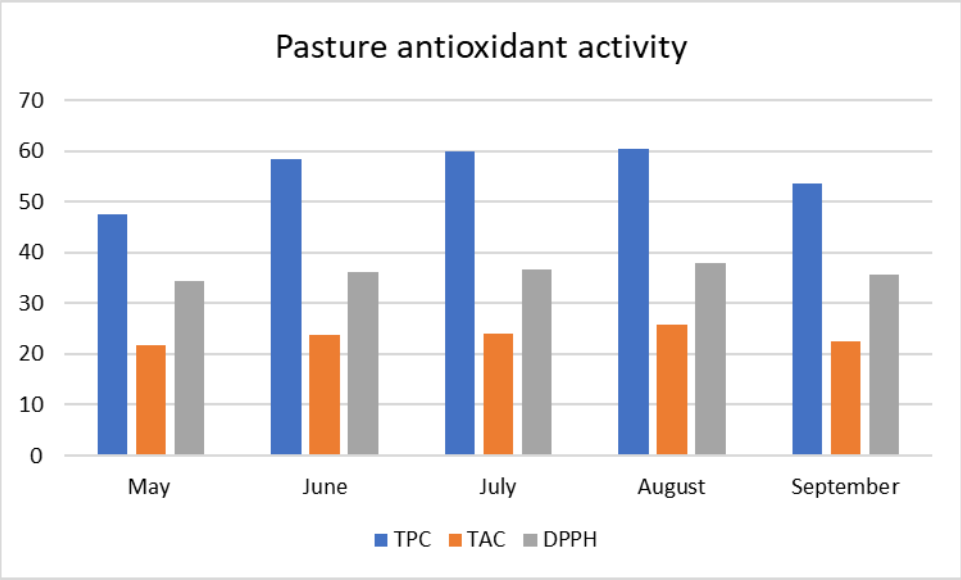


Table 2. Feed chemical composition (g/Kg DM).

Item	Pasture						Corn meal	Fennel seeds
	May	June	July	August	September	SEM		
CP	112.81±1.6^{E}	147.11±0.5 ^{D}	201.00±2.0^{A}	169.11 ±0.3^{C}	176.41 ± 2.2^{B}	0.793	100.42 ± 2.91	120.90 ± 0.7
EE	21.32 ±0.3^{C}	22.28±0.3^{B}	25.62±0.5^{A}	22.68±0.4^{A}	19.52±0.5^{D}	0.058	31.61 ± 0.16	82.41 ± 0.14
CF	269.24 ± 1.9 ^{C}	300.83 ±2.8 ^{B}	314.31 ±2.6 ^{A}	317.51 ± 3.0 ^{A}	298.41 ±7.7 ^{B}	0.455	27 ± 2.22	112.35 ± 1.7
Ash	76.45± 4.7 ^{B}	65.05±1.3 ^{B}	61.25±1.0 ^{B}	41.41±1.2 ^{C}	123.16±3.6 ^{A}	0.731	10.50 ± 0.13	14.82 ± 0.5
NDF	562.03±1.5^{A}	481.82±3.6^{B}	490.16±5.2^{B}	410.65±6.9^{C}	475.01±9.1^{C}	1.292	/	/
ADF	318.71±2.0^{E}	422.03±2.4^{D}	453.81±2.3^{C}	476.42±1.8^{A}	468.30±3.8^{B}	1.546	/	/
ADL	98.34±5.3^{D}	176.74±5.2^{B}	152.58±2.6^{C}	197.71±3.7^{C}	181.60±11.6^{A}	0.935	/	/
UFL/Kg DM	0.77	0.78	0.80	0.77	0.78	0.040	1.06	0.8

DM: dry matter; CP: crude protein; CF: crude fiber; EE: ether extract; NDF: neutral detergent fiber; ADF: acid detergent fiber; ADL: acid detergent lignin; UFL: feed unit for lactation; SEM: standard error of the mean. Values within a row with unlike superscripts differ at P <0.01 when uppercase and P<0.05 when lowercase.

Feeds fatty acid profiles are reported in Table 3. Pasture samples were rich in PUFA and in particular in omega 3, with the highest values in May and September. On the contrary, saturated fatty acids (SFA) were the lowest at the beginning and at the end of the experimental period. Fennels seeds had low values of SFA and extremely high content of MUFA, mainly composed of petroselinic acid (C18:1 n9) and very low content of C18:3 n3. Corn meal was characterized by a high content in PUFA, where C18:2n6 was the most abundant.

Table 3. Fatty acid profile (expressed as % on total fatty acids) of fennel seeds and pasture.

Item	Fennel	Corn meal	Pasture					
			May	June	July	August	September	SEM
C11:0	6.15	nd	nd	nd	nd	nd	nd	0.023
C12:0	0.01	nd	0.25^{A}	0.22 ^{B}	0.09 ^{D}	0.11 ^{C}	0.07 ^{E}	0.032
C14:0	0.04	0.07	0.58 ^{A}	nd	0.49 ^{B}	0.33 ^{D}	0.36 ^{C}	0.514
C16:0	4.18	12.02	17.65 ^{D}	19.72 ^{C}	21.56 ^{A}	20.77 ^{B}	16.45 ^{E}	0.195
C18:0	1.39	2.56	6.76 ^{A}	5.88 ^{B}	5.21 ^{C}	4.92 ^{E}	4.95 ^{D}	0.202
C22:0	0.01	nd	0.76 ^{D}	2.42 ^{A}	1.63 ^{B}	0.92 ^{C}	0.26 ^{E}	0.673
∑ SFA	12.92	15.03	27.37 ^{D}	30.21 ^{B}	30.41 ^{A}	28.61 ^{C}	23.51 ^{E}	0.087
C16:1trans	0.20	nd	0.56 ^{B}	0.89 ^{A}	0.32 ^{C}	0.13 ^{E}	0.14 ^{D}	0.034
C16:1cis	0.19	0.05	0.65 ^{A}	0.49 ^{C}	0.46 ^{D}	0.57 ^{B}	0.37 ^{E}	0.345
C18:1n9	0.33	26.02	2.27 ^{E}	3.99 ^{D}	5.76 ^{A}	4.90 ^{C}	5.48 ^{B}	0.043
C18:1n12	75.87	nd	nd	nd	nd	nd	nd	-
C20:1n9	0.14	nd	0.46 ^{C}	0.61 ^{B}	0.37 ^{D}	0.67 ^{A}	0.25 ^{E}	0.322
∑ MUFA	76.41	27.98	3.96 ^{E}	6.25 ^{D}	7.39 ^{A}	7.04 ^{B}	6.40 ^{C}	0.354
C18:2n6	9.98	53.87	12.49 ^{B}	14.81 ^{A}	11.44 ^{D}	11.23 ^{E}	11.65 ^{C}	0.952
C18:3n3	0.23	2.76	54.43 ^{B}	46.55 ^{E}	47.60 ^{D}	51.75 ^{C}	55.36 ^{A}	0.011
C20:3n3	0.20	nd	0.64 ^{A}	0.62 ^{B}	0.52 ^{C}	0.43 ^{D}	0.31 ^{E}	0.032
C20:5n3	0.06	nd	0.52 ^{A}	0.48 ^{B}	0.42 ^{C}	0.41 ^{C}	0.37 ^{D}	0.011
C22:6n3	0.02	nd	0.18 ^{B}	nd	0.12 ^{C}	0.08 ^{D}	1.51 ^{A}	0.184
∑ PUFA	10.65	56.97	68.65 ^{B}	63.53 ^{C}	62.19 ^{D}	64.33 ^{C}	70.07 ^{A}	0.823

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid
SEM: standard error of the means. Nd: not detected. Values within a row with unlike superscripts differ at P <0.01 when uppercase and P<0.05 when lowercase.

Milk yield and chemical composition

No corn refusals were detected. Goats body weight did not change between the groups. Milk yield was significantly higher in group F than in group C ($P < 0.01$) with an increase of 27.5%, whereas no modifications have been detected in fat, lactose and protein percentage (Table 4). Nevertheless, the yields of milk fat, lactose and protein were significantly higher in Fennel group with increases of 21%, 27.5% and 27.1% respectively. The sampling effect was significant for all chemical characteristics but for fat content and no interaction effect was observed.

Table 4. Body weight and milk yield and chemical composition.

Item	Control	Fennel	Group effect	Period effect	GxP	SEM
			P -value			
BW (Kg)	51.51	50.20	0.89	0.07	0.98	1.345
DMY (g)	1418.27	1809.59	<0.01	<0.01	0.90	52.843
Fat (%)	4.31	4.05	0.62	0.88	0.19	0.095
Lactose (%)	4.24	4.22	0.76	<0.01	0.39	0.062
Protein (%)	2.99	2.95	0.62	<0.01	0.98	0.041
Fat (g/d)	60.04	72.82	<0.01	<0.01	0.52	2.495
Lactose (g/d)	58.61	74.75	<0.01	<0.01	0.53	1.933
Protein (g/d)	41.26	52.48	<0.01	<0.01	0.62	1.377

BW: body weight; DMY: daily milk yield; SEM: standard error of the means.

Milk fatty acid profile of the two groups is reported in Table 5. Among the de novo synthesis fatty acids (C4:0 to C16:0), butyric (C4:0), caproic (C6:0), caprylic (C8:0), myristic (C14:0) and mirystoleic (C14:1) were different (P <0.05) between the groups.

Group F had lower content of all C18:1 isomers, except C18:1 cis11, also of C18:2 isomers, as CLAs (C18:2cis9trans11, C18:2trans10cis12). The majority of FA were affected by the sampling period and only some of them had interaction effects.

Table 5. Milk fatty acids profile expressed as g/100 g of milk fat.

Item	Group C	Group F	Group effect	Period effect	G x P	SEM
			P-value			
C4:0	3.020	2.461	<0.01	0.391	0.141	0.0979
C6:0	1.785	1.870	0.035	0.025	0.397	0.0307
C8:0	2.648	2.788	0.028	<0.01	0.301	0.0414
C10:0	10.393	10.664	0.143	<0.01	0.458	0.1110
C12:0	3.630	3.771	0.188	<0.01	0.870	0.0589
C14:0	9.191	10.044	<0.01	0.170	0.221	0.0932
C14:1	0.255	0.207	<0.01	<0.01	<0.01	0.0068
C16:0	28.896	29.540	0.047	0.490	0.812	0.1659
C16:1	0.441	0.383	<0.01	<0.01	0.612	0.0157
C18:0	12.407	12.319	0.760	<0.01	0.171	0.1616
C18:1 c6	0.099	0.058	<0.01	<0.01	0.024	0.0071
C18:1 c9	18.068	17.777	0.082	<0.01	<0.01	0.1490
C18:1 t9	0.373	0.252	<0.01	<0.01	<0.01	0.0195
C18:1 t11	1.491	1.326	<0.01	0.491	0.855	0.0267
C18:1 c10	0.338	0.301	<0.01	0.062	0.823	0.0111
C18:1 c11	0.179	0.172	0.273	<0.01	0.531	0.0035
C18:1 c12	0.114	0.092	<0.01	0.251	0.731	0.0032
C18:2 t6	0.273	0.250	0.037	<0.01	0.140	0.0091
C18:2 c6	1.615	1.496	<0.01	<0.01	0.161	0.0267
C18:2cis9trans11	0.380	0.326	<0.01	<0.01	0.451	0.0138
C18:2trans10cis12	0.220	0.196	<0.01	0.010	0.465	0.0039
C18:3 n6	0.049	0.045	0.031	0.061	0.106	0.0016
C18:3 n3	0.942	0.832	<0.01	<0.01	0.362	0.0164
C20:3 n6	0.012	0.007	0.032	<0.01	<0.01	0.0011
C20:3 n3	0.005	0.005	0.401	<0.01	0.071	0.0002
C20:4	0.138	0.113	<0.01	0.488	0.700	0.0025
C20:5 n3	0.053	0.048	0.046	0.095	0.334	0.0000
C22:0	0.133	0.115	0.020	<0.01	0.284	0.0033
C22:2 n6	0.012	0.012	0.211	0.391	0.185	0.0000
C22:6 n3	0.025	0.026	0.322	0.062	0.021	0.0010
C24:1	0.023	0.018	0.739	0.371	0.761	0.0000

SEM: standard error of the means.

Milk from group F was characterized by a higher content in saturated fatty acids *(P* < 0.01) and de novo FA whereas Group C had higher content of MUFA, PUFA, omega 3 and omega 6 FA (Table 6). For all the classes of fatty acid a significant effect of the sampling period was observed, except for mixed FA (C16:0 and C16:1); the interaction between treatment and sampling period was significant for MUFA, SFA and omega 6. Milk desaturation indexes were affected by the treatment, in particular C14:1/C14:0 and C16:1/C16:0 resulting higher in group C.

Table 6. Milk fatty acid classes and ratios expressed as g/100g of milk fat.

Item	Group C	Group F	Group effect	Period effect	G x P	SEM
			P-value			
De novo FA	32.001	32.702	<0.01	0.021	0.391	0.2331
Mixed FA	29.332	29.922	0.181	0.952	0.788	0.1642
Preformed FA	38.195	36.736	0.011	<0.01	0.381	0.2223
SFA	74.101	75.281	0.025	<0.01	<0.01	0.1562
MUFA	21.598	20.660	0.010	<0.01	<0.01	0.1531
PUFA	3.791	3.422	0.010	<0.01	0.072	0.0342
omega 6	2.146	1.962	<0.01	<0.01	0.056	0.0263
omega 3	1.056	0.932	<0.01	<0.01	0.382	0.0134
Total CLA	0.604	0.526	<0.01	<0.01	0.381	0.0122
PUFA/SFA	0.058	0.041	0.171	0.087	0.210	0.0555
omega 6/omega 3	2.065	2.142	0.09	0.196	0.721	0.0241
LA/ALA	1.736	1.840	0.010	<0.01	0.964	0.0322
AA/EPA	2.558	2.431	0.010	0.262	0.942	0.0541
C14:1/C14:0	0.027	0.020	<0.01	<0.01	<0.01	0.0007
C16:1/C16:0	0.015	0.013	<0.01	<0.01	<0.01	0.0006
C18:1/C18:0	1.479	1.482	0.946	<0.01	<0.01	0.0275
C18:2 c9t11/C18:1 t11	0.259	0.255	0.772	<0.01	0.241	0.0110

De novo FA: (C4 -C16); Mixed FA: (C16:0-C16:1); Preformed FA: (> C16); SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; CLA: conjugated linoleic acids; LA: linoleic acid; ALA: alpha linolenic acid; AA: arachidonic acid; EPA: eicosapentaenoic acid; SEM: standard error of the means.

Cheese composition and VOCs profile are reported in Tables 7 and 8. The cheese of group F had higher yield and lipid content but lower protein than control group.

The major odour compounds detected in both cheeses were dodecanoic acid and 2-Nonanone. Limonene, fenchone, estragole and trans-Anethole, not usually detected in goat cheese, were highly different between the two groups, not being detected at all in group C. These are indeed the characteristic compounds of fennel seed. Among free fatty acids nonanoic, decanoic and dodecanoic acids were higher in group C.

Cheese of fennel group had higher total antioxidant capacity than group C ($P < 0.01$). Indeed, the compounds characterizing fennel cheese, namely limonene, fenchone, estragole and trans-Anethole are known for their strong antioxidant activity.

Table 7. Cheese composition analysis (g/100g).

Item	Control	Fennel	G	P	GxP	SEM
			P-value			
Cheese yield (%)	12.40	14.11	<0.01	0.88	0.46	0.273
Protein	9.56	7.82	<0.01	<0.01	<0.01	0.674
Fat	14.95	16.98	<0.01	<0.01	0.01	0.422
Moisture	64.68	66.22	<0.01	<0.01	0.24	0.897

SEM: standard error of the means.

Table 8. Volatile organic compounds (VOCs*) and total antioxidant capacity (TAC) of cheese.

Item	Control	Fennel	Group effect	Period effect	GxP	SEM
			P-value			
Butanoic acid C4:0	3.35	3.21	0.66	0.28	0.31	0.163
Hexanoic acid	4.37	4.23	0.67	0.39	0.51	0.152
Nonanoic acid	4.70	3.78	0.02	0.88	0.45	0.204
Decanoic acid C10 :0	6.16	5.13	0.01	0.41	0.45	0.222
Dodecanoic acid C12:0	16.47	14.20	<0.01	0.09	0.08	0.393
Tetradecanoic acid C14 :0	5.85	5.64	0.52	0.54	0.40	0.157
2.3-Butanediol	7.23	7.35	0.71	0.63	0.88	0.145
1-butanol-3-methyl	6.98	7.19	0.54	0.03	0.41	0.198
Phenylethyl alcohol	6.63	6.27	0.29	0.37	0.47	0.166
Ethyl hexanoate	5.03	4.87	0.64	0.79	0.86	0.144
Ethyl octanoate	9.45	6.20	<0.01	0.00	0.00	0.585
Ethyl decanoate	6.91	7.87	0.01	0.01	0.01	0.293
2-Heptanone	5.65	5.47	0.58	0.12	0.11	0.186
2-Nonanone	12.83	15.22	<0.01	0.35	0.03	0.414
Limonene	nd	3.35	<0.01	0.02	0.22	0.532
Fenchone	nd	3.17	<0.01	0.86	0.86	0.491
Estragole	nd	2.25	<0.01	0.18	0.18	0.364
trans-Anethole	nd	2.60	<0.01	0.76	0.76	0.407
TAC, mol FeSO4 equivalents/kg	2.07	3.41	<0.01	0.36	0.53	0.254

*The values of volatile compounds are expressed as percent of total volatile compounds. TAC: total antioxidant capacity; SEM: standard error of the means.

Cheese fatty acid profile is reported in Table 9. Among the de novo synthesis FA only C14:1 was significantly different between groups, being higher in group C (P <0.05). With regard to C18:1 isomers, almost all of them were significantly different according to the treatment, to the period of sampling and to their interaction. This is the case of C18:1 cis 6 which was higher in group C while cis 9, cis 10 and cis 12 were higher in group F. Linoleic, gamma linoleic, alpha linolenic and CLA isomers (C18:2cis9trans11, C18:2trans10cis12) were higher in group F. The main FA classes had an opposite trend compared to the respective milk, disclosing significantly higher MUFA, PUFA, omega 3, omega 6 and total CLA content (P < 0.01) in group F compared to group C.

Table 9. Cheese fatty acid profile expressed as g/100 g of fat.

Item	Group C	Group F	Group effect	Period effect	G x P	SEM
			P-value			
C4:0	3.20	2.93	0.43	0.99	0.13	0.166
C6:0	2.13	1.70	0.21	0.12	0.20	0.195
C8:0	2.47	2.36	0.75	0.52	0.95	0.147
C10:0	9.73	10.27	0.12	0.56	0.63	0.161
C11:0	0.33	0.20	0.33	0.38	0.58	0.082
C12:0	3.62	3.58	0.90	0.94	0.54	0.146
C14:0	9.55	9.52	0.93	0.25	0.48	0.194
C14:1	0.34	0.21	0.02	0.96	0.79	0.033
C16:0	28.96	29.34	0.26	0.66	0.49	0.156
C16:1	0.35	0.30	<0.01	0.21	0.01	0.014
C18:0	12.04	11.83	0.53	<0.01	0.26	0.307
C18:1 c6	0.10	0.02	<0.01	<0.01	<0.01	0.025
C18:1 c9	18.55	19.54	<0.01	<0.01	<0.01	0.033
C18:t9	1.40	1.35	0.34	0.001	0.03	0.072
C18:1 t11	0.50	0.41	0.01	0.001	0.01	0.365
C18:1 c10	0.25	0.31	<0.01	<0.01	<0.01	0.034
C18:1 c11	0.16	0.17	0.78	<0.01	<0.01	0.012
C18:1 c12	0.09	0.10	<0.01	0.79	<0.01	0.015
C18:2 t6	0.24	0.32	<0.01	<0.01	<0.01	0.024
C18:2 c6	1.32	1.45	0.013	0.17	0.86	0.042
C18:2cis9trans11	0.40	0.50	<0.01	<0.01	0.08	0.021
C18:2trans10cis12	0.19	0.22	<0.01	0.02	0.42	0.015
C18:3 n6	0.04	0.06	<0.01	<0.01	<0.01	0.007
C18:3 n3	0.83	0.91	<0.01	<0.01	0.22	0.024
C22:0	0.15	0.13	0.03	<0.01	0.03	0.012
C20:3 n6	0.01	0.02	<0.01	<0.01	<0.01	0.005
C20:4	0.14	0.14	0.26	<0.01	<0.01	0.004
C22:2 n6	0.01	0.01	<0.01	<0.01	<0.01	0.002
C22:6 n3	0.02	0.03	<0.01	<0.01	<0.01	0.003
C24:1	0.02	0.02	<0.01	<0.01	<0.01	0.002
SFA	74.20	73.54	<0.01	<0.01	<0.01	0.243
MUFA	22.20	22.57	<0.01	<0.01	<0.01	0.291
PUFA	2.92	3.23	<0.01	<0.01	<0.01	0.082
omega 3	0.94	1.03	<0.01	<0.01	0.65	0.023
omega 6	2.39	2.54	<0.01	<0.01	<0.01	0.044
omega 6/omega 3	2.52	2.46	<0.01	<0.01	<0.01	0.038
Total CLA	0.60	0.72	<0.01	<0.01	0.11	0.028

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; CLA: conjugated linoleic acids; SEM: standard error of the means.

Discussion

Diet

Body weight didn't change during the trial in both the experimental groups. The diet of all the grazing goats were formulated to satisfy their energy requirements according to Tudisco et al. (2014) as follows: energy requirements for maintenance 0.0365 UFL/kg metabolic weight (MW:BW^0.75); energy requirements for milk production 0.41 UFL/kg fat-corrected milk (FCM, 4% fat). Therefore, total energy requirement was 1.25 UFL (0.68 UFL maintenance + 0.57 UFL milk production) and since the average pasture DM intake in the inlands of South Italy is 20g/kg BW (Iommelli et al., 2022), the goats, which weighed 50 kg, ingested around 1 kg of pasture, corresponding to an average of 0,78 UFL and the deficit of 0.4 UFL was met by the concentrate.

Fennel seeds resulted rich in lipids, as also previously reported by Unal et al. (2013), representing an important source of bioactive compounds enhancing their therapeutic properties (Díaz-Maroto et al., 2006).

Fennel fatty acid profile was characterized by high MUFA content, mainly represented by petroselinic acid and low content of PUFA and SFA, according to Najdoska-Bogdanov et al. (2015). Petroselinic acid (C18:1n12) is characteristic of the plant family Apiaceae and is particularly abundant in fennel oil. This compound has antimicrobial properties and is involved in the synthesis of lauric acid after its oxidation. Another peculiarity of fennel FA profile is the presence of C11:0 which, as monoacylglycerol preparation has an antimicrobial activity, especially against bacteria responsible for alimentary diseases (Doležalová et al., 2010). Additionally, fennel seeds were found to contain a high concentration of limonene, fenchone, estragole and trans-anethole, with the latter being the most prevalent as previously reported by Rather et al. (2016). Conversely, these compounds were not identified in the pasture samples, which exhibited a diverse range of compounds, including tetradecanoic acid, 1-butanol-3-methyl, and 2-nonanone as the most prevalent. Indeed, Fedele et al. (2005) demonstrated that alcohols and ketones were the most abundant compounds in spring Mediterranean pasture.

Concerning pasture chemical composition, in the present trial in May the pasture had the lowest and the highest values of protein and NDF respectively, in contrast with our previous research in the same area (Lo Presti et al., 2023). This difference could be due to the high temperatures in spring 2022, which affected plant leaves apparatus, lowering the protein content. The increase of protein in July could be due to an increase in rainfall with a recovery of leaves.

The trend of FA in pasture was characterized by lower values of PUFA and higher of MUFA during June, July and August. This changes in PUFA/MUFA ratio could be due to climatic

alterations that highly affect pasture productivity and nutritional composition. According to Martins-Noguerol et al. (2023) warming is able to increase plant productivity and at the same time it shifts the fatty acid profile, probably due to an alteration in plant community composition.

Both climatic stressors, warming and drought, might impact several plant physiology processes related to protein stability, membrane fluidity, changes in nitrogen metabolism, oxidative stress and photosynthetic activity. It was observed that the antioxidant capacity of the pasture underwent changes during the grazing season, with higher peaks of phenolic content occurring in the warmest months (July and August). In the study conducted by Bautista et al. (2016), alterations in phenolic and flavonoid concentrations were associated with the environmental circumstances of the plants and were observed to be contingent on both the taxonomy and ecology of the species under investigation. Despite species-specific differences, a positive correlation was established between plant phenolics and several environmental parameters, including altitude, as well as those related to water stress, such as temperature, evapotranspiration, and soil water deficit. These findings align with numerous prior studies on specific taxa, providing compelling evidence that the synthesis of antioxidant phenolic compounds is induced under water stress and contributes to the drought tolerance of plants in their natural habitats.

Milk

The group of goats supplemented with fennel seeds had a higher milk yield than the control group throughout the trial, as well as higher yields of fat, protein, and lactose. Similar results were reported by other authors (Moosavi-Zadeh et al., 2023; El-Hendawy et al., 2018; Fahim et al., 2022) for milk yield on cows, buffaloes and goats fed with *Foeniculum vulgare Mill.*. Nevertheless, El-Hendawy et al. (2018) and Fahim et al. (2022) recorded also increases in milk solid content contrary to Moosavi-Zadeh et al. (2023) and to our findings.

The increase in milk yield confirms the role of *Foeniculum vulgare Mill.* as galactagogue in dairy goats as already demonstrated in women, even though its mechanism of action could be differently explained in ruminants. Indeed, the chemical similarity between trans-anethole, which is abundant in fennel, and dopamine makes it a suitable antagonist for dopamine receptors thus reducing its activity and enhancing PRL action on mammary gland. Since the role of PRL in ruminants' lactation is controversial (Akers et al., 1981) other mechanisms of action could also be taken into consideration, which are mainly related to glucose metabolism.

Indeed, fennel seeds have orexigenic properties, being able to stimulate the appetite thanks to their aromatic compounds, mainly trans-anethole, estragole, and limonene, as showed in the work of Kargar et al. (2021) on calves and by Moosavi-Zadeh et al. (2023) on dairy cows. Moreover, when diet with same forage to concentrate ratio were fed to ruminants, fennel supplementation induced a reduction in the content of rumen acetate and increased the relative concentration of propionate, that is related to glucose synthesis (Moosavi-Zadeh et al., 2023). The hyperglycaemic effect of fennel depends also on its oestrogenic properties, being able to affect insulin and glucose metabolism through the suppression of adipose lipolysis which reduces circulating free FA levels enhancing insulin sensitivity (Kim et al., 2014). Moreover, acting as a dopamine antagonist, fennel increases glucocorticoid levels which are involved also in lactogenesis and galactopoiesis (Ahmadzadeh et al., 2006). Finally, another possible explanation for milk yield increase could be a regulatory effect of fennel on the expression of IGF1 (Insulin-Like Growth Factor 1), as reported by Shahsavari et al. (2022). In their study the supplementation of mice diet with fennel seeds increased the expression of IGF1, a gene involved in the metabolism of glucose and in milk synthesis (Vernon, 1989).

The changes in milk FA between the groups are probably due to the metabolic effect exerted by fennel compounds. Indeed, the lower content of preformed FA in group F and the higher content of the de novo FA suggests a lower uptake of body lipid reserve as observed also by Moosavi-Zadeh et al. (2023) on cows and may be explained by fennel properties to increase appetite and consequently intake of pasture or to improve diet digestibility. Moreover, as reported above, fennel seeds have hypolipidemic effects and stimulate insulin secretion through various mechanisms (Samadi-Noshahr et al., 2021; Kim et al., 2014) preventing body fat mobilization mainly by influencing the expression of insulin and leptin receptors (Zakernezhad et al., 2021).

Among C18:1 isomers, cis 12 was significantly higher in group C. This FA is mainly related to rumen metabolism of α-linolenic acid or mammary desaturation of the resulting isomers (Marín et al., 2015).

Among LCFAs, C20:4 and C22:0 were lower in group F. With regard to arachidonic acid (AA, C20:4) the lower content may be attributed to the higher presence of petroselinic acid, which has an inhibitory effect on the conversion of linoleic acid (LA) to AA (Heidarian Miri et al., 2013).

As also observed by Moosavi-Zadeh et al. (2023) the content of C18:0 and C18:1 cis-9 did not change between groups. According to Gross et al. (2011) these long-chain FAs in milk could be used as indicators for evaluating energy status in dairy cows and as early predictor of

negative energy balance, which suggests that in our trial all goats' energy requirements were satisfied even in group F, having higher milk production.

The effect of sampling period appeared to be particularly significant for the majority of fatty acids. As reported also by other authors (Strzałkowska et al., 2009; Tudisco et al., 2010; Bernard et al., 2021) the stage of lactation highly affects milk FA composition. Indeed, the sampling effects were observed for the de novo synthesis and the preformed FA, the latter due to the lipomobilization that generally occurs in a more intensive way during the first stages of lactation (Chilliard et al., 2006).

With regards to FA classes, group F had lower content of MUFA and PUFA and higher of SFA. Similar results were observed by Moosavi-Zadeh et al. (2023) on cows in early lactation except for SFA. On the opposite, Mahmoud et al. (2020) found lower SFA and higher MUFA in milk from cows in mid lactation supplemented with fennel.

Concerning the FA classes, SFA, PUFA and MUFA showed treatment, sampling and interaction effects. It is well known that in goats' milk SFA tend to decrease during the stage of lactation while MUFA have an opposite trend. In our trial the treatment with fennel seeds decreased the content of MUFA and PUFA and increased SFA presumably during the whole lactation stages. Finally, the desaturation indexes, C14:1/C14:0 and C16:1/C16:0 were lower in group FEN. These indexes have been suggested to be the most indicative of delta9- desaturase activity (Lock and Garnsworthy, 2003). Our findings are in agreement with the work Tudisco et al. (2019), who demonstrated that the addition to the diet of supplement rich in lipids reduce the activity of SCD in grazing goats.

Cheese

Feeding of fennel seeds in goat diet resulted in higher cheese yield and fat content compared to control group. Cheese yield is affected by many factors (milk composition, genetic variants, pasteurization, coagulant type etc.) but among them, protein and fat content are considered the most influent (A. M. Abd El- Gawad and Ahmed, 2011). As observed by Guinee et al. (2007) the increase of milk protein content during the lactation leads to an increase in cheese yield that has a similar trend to the protein content, which in our trial was reflected by the period effect.

Since these two parameters of milk did not differ between groups, it should be considered that other compounds may have affected cheese production and composition. It has already been reported by several studies that antioxidant compounds are able to affect some modification occurring during the ripening process of cheese (Evangelista et al., 2022; Lucas et al., 2006).

The changes in cheese yield, fat and protein content could be explained by the different microflora developped on F group cheese that may have led to more proteolysis than lipolysis. In the work of Ismail (2004) no cheese yield increase was found when goats were supplemented with fennel. In addition, in their work the authors found a reduction of lipolytic bacteria and a decrease of coliformis counts compared to control group.

With regard to VOCs composition, in dairy products it can be found a great variety of class of compounds, such as, alcohols, ketones, aldehydes, ester, free fatty acids, terpenes that could change according to different feeding strategies (Balivo et al., 2023). The fat appears to be the main factor affecting flavour formation (Vagenas and Roussis, 2012) due to the lipolysis occurring during the ripening which yields fatty acids. In cheeses in general, the activity of lipolysis, mainly exerted by endogenous and microbial lipase, led to a greater formation of short chain fatty acids (SCFAs) which play a more significant role in the flavour of dairy products than medium and long chain FAs. A proportion of free fatty acids, especially of short chain, also derived by the lactose and protein degradation.

In the present study, the major odour compounds detected in both cheeses were dodecanoic acid and 2-nonanone which are generally identified in goat cheese in different quantities.

The methyl ketone 2-nonanone, generally associated with fruity flavour (Chiofalo et al., 2004), resulted higher in group fed fennel whereas dodecanoic and decanoic acids were higher in group control. These two fatty acids have been recognized as the main responsible for goat flavour in dairy products (Singh et al., 2003). They generally derived from the lipolysis that occurs in cheese during the ripening and represent indicators of the process of lipolysis in milk. Moreover, during the early stage of lactation, a negative energy balance could lead to an intense lipolysis of the fatty tissue causing changes in the milk free fatty acids (FFA) content with undesirable effects in the milk and cheese taste and flavour (Strzałkowska et al., 2009; Chilliard et al., 2003). Goat milk in particular, is characterized by short chain fatty acids which tend to increase during cheesemaking and maturation. Since the cheese of group F has been characterized by a greater antioxidant activity ($P < 0.05$), milk fatty acids destined to be cut during ripening, have probably been more protected by antioxidant compounds presents in F group cheese. The use of antioxidants in cheese industry is generally aimed to reduce the phenomena of oxidation and lipolysis or proteolysis over the ripening period. Fennel seeds indeed, have been tested in cheese production for reducing the lipolytic trend in the work of Mansha Rafiq and Ghosh (2021). In their work the authors demonstrated that by adding fennel extract at a level of 1.2% of total solids at the end of cheese processing, the organoleptic properties and rate of lipolysis and proteolysis decreased. In our trial the higher yield, justified

also by a higher moisture content ($P < 0.05$), may have created a better condition for proteolytic bacteria proliferation, leading to a reduction of protein content and an increase of protein derived products.

Fennel seeds supplementation highly affected cheese VOCs profile, providing 4 specific aromatic compounds, namely limonene, fenchone, estragole and trans-Anethole which are usually not detected in goats cheese and that are the main responsible of fennel well known antioxidant properties (Rather et al., 2016). In particular, limonene has been associated to fresh fruity flavour, fenchone to camphor flavour and trans-anethole to anise flavour. In the work of Ismail (2004), authors used a group of panellists to rate the likeability of cheese, with positive results for cheese obtained from goats supplemented with fennel seeds.

The FA profile of cheese also resulted highly affected by fennel supplementation, revealing several differences between the groups. The FA profile of the cheese did not reflect the same composition as the respective milk. The cheese from the fennel group had higher levels of MUFA, PUFA, omega 3, omega 6, and total CLA content compared to group C. In contrast, the milk from the fennel group had lower levels of these FAs than group C. With regard to the other main differences, the cheese of group F had higher content of C18:1 cis10, cis 12, C18:2 cis 6 (LA), and C 18:3 n3 (ALA) and two CLA isomers (C18:2 cis9trans11 and C18:2 trans10cis12), the last known as beneficial for human health (Cavaliere et al., 2018). During cheese ripening, as for others processed and stored foods, oxidation of FA occurs, more intensively on PUFA (Tao, 2015). The results obtained on cheese may suggest that thanks to its antioxidant properties, fennel seeds, specifically their compounds which had been transferred in cheese (trans-anethole, limonene, fenchone and estragole), were able to protect long chain and unsaturated FA from oxidation, revealing significant differences from control group. Nevertheless, the higher TAC in cheeses of the F group, might be also due to its high fat percentages and may not indicate an antioxidant improvement. Therefore, the analysis of oxidative damage should be explored in further research to better assess the antioxidant efficacy of such compounds on cheese.

Chapter V

Effects of milk thistle and green anise supplementation in grazing goats' diet on milk and cheese nutritional characteristics and on the expression of a panel of genes

Experimental Design

The trial was performed at Azienda Zootecnica Antonio Amato located in Casaletto Spartano (SA, Italy; 832 m a.s.l. (40°09' N; 15°37'E)) on 24 multiparous lactating Murciana goats from May to June 2023.

Sixty days after kidding (February), the goats, homogeneous for partity (3^{rd}), body weight (BW = 50 kg ± 2.0 kg) and DMY (1980 ± 120 g/day), were randomly allocated into three groups of 8 goats each (C: control, T: thistle and A: anise). All the animals were fed on a permanent pasture from 9.00 to 16.00 and received 400 g/head/day of corn meal in individual pen; in addition, group T and A received 15 g/head/day of dried milk thistle leaves (*Sylibum marianum*) and anise seed powder (*Pimpinella anisum L.*), respectively, which was mixed into the concentrate before being offered to the animals. All the animals had free access to fresh water.

Analysis

The analysis on feed, milk and cheese were the same conducted in the trial on fennels seeds described in chapter IV.

Gene expression analysis

Milk fat sampling and RNA isolation

At the end of the trial, samples for gene expression analysis were collected as follows. The udder was washed, dried and disinfected and a total of 50 mL of milk, obtained from the morning milking, were individually collected from each animal in sterilized, RNAse free falcon tubes and immediately centrifugated at 4000 g x 10 minutes at 4 °C. One gram of milk fat layer was then scooped out in 15 ml tubes with 3 ml of TRIzol™ LS Reagent (Invitrogen Life Technologies) and vigorously shaken and vortexed for 30 seconds. The samples were immediately transferred in dry ice, transported to the lab and kept at -80 °C until further analysis.

The RNA extraction was performed following TRIzol™ LS Reagent protocol: after thawing in ice, the samples were vortexed and centrifugated at 10000 g x 10 minutes at 4 °C in order to separate the liquid phase, which was transferred into four 2-ml sterilized tubes and added with

200 µL of chloroform. After centrifugation (10000 g x 15 minutes at 4 °C) only the upper phase was taken ad added in a new tube with isopropanol (500 µL) and left at -20 °C over-night for RNA precipitation. After brief vortexing, the samples were centrifugated at 10000 g x 10 minutes at 4 °C to obtain a small pellet by removing gently the isopropanol. One step of ethanol washing was performed by adding 1 mL of ethanol (75%) in each tube and completely removing it after a brief centrifugation (7500 g x 5 minutes at 4 °C). The pellets obtained were then dissolved in 40 µl of sterilized RNAse free water and the kept at -80°C. Two aliquots of RNA samples were subjected to quantitative and qualitative analysis respectively with NanoDrop (ND-1000 spectrophotometer; NanoDrop, Labtech) to measure the absorbance at 260, 280 and 320 and a 2100 Bioanalyzer (Agilent Technologies) to determine RNA integrity. The mean value of RNA integrity (RIN) was 4.5 (± 0.8), which is consistent with the results of other studies on milk fat compared to other matrices (Li et al., 2016).

Reverse Transcription and RT-qPCR

Reverse transcription (RT) was performed from 1 µg of total RNA by using High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems) in a total volume of 20 µL, composed by 6 µL of RNA, 3 µL (2ng/µL) of Luciferase RNA Control (Promega) and 11 µL of Master Mix, according to the manufacturer's instructions. The complementary DNA (cDNA) obtained was then stored at -20 °C for RT-qPCR.

Samples of cDNA were finally subjected to RT-qPCR in duplicate with MicroAmp™ Fast Optical 96-Well Reaction Plates (Applied Biosystems) using Power SYBR™ Green PCR Master Mix (Applied Biosystems).

The candidate genes (*LPL, FASN, SCD1, ACACA, TLR4, SOD, LEP, LEPR, GLUT1*) were investigated and normalized by adopting Luciferase as reference gene (Jiwaji et al., 2010). Each well was filled with 5 µL of cDNA (previously diluted 1:50 with nuclease free water) and 15 µL of Master Mix assembled by 10 µL of Mix SYBR, 2 µL of specific primers (1 µL Forward and 1 µL Reverse) and adjusted to volume of 15 µL with nuclease free water.

RT-qPCR reaction was performed using 7300 Real-Time PCR System (Applied Biosystems) set up with the following program: denaturation at 95°C for 10 minutes; 40 cycles of PCR reactions at 95°C for 15 seconds and 60 °C for 45 seconds according to the hybridization temperature.

The samples were run in duplicate and compared to reference gene to obtain ΔCt. Validation of reference gene stability was obtained with Normfinder software.

Primers were specifically designed on goat reference mRNA sequences and were purchased by Merck (Sigma-Aldrich). Gene primers are reported in table 1.

Table 1. Primer sequences and conditions used for real-time qPCR.

Gene symbol	Encoded protein	Accession no[1]	Nucleotide sequence (5' – 3')[2]	Product size (bp)	Source
LPL	Lipoprotein Lipase	DQ997818.2	F: TTCAGAGGCTATTACTGGAAATCC R: ATGTCAATCACAGCATTCATTCTACT	235	Bernard et al. 2005a
FASN	Fatty Acid Synthase	DQ223929	F: ACAGCCTCTTCCTGTTTGACG R: CTCTGCACGATCAGCTCGAC	247	Ollier et al. 2009
SCD1	Stearoyl-CoA Desaturase	NM_173959	F: TGCTGACAACTTATCTGGATGC R: AAGGAATCCTGCAAACAGCTA	179	Bernard et al. 2005a
ACACA	Acetil Co-A carboxylase	NM_174224.2	F: CATGGAAATGTACGCGGACC R: GGTGGTAGATGGGAAGGAGGA	120	Bernard et al. 2005a
TLR4	Toll Like Receptor 4	JF825527	F: CTTGCGTCCAGGTTGTTCCTAA R: CTGGGAACCTGGAGAAGTTATG	153	Paul et al. 2015
SOD	Super oxide dismutase	AB201469.1	F: AGGCAAAGGGAGATAAAGTCGTCG R: TGCACTGGTACAGCCTTGTGTATTG	113	He et al. 2012
LEP	Leptin	GU944974	F: GCCTATGTGGGCATCCTTTA R: TGGAACAGGGAGGAAGACTG	199	Batista et al. 2013
LEPR	Leptin receptor	AY846770	F: GCTCTGCTTTTGACGACTCC R: ATAAGCCCTTGCTCCTCCTC	196	Batista et al. 2013
GLUT	Glucose transporter	NM_174602.2	F: TGCTCATTAACCGCAACGAG R: GAACAGCTCCAGGATGGTG	149	Komatsu et al. 2005
Luciferase	Luciferase	-	F: AGAGATACGCCCTGGTTCCT R: ATAAATAACGCGCCCAACAC	202	Bui et al. 2009

[1]Accession number of the sequence used to design primers.
[2]Sequences: F = forward primer; R = reverse primer.

Statistical analysis

Data from milk results were analysed by the one-way ANOVA with SPSS IBM software (V29.0.1.0); the means were compared using Tukey's test. Differences were considered significant at $P < 0.05$.

Relative mRNA levels were then calculated for each gene using $2^{-\Delta Ct}$ method. Ct of reference gene was subtracted from Ct of each gene to obtain ΔCt and then relative mRNA value was calculated by the formula $2^{-\Delta Ct}*100$. Data were analysed with one way ANOVA and means compared using Tukey's test.

Results

The VOCs composition and antioxidant activity of pasture and the two herbs used in this trial were disclosed in Table 2. Milk thistle leaves were characterized by high content in butanoic acid, 2-butanol and acetic acid whereas some compounds abundant in anise seeds powder, such as fenchone, estragole and trans-anethole, were non detected in thistle. With regards to TPC, milk thistle was characterized by a content of phenolic compounds almost triple than anise while its antioxidant activity, measured in DPPH and ABTS was slightly lower than anise. Pasture was characterized by a higher variability of VOCs, where tetradecanoic acid, 1-butanol-3-methyl and 2-Nonanone were predominant. Regarding antioxidant activity, pasture had high content in TPC and values od DPPH similar to that of aniseed powder.

Table 2. Anise and thistle antioxidant activity and VOCs.

Item %	Supplementation		Pasture
	Anise seeds powder	Milk thistle aereal part	
Butanoic acid	0.10	7.20	nd
Hexanoic acid	0.10	1.60	3.07
Nonanoic acid	0.10	0.20	2.24
Decanoic acid	0.10	0.20	0.24
Dodecanoic acid	0.10	0.20	2.24
Tetradecanoic acid	0.20	0.20	20.40
2.3-Butanediol	0.10	3.00	0.87
1-butanol-3-methyl	0.10	2.00	31.42
Phenylethyl alcohol	0.10	2.00	13.16
Ethyl hexanoate	0.50	1.20	0.80
Ethyl octanoate	0.40	1.20	nd
Ethyl decanoate	0.20	1.0	0.54
2-Heptanone	0.20	0.80	1.05
2-Nonanone	0.20	0.30	23.05
Limonene	1.80	0.40	0.24
Fenchone	0.10	nd	nd
Estragole	1.50	nd	nd
trans-Anethole	94.10	nd	nd
2-Butanol	nd	47.20	nd
Acetic acid	nd	31.30	0.61
Antioxidants			
TPC. mg GAE/g	8.75	23.82	52.96
ABTS. mg/g TE	8.01	5.43	22.79
DPPH. EC50 µg/ml	37.74	22.61	35.32

TPC: total phenolic content; ABTS: 2,20-azino-bis(3-ethylbenzothiazoline-60-sulfonic acid) diammonium salt; DPPH: 2,2-diphenyl-1-picrylhydrazyl ; ND : not detected.

Feed chemical composition is reported in table 3. Crude fiber (CF) resulted to be higher in anise seeds than thistle whereas ether extract was higher in thistle. The higher content of fiber in anise seeds powder could be related to the external part of the seeds whereas for thistle only the leaves were used, which are characterized by lower fiber and higher protein content (24,84 g/100g). Pasture was characterized by a low content in protein whereas it had high EE and NDF. Corn meal, on the opposite had low content in CF, good nutritional value in term of EE and UFL.

Table 3. Feed chemical composition expressed as g/Kg DM.

Item	Anise	Thistle	Corn meal	Pasture
CP	120.31±1.7	248.47±2.6	101.20 ± 2.3	129.56 ± 1.3
EE	154.52±1.3	203.36±2.1	30.80 ± 0.14	21.78 ± 0.5
CF	154.85±1.2	74.85±0.7	26.1 ± 2.12	280.90 ± 0.42
Ash	57.01±0.6	51.21±0.5	10.41 ± 0.11	70.05 ± 1.3
NDF	/	/	/	504.02 ± 3.4
ADF	/	/	/	371.02 ± 2.8
ADL	/	/	/	136.21 ± 2.1
UFL/Kg DM	0.68	0.79	1.05	0.76

DM: dry matter; CP: crude protein; CF: crude fiber; EE: ether extract; NDF: neutral detergent fiber ; ADF: acid detergent fiber; ADL: acid detergent lignin; UFL: feed unit for lactation; SEM: standard error of the mean.

The fatty acid profile of anise and thistle highly differed for the content of SFA and MUFA while a lower difference was observed for total PUFA (table 4). SFA content of thistle was higher than the anise one, and it was mainly determined by the high content of C16:0 (palmitic acid) and C18:0 (stearic acid). On the opposite, anise had a low content in SFA and higher in MUFA, which were mainly composed by C18:1cis9 (oleic acid). With regards to PUFA, the two herbs had a similar content (anise: 56,44 and thistle: 55,99) but were highly different for their composition especially for two fatty acids. Anise, indeed was characterized by a high content of C18:2cis6 (linoleic acid) whereas thistle had higher C18:3 (γ linolenic acid). Pasture samples were rich in PUFA and in particular in omega 3, wwhich was the most abundant whereas corn meal was characterized by a high content in PUFA, mainly C18:2n6.

Table 4. Feed fatty acid analysis (expressed as g/100g lipid).

Item	Anise	Thistle	Corn	Pasture
C6:0	nd	0.48	nd	nd
C8:0	nd	0.27	nd	nd
C10:0	0.05	nd	nd	nd
C11:0	nd	nd	nd	nd
C12:0	0.02	nd	nd	0.23
C14:0	0.11	0.52	0.06	0.57
C16:0	12.32	29.39	11.98	18.82
C17:0	0.09	0.28	nd	nd
C18:0	4.21	8.07	2.65	6.73
C21:0	nd	0.04	nd	nd
C22:0	nd	0.26	nd	nd
C24:0	nd	0.05	nd	nd
∑ SFA	16.83	39.86	14.71	26.35
C16:1trans	0.14	0.94	nd	0.71
C16:1cis	0.09	0.77	0.08	0.53
C17:1	0.03	0.14	nd	nd
C18:1n9	24.43	2.18	27.78	4.35
C20:1n9	0.37	nd	nd	0.77
∑ MUFA	25.08	4.14	27.90	6.36
C18:2n6	51.00	15.47	54.52	13.76
C18:3n6	4.70	38.77	nd	nd
C18:3n3	0.19	0.14	2.85	52.18
C20:3n3	0.37	0.34	nd	0.62
C20:5n3	0.16	0.46	nd	0.58
C22:5n3	nd	0.12	nd	nd
C22:6n3	nd	0.66	nd	0.15
∑ PUFA	56.44	55.99	57.37	67.29

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.
ND: not detected.

Milk yield and chemical composition

The supplementation of these two herbs resulted in a higher milk yield both in the months of May and June (Table 5). Milk fat content was different among the groups only in month of June, with higher content in the group Control. Lactose content both in months of May and June resulted higher in group C, being not different to anise group in the month of May.

Table 5. Body weight and mik chemical composition.

Item	May					June			
	Control	**Thistle**	**Anise**	**SEM**		**Control**	**Thistle**	**Anise**	**SEM**
BW (Kg)	51.30	50.10	51.20	1.362		50.80	49.70	50.60	1.241
DMY (g)	1478.70^{B}	1758^{A}	1821.20^{A}	91.511		1200^{B}	1632.50^{A}	1672.50^{A}	81.691
Fat (%)	4.11	3.52	3.72	0.120		5.10^{a}	4.25^{ab}	4.051^{b}	0.181
Lactose (%)	4.59^{A}	4.30^{B}	4.41^{AB}	0.038		4.70^{A}	4.42^{B}	4.46^{B}	0.041
Protein (%)	3.07	2.96	3.01	0.025		2.92	2.89	2.97	0.031
Fat g/d	60.59^{b}	62.00^{b}	68.19^{a}	3.456		59.55	70.59	67.33	3.55
Lactose g/d	67.84^{B}	75.63^{A}	80.48^{A}	3.836		56.36	72.19	74.43	3.42
Protein g/d	45.37^{B}	52.03^{A}	54.83^{A}	2.663		35.37^{B}	46.93^{A}	49.68^{A}	2.39

DMY: daily milk yield; BW: body weight; SEM: standard error of the mean. Values within a row with unlike superscripts differ at $P < 0.01$ when uppercase and $P < 0.05$ when lowercase.

Milk fatty acids are reported in table 6. In the samples of May, short chain fatty acids did not change among groups with the exception of C11:0, C14:1 and C15:0, the last two being higher in group C compared to the other groups. The difference in short fatty acids, was more remarkable in the samples of June in which samples disclosed different concentration in several fatty acids such as C4:0 which was higher in Thistle group, C6:0 higher in C and T groups whereas C14:1, C15:0 and C16:0 were higher in group control as in the month of May. Preformed FA (> C16:0) showed higher differences among groups both in the month of May and June. In the month of May, the milk of anise a thistle groups had lower content of C17:0, C18:0, C18:1t11, C18:1c12, C18:2t6, C18:3 (ALA), C22:0, C20:4, C24:0, C20:5, C22:5 than C group. Isomers of C18:1 such as C18:1c10 were in lower content only in group C, while C18:1c11 was lower only in group A. The CLA isomer C18:2c9t11 was higher in group Control and Anise and C20:2n6, C22:1, C24:1 were higher in group T. In month of June the group C, as for May, had higher content of C17:0, C18:1c10 and c11, C22:0, C22:5 and lower content of C20:3 and C22:6. In addition C and A group had higher content of C18:0, C18:2 (LA), C18:3 (ALA), C20:4 and C20:5.

Table 6. Milk fatty acid composition expressed as g/100 g of milk fat.

Item	May				June			
	Control	**Thistle**	**Anise**	**SEM**	**Control**	**Thistle**	**Anise**	**SEM**
C4:0	3.386	2.600	3.824	0.2261	3.032^{C}	6.363^{A}	3.989^{B}	0.3231
C6:0	1.455	1.569	1.409	0.0382	1.541^{A}	1.592^{A}	1.232^{B}	0.0492
C8:0	2.268	2.529	2.451	0.0800	2.325	2.213	2.043	0.0595
C10:0	8.628	9.929	9.025	0.2430	8.886	8.216	8.356	0.1976
C11:0	0.113^{b}	0.149^{a}	0.138^{ab}	0.0055	0.103	0.114	0.122	0.0054
C12:0	3.164	3.432	3.447	0.1060	3.181	2.648	3.016	0.0955
C13:0	0.031	0.029	0.033	0.0022	0.034	0.022	0.026	0.0032
C14:0	8.437	9.092	9.392	0.1936	8.178	8.215	8.468	0.1061
C14:1	0.243^{A}	0.175^{B}	0.194^{B}	0.0091	0.194^{A}	0.146^{B}	0.155^{B}	0.0071
C15:0	0.749^{A}	0.555^{B}	0.600^{B}	0.0242	0.627^{A}	0.503^{B}	0.539^{B}	0.0172
C16:0	28.343	29.252	28.582	0.3114	28.318	28.813	28.481	0.3555
C16:1	0.388	0.335	0.291	0.0235	0.370^{A}	0.301^{B}	0.321^{B}	0.0095
C17:0	0.554^{A}	0.425^{B}	0.421^{B}	0.0168	0.490^{A}	0.352^{C}	0.427^{B}	0.0151
C17:1	0.032	0.034	0.029	0.0027	0.026	0.039	0.028	0.0032
C18:1 cis 6	0.024	0.020	0.023	0.0014	0.027	0.029	0.026	0.0016
C18:0	14.886^{a}	13.133^{b}	13.264^{b}	0.2814	15.202^{a}	13.443^{b}	15.425^{a}	0.3128
C18:1 t9	0.498	0.318	0.605	0.0661	0.396^{B}	0.671^{A}	0.320^{B}	0.0504
C18:1 t11	1.336^{a}	1.055^{b}	1.063^{b}	0.0501	1.903	1.738	1.699	0.0535
C18:1 c9	19.748	20.575	20.340	0.2455	19.342	19.667	19.459	0.2044
C18:1 c10	0.318^{A}	0.189^{B}	0.244^{AB}	0.0188	0.358^{a}	0.265^{b}	0.331^{ab}	0.0166
C18:1 c11	0.206^{A}	0.166^{AB}	0.142^{B}	0.0089	0.199^{A}	0.145^{B}	0.152^{B}	0.0075
C18:1 c12	0.079^{a}	0.055^{ab}	0.050^{b}	0.0057	0.101	0.078	0.086	0.0052
C18:2 t6	0.272^{A}	0.186^{B}	0.198^{B}	0.0128	0.307	0.282	0.274	0.0075
C18:2 c6	1.837	1.695	1.654	0.0441	1.859^{A}	1.532^{B}	1.865^{A}	0.0502
C20:0	0.252	0.248	0.234	0.0092	0.234	0.210	0.254	0.0090
C20:1	0.060	0.049	0.052	0.0021	0.050	0.054	0.042	0.0031
C18:3 n3	0.987^{A}	0.713^{B}	0.718^{B}	0.0350	1.053^{A}	0.795^{B}	0.918^{AB}	0.0320
C18:2 cis9 trans11	0.270^{A}	0.198^{B}	0.263^{A}	0.0110	0.405	0.384	0.404	0.0121
C18:2 trans10 cis12	0.216	0.211	0.196	0.0050	0.235	0.227	0.212	0.0062
C20:2 n6	0.026^{A}	0.024^{A}	0.015^{B}	0.0015	0.028	0.029	0.028	0.0016
C22:0	0.133^{A}	0.097^{B}	0.102^{B}	0.0055	0.124^{a}	0.100^{b}	0.108^{ab}	0.0041
C20:3 n6	0.008	0.008	0.010	0.0001	0.006^{b}	0.010^{a}	0.006^{b}	0.0010
C22:1	0.006^{b}	0.009^{a}	0.008^{ab}	0.0000	0.007	0.007	0.006	0.0001
C20:4 n6	0.179^{A}	0.128^{B}	0.120^{B}	0.0071	0.151^{A}	0.109^{B}	0.123^{AB}	0.0062
C22:2 n6	0.026	0.026	0.021	0.0022	0.014	0.018	0.014	0.0015
C24:0	0.055^{a}	0.050^{ab}	0.036^{b}	0.0035	0.046	0.036	0.039	0.0031
C20:5 n3	0.069^{A}	0.039^{B}	0.042^{B}	0.0035	0.067^{A}	0.039^{B}	0.055^{A}	0.0032
C24:1	0.022^{ab}	0.0238^{a}	0.017^{b}	0.0011	0.020	0.020	0.020	0.0015
C22:4 n6	0.021	0.024	0.022	0.0018	0.017	0.014	0.016	0.0016
C22:5 n3	0.119^{A}	0.024^{B}	0.020^{B}	0.0109	0.122^{A}	0.018^{B}	0.017^{B}	0.0112
C22:6 n3	0.035^{B}	0.074^{A}	0.077^{A}	0.0054	0.032^{C}	0.066^{B}	0.083^{A}	0.0052

SEM: standard error of the mean. Values within a row with unlike superscripts differ at $P < 0.01$ when uppercase and $P < 0.05$ when lowercase.

The main milk FA classes, ratios and desaturation indexes are reported in table 7. Both in the months of May and June no differences were found in the content of milk SFA and MUFA, whereas the content of PUFA was highly different. In the month of May, group C had higher content of PUFA, omega 6 and 3, PUFA/SFA ratio and C14:1/C14:0 desaturation index than the other two groups. In May higher content of CLA were observe both for C and A groups, the ratio LA/ALA was higher both in T and A groups and the desaturation index C18:1/C18:0 was higher in the supplemented groups. In the month of June group C and A recorded higher value of PUFA, omega 6, PUFA/SFA compared to group T. Group C had higher content of omega 3 and higher value of the desaturation index C14:1/C14:0 and C16:1/C16:0. Group T had higher value for the ratio AA/EPA and the desaturation index C18:1/C18:0 compared to the other groups.

Table 7. Milk fatty acid classes and ratios.

Item	May					June			
	Control	**Thistle**	**Anise**	**SEM**		**Control**	**Thistle**	**Anise**	**SEM**
SFA	72.464	73.092	72.961	0.2731		72.329	72.843	72.528	0.2561
MUFA	22.963	23.005	23.061	0.2372		22.995	23.160	22.647	0.1972
PUFA	4.132^{A}	3.353^{B}	3.359^{B}	0.0985		4.346^{A}	3.525^{B}	4.020^{A}	0.0890
omega 6	2.434^{A}	2.091^{B}	2.041^{B}	0.0586		2.431^{A}	1.995^{B}	2.327^{A}	0.0542
omega 3	1.211^{A}	0.852^{B}	0.858^{B}	0.0445		1.275^{A}	0.919^{B}	1.076^{B}	0.0395
CLA	0.486^{a}	0.409^{b}	0.459^{ab}	0.0124		0.639	0.611	0.616	0.0156
PUFA/SFA	0.057^{A}	0.045^{B}	0.046^{B}	0.0018		0.060^{A}	0.048^{B}	0.055^{A}	0.0014
omega 6/omega 3	2.028	2.493	2.433	0.0865		1.912	2.211	2.198	0.0698
LA/ALA	1.882^{b}	2.417^{a}	2.383^{a}	0.1012		1.775	1.959	2.075	0.0735
AA/EPA	2.586	3.490	2.941	0.1721		2.255^{B}	2.938^{A}	2.247^{B}	0.1116
De novo	28.474	30.059	30.51	0.4352		28.10	30.03	27.94	0.4159
Mixed	28.730	29.58	28.87	0.3155		28.68	29.11	28.80	0.3544
Preformed	42.345^{A}	39.80^{B}	39.99^{B}	0.3976		42.87^{A}	40.38^{B}	42.44^{A}	0.3762
C14:1/C14:0	0.028^{A}	0.019^{B}	0.020^{B}	0.0018		0.023^{a}	0.018^{b}	0.018^{b}	0.0016
C16:1/C16:0	0.014	0.011	0.010	0.0017		0.013^{A}	0.010^{B}	0.011^{B}	0.0004
C18:1/C18:0	1.330^{b}	1.577^{a}	1.556^{a}	0.0445		1.280^{b}	1.472^{a}	1.276^{b}	0.0362
C18:2 c9t11/C18:1 t11	0.202	0.194	0.267	0.0151		0.212	0.224	0.242	0.0080

De novo FA: (C4 -C16); Mixed FA: (C16:0-C16:1); Preformed FA: (> C16); SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; CLA: conjugated linoleic acids; LA: linoleic acid; ALA: alpha linolenic acid; AA: arachidonic acid; EPA: eicosapentaenoic acid; SEM: standard error of the mean. Values within a row with unlike superscripts differ at $P < 0.01$ when uppercase and $P < 0.05$ when lowercase.

Cheese results

The cheeses were obtained using the milk of May and June and were collected after 25 and 50 days of ripening. Therefore, below the will be reported two different table, one for may, with both the ripening period and another table for the month of June.

The chemical composition of cheese obtained in May and antioxidant activity are reported in table 8. For all the parameters considered, significant differences were found. The cheese of group A ripened for 25 days obtained with the milk of may was characterized by higher content of fat, crude protein, TPC and TAC and with the cheese of group T had higher value of DPPH. The cheese of group T ripened 25 days had the lowest content of fat and had higher values of crude protein, TPC and TAC than group C and lower than group A. Group C showed the highest content of cholesterol. The differences observed in 25 days ripened cheese were similar to that of 50 days ripened cheese except for the content of protein which was higher in group T.

Table 8. chemical composition and antioxidant activity of cheese obtained in May at different ripening period.

Item	May 25 days ripening					May 50 days ripening			
	Control	**Thistle**	**Anise**	**SEM**		**Control**	**Thistle**	**Anise**	**SEM**
Fat (g/100g)	36.35^{B}	30.62^{C}	38.54^{A}	1.184		41.53^{B}	32.56^{C}	46.41^{A}	2.029
Protein (g/100g)	26.12^{C}	26.69^{B}	27.74^{A}	0.240		28.55^{C}	29.55^{A}	29.20^{B}	0.146
Total chol. mg/100 g	27.88^{A}	25.18^{B}	25.18^{B}	0.449		38.71^{A}	33.56^{C}	34.75^{B}	0.778
TPC. mg GAE/kg	142.00^{C}	152.66^{B}	154.76^{A}	1.978		196.16^{C}	200.90^{B}	311.10^{A}	18.774
TAC. mol FeSO4 eq/kg	1.80^{C}	2.74^{B}	3.13^{A}	0.197		1.70^{C}	3.20^{B}	3.70^{A}	0.300
DPPH. % scavenging activity	54.32^{B}	56.83^{A}	56.81^{A}	0.419		52.97^{C}	57.88^{B}	58.39^{A}	0.864

TPC: total phenolic content; TAC: total antioxidant capacity; DPPH: 2,2-diphenyl-1-picrylhydrazyl; SEM: standard error of the mean. Values within a row with unlike superscripts differ at $P < 0.01$ when uppercase and $P < 0.05$ when lowercase.

In Table 9 The chemical composition of cheese obtained in June and antioxidant activity are reported. The results related to June 25 days ripened cheese samples are similar to what observed in May. Anise group cheese had the highest values of CP, TPC, TAC and DPPH while, on the contrary of what observed in May, the highest content of fat was registered for the cheese of group C, both for the 25- and 50-days ripened samples. In the samples obtained after 50 days of ripening the T group recorded the highest value of TAC and the lowest of Cholesterol.

Table 9. chemical composition and antioxidant activity of cheese obtained in June at different ripening period.

Item	June 25 days ripening					June 50 days ripening			
	Control	**Thistle**	**Anise**	**SEM**		**Control**	**Thistle**	**Anise**	**SEM**
Fat (g/100g)	34.76^{A}	28.27^{C}	31.35^{B}	0.936		55.20^{A}	33.80^{C}	43.17^{B}	3.097
Protein (g/100g)	27.92^{A}	26.58^{B}	28.01^{A}	0.232		30.45^{B}	30.52^{B}	31.34^{A}	0.144
Total chol. mg/100 g	28.14^{A}	27.57^{B}	26.79^{C}	0.198		40.42^{A}	36.71^{C}	38.72^{B}	0.536
TPC. mg GAE/kg	237.96^{C}	288.53^{B}	300.16^{A}	9.549		143.80^{C}	316.10^{B}	328.10^{A}	29.767
TAC. mol FeSO4 eq/kg	1.82^{B}	2.86^{A}	2.93^{A}	0.179		2.09^{C}	3.29^{A}	3.16^{B}	0.189
DPPH. % scavenging activity	55.08^{C}	58.64^{B}	58.25^{A}	0.564		54.46^{C}	56.82^{B}	58.84^{A}	0.635

TPC: total phenolic content; TAC: total antioxidant capacity; DPPH: 2,2-diphenyl-1-picrylhydrazyl; SEM: standard error of the mean. Values within a row with unlike superscripts differ at $P < 0.01$ when uppercase and $P < 0.05$ when lowercase.

In table 10 the fatty acids of the cheese obtained from the milk of may are reported for 25 and 50 days of ripening. The 35 days ripened cheese showed more differences in short chain fatty acids than the 50 days ripened one. In particular group A and T differed from T for the content of C11:0 and C14:0, whereas group C and T had higher content of C14:0 than group A. Among long chain fatty acids (> C16:0), C16:1, C17:0, C18:0, C18:1t11, C18:1c10, C18:2t6, C20:0, C22:2 were higher in group control than the others, while C18:1t9 was higher both in group A and C and C18:1c11, C20:1, C24:1, C22:4 were higher in group A and only C20:3 and C22:1 were higher in group T compared to the other groups. The main FA classes and ration did not change in 25 days ripened cheese among the groups. With regard to the 50 days ripened cheese, among short chain FA only C4:0 was different, being higher both in groups C and T while C16:0 was higher in groups A and T. Among long chain FA, C17:0, C18:0, C18:1t11, C18:1c12, C20:4, C22:2 were higher in group C. Group T and C had also higher content of C17:1, C18:1t9, C18:1c11, C20:5, C22:5 and C22:6 and only group T had higher content of C20:1, C20:4, C24:1 and C22:4. Among FA classes and ratios the highest T group showed the highest MUFA and the lowest SFA content as also lower content of omega 6 and PUFA/SFA and omega6/omega3 ratio than group A.

Table 10. Fatty acid profile of cheese obtained with the milk of May (expressed as g/100g of fat).

Item	May 25 days ripening					May 50 days ripening			
	Control	**Thistle**	**Anise**	**SEM**		**Control**	**Thistle**	**Anise**	**SEM**
C4:0	2.574	2.651	2.985	0.1026		3.444^{A}	3.306^{A}	2.253^{B}	0.1667
C6:0	1.529	1.702	1.731	0.0489		1.761	1.806	1.616	0.0516
C8:0	2.397^{b}	2.809^{a}	2.500^{ab}	0.0745		2.713	2.705	2.366	0.0835
C10:0	8.994	9.103	9.056	0.0984		8.921	8.798	9.003	0.1312
C11:0	0.129^{B}	0.169^{A}	0.159^{A}	0.0061		0.132	0.161	0.152	0.0070
C12:0	3.561	3.782	3.518	0.1178		3.572	3.283	3.614	0.0751
C13:0	0.037	0.037	0.040	0.0008		0.035	0.033	0.043	0.0020
C14:0	8.207^{B}	8.879^{A}	8.794^{A}	0.1039		8.559	9.161	8.994	0.1479
C14:1	0.157^{A}	0.153^{A}	0.134^{B}	0.0033		0.141	0.147	0.139	0.0056
C15:0	0.544	0.573	0.573	0.0117		0.533	0.493	0.523	0.0084
C16:0	27.063	27.135	27.446	0.1251		25.828^{B}	27.334^{A}	27.986^{A}	0.2938
C16:1	0.346^{A}	0.294^{B}	0.290^{B}	0.0095		0.362	0.476	0.343	0.0548
C17:0	0.458^{A}	0.413^{B}	0.393^{B}	0.0094		0.552^{A}	0.397^{B}	0.374^{B}	0.0255
C17:1	nd	nd	nd	-		0.017^{A}	0.015^{A}	0.022^{B}	0.0009
C18:1 c6	0.029^{AB}	0.025^{B}	0.031^{A}	0.0010		0.168	0.085	0.0728	0.0306
C18:0	16.150^{A}	15.018^{B}	14.845^{B}	0.1896		15.722^{a}	13.214^{b}	15.206^{ab}	0.4553
C18:1 t9	0.326^{A}	0.256^{B}	0.360^{A}	0.0144		0.423^{A}	0.442^{A}	0.354^{B}	0.0131
C18:1 t11	1.886^{A}	1.501^{B}	1.604^{B}	0.0526		2.493^{A}	1.813^{B}	1.538^{C}	0.1241
C18:1 c9	18.660	19.053	18.575	0.1015		17.860^{B}	19.624^{A}	18.629^{AB}	0.2565
C18:1 c10	0.500^{A}	0.335^{B}	0.387^{B}	0.0219		0.467^{A}	0.259^{B}	0.408^{A}	0.0289
C18:1 c11	0.199^{b}	0.208^{b}	0.252^{a}	0.0098		0.222^{A}	0.207^{A}	0.156^{B}	0.0093
C18:1 c12	0.114	0.108	0.101	0.0054		0.101^{A}	0.071^{B}	0.079^{B}	0.0040
C18:2 t6	0.308^{A}	0.244^{B}	0.253^{B}	0.0103		0.323^{A}	0.130^{B}	0.301^{A}	0.0315
C18:2 c6	1.738	1.863	1.853	0.0277		1.806^{B}	1.838^{B}	2.148^{A}	0.0519
C20:0	0.337^{A}	0.276^{B}	0.282^{B}	0.0102		0.300	0.281	0.297	0.0091
C20:1	0.035^{B}	0.030^{B}	0.048^{A}	0.0023		0.040^{B}	0.057^{A}	0.039^{B}	0.0028
C18:3n3	0.934	0.883	0.947	0.0187		0.968^{ab}	0.826^{b}	1.047^{a}	0.0358
C18:2cis9trans11	0.329	0.290	0.362	0.0145		0.316	0.300	0.304	0.0068
C18:2trans10cis12	0.248^{a}	0.262^{a}	0.215^{b}	0.0083		0.260	0.272	0.250	0.0086
C20:2 n6	0.048	0.038	0.048	0.0021		0.040^{a}	0.026^{b}	0.029^{ab}	0.0023
C22:0	0.151	0.146	0.132	0.0055		0.123	0.118	0.129	0.0036
C20:3 n6	0.013^{B}	0.017^{A}	0.013^{B}	0.0006		0.012	0.014	0.014	0.0006
C22:1	0.016^{A}	0.017^{A}	0.010^{B}	0.0010		0.006^{B}	0.013^{A}	0.012^{A}	0.0011
C20:4 n6	0.117	0.096	0.112	0.0049		0.156^{A}	0.101^{B}	0.120^{B}	0.0074
C23:0	0.049	0.052	0.056	0.0014		0.078	0.027	0.017	0.0179
C22:2 N6	0.016^{A}	0.010^{B}	0.011^{B}	0.0009		0.061^{A}	0.019^{B}	0.017^{B}	0.0064
C24:0	0.095	0.093	0.090	0.0018		0.060^{B}	0.113^{A}	0.046^{B}	0.0091
C20:5 n3	0.118	0.128	0.132	0.0067		0.118^{A}	0.109^{A}	0.074^{B}	0.0066
C24:1	0.148^{B}	0.160^{B}	0.218^{A}	0.0101		0.050^{B}	0.201^{A}	0.087^{B}	0.0214
C22:4 n6	0.030^{C}	0.054^{B}	0.090^{A}	0.0077		0.018^{C}	0.106^{A}	0.067^{B}	0.0109
C22:5 n3	0.022^{b}	0.031^{a}	0.026^{ab}	0.0016		0.029^{a}	0.027^{a}	0.022^{b}	0.0012
C22:6 n3	0.082	0.120	0.134	0.0105		0.138^{A}	0.165^{A}	0.084^{B}	0.0107
SFA	72.283	72.845	72.606	0.1671		72.339^{ab}	71.235^{b}	72.627^{a}	0.2565
MUFA	22.422	22.144	22.015	0.0972		22.373^{B}	23.433^{A}	21.907^{B}	0.2349
PUFA	4.007	4.043	4.202	0.0449		4.250^{ab}	3.940^{b}	4.482^{a}	0.0846
omega 6	2.273	2.325	2.383	0.0254		2.419^{B}	2.238^{B}	2.698^{A}	0.0633
omega 3	1.156	1.164	1.241	0.0299		1.255	1.128	1.229	0.0300

CLA	0.577	0.552	0.578	0.0110		0.576	0.573	0.554	0.0080
PUFA/SFA	0.055	0.055	0.057	0.0006		0.058^{ab}	0.055^{b}	0.061^{a}	0.0010
omega 6/omega 3	1.975	1.998	1.944	0.0503		1.927^{b}	1.984^{ab}	2.212^{a}	0.0511
LA/ALA	1.862	2.110	1.977	0.0577		1.865	2.234	2.072	0.0678
AA/EPA	0.995	0.763	0.876	0.0474		1.333^{A}	0.955^{B}	1.608^{A}	0.0909

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; CLA: conjugated linoleic acids; LA: linoleic acid; ALA: alpha linolenic acid; AA: arachidonic acid; EPA: eicosapentaenoic acid; nd: not detected; SEM: standard error of the means. Values within a row with unlike superscripts differ at $P < 0.01$ when uppercase and $P < 0.05$ when lowercase.

In table 11 the FA of cheese obtained at 25 and 50 days of ripened with the milk of June are reported. In the 25-days ripened cheeses several differences in short chain fatty acids were observed among groups. In particular C4:0, C8:0, C:10, C11:0 and C12:0 were higher in group Anise and similar between C and T group. Among long chain FA group C and T had higher values than group A for C18:0, C18:1t9, C18:1c9, C18:1c10, C18:1c11, C18:1c12, C18:2t6, C20:0 and C20:1. The highest content of ALA (C18:3n3) was observed in the cheese of group C. Among the main FA classes and ratios anise group scored the highest content of SFA whereas MUFA, omega6/omega3 and LA/ALA were higher in group T compared to the other groups. Similarly, the cheese obtained with the milk of June showed the lowest value of short chain fatty in the group C. among long chain fatty acids C17:0, C17:1, C18:1c10 and C18:1c12 were higher in group C whereas both in group C and T were recorded higher values of C18:0, C18:1c9 and C18:c11 than group A. on the opposite of what observed in the 25 days ripened cheese, the content of C18:2c9t11 was higher in group A than the other groups. Very long chain FA were higher in group T, specifically C22:2, C24:0, C20:5, C24:1 and C22:5. As for the ratios and classes, similarly to the 25-days ripened cheese, Anise group scored the highest SFA content and T group scored the highest content of MUFA but the lowest of total n3 FA.

Table 11. Fatty acid profile of cheese obtained with the milk of June (expressed as g/100g of fat).

Item	June 25 days ripening				June 50 days ripening			
	Control	**Thistle**	**Anise**	**SEM**	**Control**	**Thistle**	**Anise**	**SEM**
C4:0	3.065^{A}	1.320^{B}	2.878^{A}	0.2419	1.908^{C}	3.167^{A}	2.597^{B}	0.1589
C6:0	1.792	1.911	1.887	0.0253	1.813	1.612	1.695	0.0412
C8:0	2.459^{B}	2.531^{B}	2.759^{A}	0.0478	2.537	2.665	2.753	0.0607
C10:0	8.673^{B}	8.332^{B}	10.096^{A}	0.2419	8.654^{B}	7.408^{C}	9.980^{A}	0.3258
C11:0	0.143^{b}	0.150^{ab}	0.175^{a}	0.0054	0.142^{B}	0.249^{A}	0.138^{B}	0.0163
C12:0	3.077^{B}	2.930^{B}	3.620^{A}	0.0926	3.173^{B}	3.089^{B}	3.989^{A}	0.1388
C13:0	0.038	0.035	0.039	0.0010	0.031	0.026	0.027	0.0011
C14:0	8.115	8.585	8.521	0.1006	8.193	8.781	8.803	0.1233
C14:1	0.180	0.183	0.165	0.0049	0.168^{a}	0.137^{ab}	0.133^{b}	0.0062
C15:0	0.690	0.628	0.622	0.0147	0.648	0.633	0.606	0.0154
C16:0	25.333^{C}	26.437^{A}	25.845^{B}	0.1501	25.667^{b}	26.197^{ab}	26.335^{a}	0.1163
C16:1	0.352	0.452	0.375	0.0307	0.381^{A}	0.289^{B}	0.304^{B}	0.0137
C17:0	0.531	0.524	0.483	0.0152	0.508^{a}	0.366^{b}	0.403^{ab}	0.0238
C17:1	nd	nd	nd	-	0.029^{A}	0.020^{B}	0.020^{B}	0.0013
C18:1 c6	0.030	0.030	0.039	0.0018	0.064	0.171	0.0278	0.0315
C18:0	17.081^{A}	16.161^{B}	15.389^{C}	0.2176	16.762^{A}	16.100^{A}	14.652^{B}	0.2818
C18:1 t 9	0.433^{A}	0.371^{B}	0.371^{B}	0.0104	0.488	0.466	0.486	0.0154
C18:1 t 11	1.674	1.766	1.756	0.0317	1.966	1.609	1.804	0.0870
C18:1 c9	19.835^{B}	21.073^{A}	18.658^{C}	0.3045	20.262^{A}	20.995^{A}	18.870^{B}	0.2859
C18:1 c10	0.404^{A}	0.290^{B}	0.315^{B}	0.0157	0.387^{A}	0.187^{C}	0.302^{B}	0.0260
C18:1 c11	0.275^{a}	0.233^{b}	0.237^{b}	0.0076	0.271^{A}	0.277^{A}	0.192^{B}	0.0138
C18:1 c12	0.109^{ab}	0.115^{a}	0.080^{b}	0.0065	0.091^{A}	0.051^{C}	0.063^{B}	0.0052
C18:2 t6	0.279^{A}	0.257^{A}	0.212^{B}	0.0093	0.268	0.230	0.259	0.0075
C18:2 c6	1.736	1.894	1.905	0.0339	1.755	1.941	1.925	0.0365
C20:0	0.339^{A}	0.298^{AB}	0.276^{B}	0.0097	0.357^{A}	0.289^{B}	0.248^{B}	0.0156
C20:1	0.045^{A}	0.036^{A}	0.017^{B}	0.0040	0.039^{B}	0.050^{A}	0.040^{B}	0.0018
C18:3 n3	0.935^{a}	0.856^{b}	0.853^{b}	0.0158	1.033^{A}	0.693^{C}	0.869^{B}	0.0449
C18:2cis9trans11	0.310	0.334	0.310	0.0119	0.336^{B}	0.365^{B}	0.427^{A}	0.0134
C18:2trans10cis12	0.298^{a}	0.269^{a}	0.194^{b}	0.0167	0.277	0.241	0.268	0.0075
C20:2 n6	0.048^{A}	0.029^{B}	0.036^{B}	0.0026	0.032^{A}	0.037^{A}	0.022^{B}	0.0021
C22:0	0.159	0.153	0.156	0.0053	0.157^{A}	0.150^{A}	0.111^{B}	0.0070
C20:3 n6	0.012	0.010	0.013	0.0005	0.013	0.012	0.015	0.0005
C22:1	0.011	0.008	0.011	0.0005	0.010	0.010	0.010	0.0003
C20:4 n6	0.127^{A}	0.122^{A}	0.098^{B}	0.0041	0.163^{A}	0.106^{B}	0.101^{B}	0.0094
C23:0	0.046	0.031	0.131	0.0299	0.015^{B}	0.013^{B}	0.030^{A}	0.0024
C22:2 n6	0.014^{B}	0.016^{B}	0.057^{A}	0.0059	0.025^{B}	0.036^{A}	0.020^{B}	0.0022
C24:0	0.106	0.094	0.090	0.0038	0.060^{B}	0.138^{A}	0.047^{B}	0.0121
C20:5 n3	0.126	0.125	0.108	0.0048	0.122^{A}	0.115^{A}	0.084^{B}	0.0061
C24:1	0.167	0.137	0.153	0.0070	0.079^{A}	0.091^{A}	0.064^{B}	0.0037
C22:4 n6	0.092	0.102	0.109	0.0032	0.077	0.083	0.088	0.0027
C22:5 n3	0.018^{b}	0.031^{a}	0.021^{ab}	0.0023	0.021^{B}	0.038^{A}	0.027^{B}	0.0023
C22:6 n3	0.137	0.140	0.159	0.0050	0.098	0.096	0.110	0.0094
SFA	71.656^{B}	70.128^{C}	72.973^{A}	0.3767	70.630^{B}	70.890^{B}	72.421^{A}	0.2928
MUFA	23.519^{B}	24.700^{A}	22.181^{C}	0.3166	24.270^{A}	24.380^{A}	22.345^{B}	0.2937
PUFA	4.135	4.191	4.079	0.0479	4.227	4.000	4.221	0.0467
omega 6	2.309	2.433	2.432	0.0313	2.335	2.447	2.432	0.0313
omega 3	1.217	1.153	1.142	0.0163	1.276^{A}	0.945^{C}	1.092^{B}	0.0445

CLA	0.608	0.604	0.504	0.0224		0.614^{ab}	0.607^{b}	0.695^{a}	0.0168
PUFA/SFA	0.057	0.059	0.055	0.0008		0.059	0.056	0.058	0.0006
omega 6/omega 3	1.898^{B}	2.109^{A}	2.131^{A}	0.0362		1.834^{B}	2.601^{A}	2.231^{A}	0.1065
LA/ALA	1.855^{B}	2.211^{A}	2.233^{A}	0.0545		1.708^{C}	2.799^{A}	2.225^{B}	0.1424
AA/EPA	1.020	0.977	0.932	0.0379		1.369	0.920	1.201	0.0849

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; CLA: conjugated linoleic acids; LA: linoleic acid; ALA: alpha linolenic acid; AA: arachidonic acid; EPA: eicosapentaenoic acid; nd: not detected; SEM: standard error of the means. Values within a row with unlike superscripts differ at $P < 0.01$ when uppercase and $P < 0.05$ when lowercase.

In table 12 the volatile organic compounds of cheeses obtained in May are presented. The cheese obtained by the group supplemented with thistle was characterized by higher butanoic acid, acetic acid and 2 butanol. The last two compounds were not detected in the other two cheese. In the cheese ripened 50 days the T group was also characterized by higher hexanoic and decanoic acids than the other groups. The cheese from group A after 25 days of ripening presented higher content of dodecanoic acid, 2-nonanone, limonene and trans-anethole. Also in this case, the last two compounds were not detected in the other cheese. After 50 days of ripening the A group cheese scored in addition higher values of 1-butanol-3-methyl than other groups. Group P scored the highest values of Tetradecanoic acid, 2,3-Butanediol and 1-butanol-3-methyl and Ethyl decanoate both at 25 and 50 days of ripening.

Table 12. VOCs in the cheese obtained with the milk collected in May.

Item (%)	May 25 days ripening				May 50 days ripening			
	Control	**Thistle**	**Anise**	**SEM**	**Control**	**Thistle**	**Anise**	**SEM**
Butanoic acid	3.26^{B}	6.56^{A}	3.56^{B}	0.536	4.06^{B}	5.46^{A}	2.86^{C}	0.389
Hexanoic acid	4.06	4.06	4.36	0.113	3.76^{B}	5.16^{A}	2.86^{C}	0.350
Nonanoic acid	4.96	4.36	4.06	0.167	3.76^{B}	5.06^{A}	5.16^{A}	0.247
Decanoic acid	6.16	5.96	5.76	0.117	5.86^{B}	6.86^{A}	5.46^{B}	0.232
Dodecanoic acid	15.86^{B}	16.26^{B}	17.36^{A}	0.246	16.86^{A}	17.36^{A}	16.06^{B}	0.215
Tetradecanoic acid	4.96^{A}	3.56^{B}	3.86^{B}	0.236	5.56^{A}	3.96^{B}	4.46^{B}	0.257
2.3-Butanediol	6.96^{A}	4.06^{B}	4.36^{B}	0.471	7.16^{A}	3.86^{B}	4.46^{B}	0.517
1-butanol-3-methyl	7.56^{A}	5.86^{B}	6.16^{B}	0.281	7.36^{A}	5.76^{B}	6.66^{A}	0.253
Phenylethyl alcohol	5.76	5.76	6.06	0.113	6.16^{a}	6.16^{a}	5.16^{b}	0.195
Ethyl hexanoate	4.96	4.16	4.36	0.157	5.06^{a}	4.06^{b}	4.46^{ab}	0.177
Ethyl octanoate	8.16^{a}	6.86^{b}	7.36^{b}	0.215	7.06	7.36	6.86	0.125
Ethyl decanoate	6.86^{A}	5.26^{B}	5.56^{B}	0.266	8.26^{A}	5.86^{B}	7.56^{A}	0.370
2-Heptanone	6.86	6.16	6.86	0.155	6.16^{a}	5.16^{b}	5.86^{ab}	0.180
2-Nonanone	13.06^{B}	13.06^{B}	15.76^{A}	0.461	12.36^{B}	13.06^{B}	16.76^{A}	0.690
Limonene	nd	nd	0.86^{A}	0.156	0.00	0.00	0.86^{A}	0.156
Fenchone	nd	nd	nd	-	nd	nd	nd	-
Estragole	nd	nd	nd	-	nd	nd	nd	-
trans-Anethole	nd	nd	3.06^{A}	0.514	nd	nd	3.86^{A}	0.647
2-Butanol	nd	4.76^{A}	nd	0.797	nd	2.76^{A}	nd	0.465
Acetic acid	nd	2.66^{A}	nd	0.448	nd	1.46^{A}	nd	0.251

ND: not detected; SEM: standard error of the mean. Values within a row with unlike superscripts differ at $P < 0.01$ when uppercase and $P < 0.05$ when lowercase.

In table 13 the VOCs of cheeses obtained with the milk of June are reported. The samples obtained from the group supplemented with thistle scored higher content of Butanoic acid, 2-butanol and acid acetic as observed in the samples of May and lower content of Phenylethyl alcohol compared with the other groups. In the samples ripened 50 days, Ethyl octanoate also resulted higher in group T. The cheeses of Anise group had higher content of Ethyl hexanoate in the 50 days ripened samples and in both the ripening period it showed higher content of limonene and trans-anethole. The cheese from group C were instead characterized by higher content of Decanoic acid and 2,3-Butanediol, 1-butanol-3-methyl, Ethyl decanoate in both the ripening period and 2-Heptanone resulted higher only in samples ripened 25 days.

Table 13. VOCs in the cheese obtained with the milk collected in June.

Item (%)	June 25 days ripening					June 50 days ripening			
	Control	**Thistle**	**Anise**	**SEM**		**Control**	**Thistle**	**Anise**	**SEM**
Butanoic acid	3.86^{B}	5.76^{A}	4.06^{B}	0.318		3.86^{B}	7.36^{A}	3.76^{B}	0.600
Hexanoic acid	4.56	4.06	4.76	0.145		3.56^{b}	4.06^{ab}	4.86^{a}	0.215
Nonanoic acid	4.56^{ab}	3.86^{b}	5.06^{a}	0.201		3.46^{b}	3.76^{ab}	4.46^{a}	0.180
Decanoic acid	5.96^{A}	3.86^{B}	3.86^{B}	0.364		5.76^{ab}	5.46^{b}	6.56^{a}	0.193
Dodecanoic acid	15.16^{B}	16.46^{A}	16.76^{A}	0.266		16.36	15.86	16.26	0.127
Tetradecanoic acid	4.96^{a}	5.16^{a}	3.96^{b}	0.211		5.86^{A}	3.96^{C}	4.86^{B}	0.292
2.3-Butanediol	6.76^{A}	4.76^{B}	4.16^{B}	0.406		8.06^{A}	3.76^{B}	4.36^{B}	0.680
1-butanol-3-methyl	8.16^{A}	6.96^{B}	5.66^{C}	0.375		7.46^{a}	6.86^{ab}	6.16^{b}	0.213
Phenylethyl alcohol	6.56^{A}	5.06^{B}	6.56^{A}	0.270		6.26^{A}	4.76^{B}	6.06^{A}	0.256
Ethyl hexanoate	4.77	4.96	5.16	0.117		5.16^{B}	5.06^{B}	6.56^{A}	0.262
Ethyl octanoate	7.76	7.26	6.86	0.165		5.46^{B}	7.36^{A}	3.36^{C}	0.586
Ethyl decanoate	7.76^{A}	5.96^{B}	5.06^{C}	0.410		7.76^{A}	5.06^{B}	3.56^{C}	0.623
2-Heptanone	6.86^{A}	5.76^{B}	5.76^{B}	0.209		8.16	8.06	7.46	0.149
2-Nonanone	11.76^{C}	13.56^{B}	16.86^{A}	0.754		12.26^{C}	13.26^{B}	16.06^{A}	0.578
Limonene	0.00	0.00	1.06^{A}	0.187		0.00	0.00	0.86^{A}	0.156
Fenchone	nd	nd	nd	-		nd	nd	nd	-
Estragole	nd	nd	nd	-		nd	nd	nd	-
trans-Anethole	nd	nd	3.76^{A}	0.631		nd	nd	4.16^{A}	0.697
2-Butanol	nd	4.16^{A}	nd	0.697		nd	2.86^{A}	nd	0.481
Acetic acid	nd	1.76^{A}	nd	0.300		nd	1.86^{A}	nd	0.317

ND: not detected; SEM: standard error of the mean. Values within a row with unlike superscripts differ at P <0.01 when uppercase and P<0.05 when lowercase.

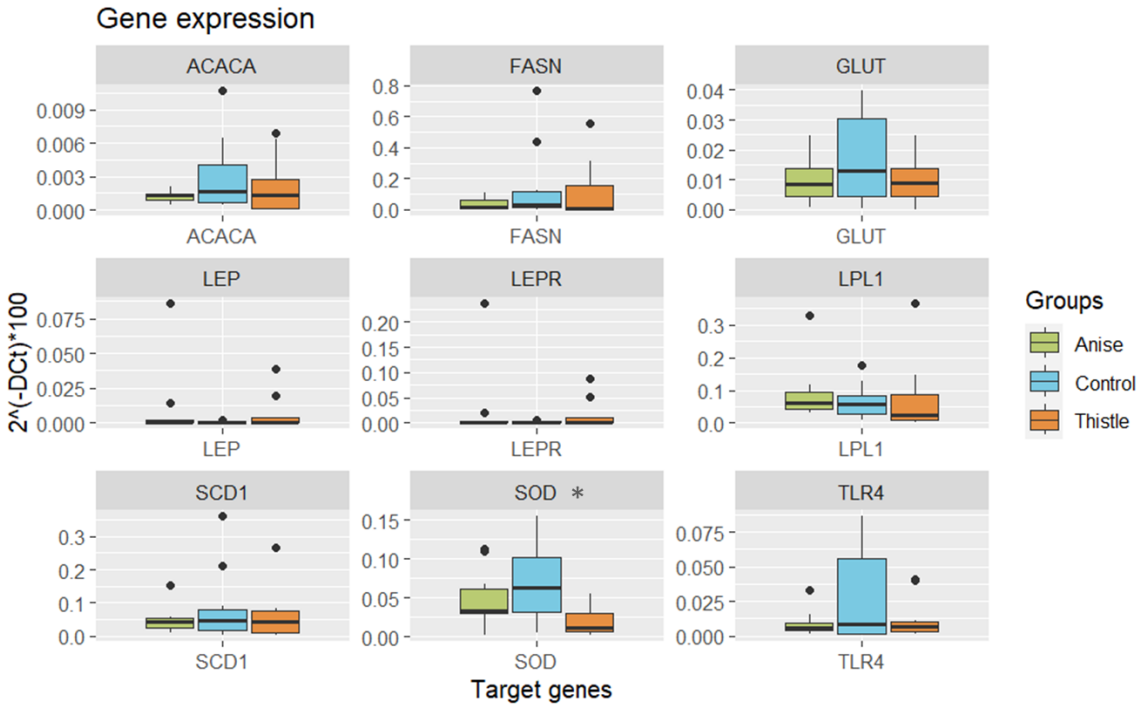
In table 14 the results of the gene expression analysis are presented. The lipogenic genes (*ACACA, FASN* and *SCD1*) exhibited high expression levels in the pasture group, although the observed differences were not statistically significant when compared to the other groups. The metabolic genes *LEP* and *LEPR* exhibited higher expression levels in both treated groups, as well as *LPL1*. However, these differences did not reach a statistically significant value. The expression of *SOD* was found to differ significantly between the groups ($P < 0.05$). Indeed, the pasture group exhibited the highest level of expression, while the thistle group demonstrated the lowest. As illustrated in Figure 1, despite all groups being fed on pasture, only the Pasture group exhibited considerable variability in candidate gene expression.

Table 14. Candidate gene expression.

Target genes	Control	Thistle	Anise	SEM	P value
ACACA	0.0030	0.0021	0.0012	0.00045	0.288
FASN	0.1545	0.1121	0.0384	0.03368	0.376
GLUT	0.0168	0.0100	0.0101	0.00200	0.290
LEP	0.0005	0.0066	0.0104	0.00313	0.442
LEPR	0.0017	0.0159	0.0265	0.00839	0.495
LPL1	0.0676	0.0727	0.0909	0.01566	0.826
SCD1	0.0863	0.0603	0.0484	0.01493	0.585
SOD	0.0703[a]	0.0196[b]	0.0487[ab]	0.00771	0.020
TLR4	0.0294	0.0123	0.0090	0.00435	0.120

SEM: standard error of the mean. Values within a row with unlike superscripts differ at P <0.01 when uppercase and P<0.05 when lowercase.

Figure 1. Candidate gene expression boxplot. Candidate genes with (*) are statistically different ($P < 0.05$) among groups.



Discussion

Diet

The chemical composition of the pasture was found to be similar to that previously reported by Iommelli et al. (2024) in the research conducted in the same area. Specifically, the NDF value was comparable, whereas the protein content was lower. This discrepancy may be attributed to the disparate sampling periods or the varying botanical composition at the time of the trial. These parameters have been demonstrated to affect the nutritional composition of pasture (Martins-Noguerol et al., 2023). The nutritional value of thistle and anise was found to be particularly high in terms of protein and lipid content. Thistle exhibited the highest protein content among the feed samples analysed, a finding that is consistent with the observations of Marceddu et al. (2022). Furthermore, milk thistle had the highest content of C18:3n6 (gamma linolenic acid), which contrasts with the findings of Malekzadeh et al. (2011), who identified the highest quantity of oleic and linoleic acid in the seeds. Conversely, anise seeds were found to contain a high quantity of C18:2n6 (linoleic acid) and C18:1n9 (oleic acid), a finding that aligns with the observations of Topčagić et al. (2022). In contrast, pasture exhibited the highest concentration of C18:3n3.

With regard to the VOC profile, anise was characterised mainly by trans-anethole, which is the major odour compound of this plant (Singletary, 2022). In contrast, milk thistle exhibited a distinct VOC profile, with 2-butanol, butanoic acid and acetic acid representing the key odour compounds. These compounds were not identified in the pasture samples, which revealed a high degree of chemical diversity. Among the most prevalent compounds were tetradecanoic acid, 1-butanol-3-methyl, and 2-nonanone. Indeed, Fedele et al. (2005) have demonstrated that alcohols and ketones represent the most abundant compounds in spring Mediterranean pasture.

Milk yield and chemical composition

The process of lactation in dairy ruminants is a complex phenomenon involving a multitude of organs and hormones, which may be influenced by nutrients or specific compounds ingested by animals (Musco et al., 2022). In particular, the plants selected for this trial have been demonstrated to exert their effects on the liver and the mammary gland. The effects of milk thistle (*Silybum marianum L.*) on the liver are exerted through silymarin. The hepatoprotective effects of silymarin have been attributed to a number of mechanisms, including antioxidant, anti-lipid peroxidation and anti-inflammatory properties. Furthermore, these complex compounds facilitate liver detoxification by inhibiting phase I detoxification, while simultaneously enhancing the process of glucuronidation and protecting against glutathione

depletion. Additionally, silymarin acts as an anti-inflammatory compound, inhibiting leukotriene and prostaglandin synthesis, and increases hepatocyte protein synthesis, thereby promoting hepatic tissue regeneration (Porwal et al., 2019).

Silymarin has been employed for its hepatoprotective effects, particularly in peripartum animals, with the objective of mitigating the risks associated with this period, such as negative energy balance and the consequent risk of ketosis and hepatic steatosis (Tedesco et al., 2004). Although the administration of a hepatoprotective agent may be particularly beneficial during this phase, it is nevertheless important to maintain a healthy liver throughout lactation. The liver plays a role in lactogenesis, synthesising plasma proteins and processing fats to transform these components into milk. Additionally, the liver is responsible for the production of hormones and molecular messengers that are directly involved in the process of lactation. Recent studies have demonstrated that the liver also functions as an endocrine organ. Insulin-like growth factor 1 (IGF-1) is a well-documented endocrine factor derived from the liver. The anabolic role of plasma IGF-1 is to stimulate the uptake of amino acids and glucose by cells, with an action similar to that of insulin (Magistrelli et al., 2005). Furthermore, it has been demonstrated that higher milk yield in goats is associated with higher serum levels of IGF-1 (Yakan et al., 2018). Additionally, Capasso et al. (2009) demonstrated that the administration of silymarin to milking rats resulted in an increased level of serum PRL. Moreover, the supplementation of polyphenol-rich additives, such as thistle, can increase milk yield by improving feed efficiency through the modulation of rumen fermentation (Kelleheret al., 2024). The benefits of a diet rich in polyphenols may be derived from their antioxidant, anti-inflammatory, hormone-regulating and autophagy-promoting properties (Serino and Salazar, 2018). Such effects may also enhance lactation performance and milk production. It is evident that lactation represents a period of elevated energy demand, which is met through the enhancement of mitochondrial function. However, impaired nutrient sensing and signalling can result in an insufficient energy supply, which can impair lactation. Energy production is associated with robust reactive oxygen species (ROS) generation, the effects of which may be mitigated by antioxidant activity. However, if antioxidant activity is inadequate to counterbalance ROS generation, this may lead to increased oxidative stress, impaired lactation, and/or inflammation. Systemic and localized ROS generation, whether or not it occurs in the mammary gland, may impair milk production and result in a low milk supply.

In addition to the previously discussed effects of integration with a rich source of phenolic compounds, it is also important to consider the role of individual compounds in the observed increase in production in the anise-supplemented group. In the case of anise, it is particularly

relevant given that it is a rich source of trans-anethole, a compound that has been demonstrated to increase the level of prolactin (PRL), thereby increasing milk production. As with numerous other plant compounds, anethole bears an analogous structure to the catecolamines epinephrine, norepinephrine, and dopamine. This structural similarity appears to underlie the numerous sympathomimetic effects attributed to fennel and anise. Dopamine acts to inhibit the secretion of the prolactin hormone and, consequently, an alternative means by which anethole might exert an influence on milk secretion is by competing for receptor sites, thus preventing the inhibition of prolactin secretion by dopamine (Albert-Puleo, 1980).

In the present trial, the lactose content was found to be lower in both supplemented groups in June, with only the anise group exhibiting a similar result to the control group in May. In contrast to the findings of Karaiskou et al. (2021), who observed an increase in lactose and a reduction in protein content following the administration of milk thistle oil to dairy ewes, the thistle group exhibited a different outcome. As these authors proposed, the discrepancies in milk chemical composition may be attributed to the interaction of secondary compounds, which are present in varying quantities in milk thistle oil and leaves, with rumen microbiota and the mechanism that leads to lactose and protein synthesis. Consequently, further investigations are required to elucidate these interactions.

Milk and cheese FA profile

Among the various factors influencing the milk fat profile, dietary treatment is the most significant. It has been demonstrated that different dietary regimens or feed integration can modify the milk fatty acid profile of both milk (Tudisco et al., 2014) and cheese (Corazzin et al., 2019). Among the aforementioned factors, grazing systems are widely acknowledged as the most effective means of obtaining a high-quality milk product in terms of beneficial fatty acids (Lo Presti et al., 2023), a conclusion that is also supported by the findings of the present study. All three groups were primarily fed on pasture, and all demonstrated optimal levels of MUFA PUFA, CLA, and omega-6/omega-3 ratios within the recommended range for a healthy diet (Simopoulos, 2021).

The cheese obtained from the thistle-supplemented group exhibited a higher content of C4:0 and C6:0. Butyric acid (C4:0) is well documented for its anticancer effects, as it induces morphological and biochemical differentiation in a variety of cells, leading to the concomitant suppression of neoplastic properties (Dwidar et al., 2012). Caproic acid (C6:0) has recently been demonstrated to exert antineoplastic properties (Burdick et al., 2022). Additionally, as

reported by Woo and Lindsay (1984), these FA are also responsible for the development of the aroma of some cheeses.

Polyphenols have been shown to have a generally mitigating effect on rumen fermentation, offering several potential advantages with regard to methane emissions and biohydrogenation. It has been demonstrated that certain polyphenols are capable of protecting polyunsaturated fatty acids (PUFAs) from complete biohydrogenation, which results in a reduction in the content of C18:0 in milk (Gadeyne et al., 2015). This phenomenon may have occurred in the present trial, as evidenced by the milk samples from the supplemented groups. However, the supplemented groups produced milk with a lower concentration of alpha-linolenic acid in comparison to the control group. The two plants that were supplemented were found to be richer in the n6 series of FA, specifically linoleic acid in anise and gamma linolenic acid in thistle.

The principal CLA isomer, C18:2c9t11, was identified at elevated levels in groups C and A during the month of May, while no significant differences were observed in the month of June. As previously stated, anise seeds powder was found to contain a higher linoleic acid concentration than milk thistle, which is recognized to be one of the main precursors of CLA.

The very long-chain FA were found to be present at an elevated level in the control group, a finding that was confirmed by the higher preformed FA content. In the month of June, the cheese of group A displayed a higher level of preformed FA than that of group T. These FA derive from feed metabolism and are typically associated with an increase in feed intake or enhanced feed efficiency (Moate et al., 2008).

It can be observed that there is a high degree of correlation between the chemical profile of cheese and that of the milk from which it was produced (Falchero et al., 2010). However, as the compounds present in the cheese are of greater concentration, some differences are more evident. In particular, cheese FA can be subjected to changes due to a number of factors (Thierry et al., 2017), including: i) the different microbic selection that occurs during the ripening period; ii) the type of rennet employed and iii) the presence of bioactive compounds (Giorgio et al., 2019). Furthermore, phenolic compounds present in milk can influence the process of lipolysis, thereby protecting PUFA from complete peroxidation and facilitating the release of some short FAs. With regard to the PUFA, which were present in greater quantity in the milk of the Control group, this difference was not evident in the cheese, where the PUFA content remained unchanged across the groups. Accordingly, as proposed by Picciotti et al. (2022), the bioactive compounds, including those present in milk thistle and aniseed, may have served as a protective measure against lipolysis in the supplemented groups, resulting in a reduced oxidation of PUFA, which are known to be particularly susceptible to this phenomenon.

Cheese chemical composition and antioxidant activity

The chemical properties of milk play an important role in the cheese-making process and influence the coagulation properties of milk (Skeie, 2007). It is well established that the primary components influencing the cheese-making process are the fat and protein content of the milk, which were found to be similar across the groups in the present study. However, the chemical composition of the cheeses exhibited notable differences in terms of fat, protein, and cholesterol content between the samples across both ripening periods. Notably, the elevation in fat content observed in Groups A and C, resulting from the concentration of compounds during the ripening process, was less pronounced in Group T, where this discrepancy was more significant.

With regard to cholesterol levels, both the aniseed and thistle groups displayed lower concentrations in comparison to the control group. The underlying causes of this phenomenon are diverse. With respect to aniseed, it has been demonstrated that aniseed is capable of reducing serum cholesterol levels in human (Singletary, 2022) and has also been shown to reduce cholesterol levels in broiler chickens (Soltan et al., 2008). As proposed by Helal et al. (2019), the antioxidant properties and radical scavenging activity are the mechanisms by which anise oil exerts its beneficial effects on cholesterol, triglycerides and LDL. Indeed, the polyphenols present in anise seeds possess the capacity to donate electrons and react with free radicals, thereby converting them into stable products and terminating the free radical chain. Meanwhile, other compounds in the oil act as chain breakers in lipid peroxidation. With regard to the thistle group, the reduction in cholesterol levels can be attributed to the action of silymarin, which has been demonstrated to enhance the protein expression of ABC transporters and cytochrome P450, including CYP7A1 and CYP4A. The latter is involved in omega and omega-1 hydroxylation of fatty acids, which is a requisite step for TG synthesis. Both CYP7A1 and ABC transporters are implicated in the clearance of cholesterol and the reduction of total cholesterol (TC) (Tajmohammadi et al., 2018).

The antioxidant activity of the cheese, as determined by TAC and DPPH assays, was higher in both supplemented groups, which can be attributed, at least in part, to the elevated TPC. It is well established that polyphenols are potent antioxidants. Previous research has demonstrated that their supplementation in animal diets enhances the antioxidant properties of the resulting food products (Clarke et al., 2019; Hilario et al., 2010; Ianni et al., 2019). This represents a novel approach to modifying the nutritional attributes of dairy products through the utilisation of natural compounds that are perceived as more acceptable to consumers than synthetic additives. It is proposed that foods with higher antioxidant compounds be incorporated into

human nutrition as a means of improving dietary quality and preventing ROS damage (Lobo et al., 2010).

The milk thistle leaves employed in the present study exhibited a higher TPC than the aniseed powder, which led to the unexpected observation of a higher TPC in the aniseed cheese group. However, an alternative explanation could be that since the majority of phenolic compounds are known to bind to the lipid fraction of a matrix, the aniseed cheese group, which has a higher fat content, also demonstrated a higher TPC.

Cheese VOCs composition

The volatile organic compound (VOC) composition of cheese is associated with its flavour, and is influenced by a number of factors, including the enzymes present in the milk, the thermic treatment applied, microbial activity, and the type of rennet used. Furthermore, the maturation of cheese results in the release of a greater variety of compounds due to the lipolytic and proteolytic processes that occur during this period. To date, more than 600 volatile organic compounds (VOCs) have been identified in different types of cheese, with many of them having been linked to specific and distinctive flavour notes (Curioni and Bosset, 2002; Molimard and Spinnler, 1996; Sablé and Cottenceau, 1999). A further significant factor influencing cheese flavour is the diet of the animal from which the milk is derived. A number of studies (Clarke et al., 2019; Hilario et al., 2010; Ianni et al., 2019) have demonstrated that the profile of volatile organic compounds (VOCs) present in milk and cheese can be influenced by dietary and nutritional supplementation practices in different ruminant species. The two groups that received supplementation differed with respect to a number of specific compounds. The cheese obtained from the group that had been supplemented with thistle exhibited a distinctive profile, with the presence of 2-butanol and butanoic acid, which were also detected in significant quantities in milk thistle leaves. The latter compound is typically associated with strong unpleasant odour and it has already been detected in several ewes' cheeses including the Oscypek PDO cheese, Canestrato Pugliese PDO cheese, Fiore Sardo PDO cheese, Torta del Casar PDO cheese, Terrincho PDO cheese, Roncal PDO cheese, Manchego PDO cheese, and Pecorino Romano PDO cheese (Barron et al., 2005, Massouras et al., 2006, Majcher et al., 2011, Sádecká et al., 2014, Delgado-Martínez et al., 2019) and Queijo de Azeitão PDO cheese, the latter obtained with milk thistle rennet (Cardinali et al., 2021). The presence of 2-butanol can be attributed to its high concentration in thistle leaves. This compound is typically present in Cheddar cheese and is associated with the development of undesirable flavours. The primary source of 2-butanol is the reduction of 2-butanone, which, conversely, contributes to the

desirable flavour complex of Cheddar cheese (Keen et al., 1974). Additionally, 2-butanol has been identified in Castellano cheese by Fernández-García et al. (2004) and these authors proposed that this compound is not a significant contributor to the aroma profile due to its high flavour thresholds.

The quantity of hexanoic acid present was found to be greater in the thistle-supplemented and anise groups of cheese that had undergone 50 days of ripening. This compound is commonly associated with waxy, soapy, goat-like, and sweaty odours (Ferreira et al., 2009). Previous research has identified it in raw milk Feta PDO and Piacentinu Ennese PDO cheeses (Horne et al., 2005). Hexanoic acid is primarily produced by lactic acid bacteria and has been shown to exert an antagonistic effect on fungal growth and mycotoxin production (Taheur et al., 2019).

The cheese of the group that had been supplemented with anise was distinguished by the presence of trans-anethole and limonene, which were not detected in the other groups. These compounds are typically present in *Pimpinella anisum L.* and in *Foeniculum vulgare Mill.* and were found in great quantity in aniseed powder. In particular, limonene has been associated with a fresh fruity flavour, while trans-anethole has been linked to aniseed flavour. Additionally, the anise group exhibited a higher concentration of 2-nonanone, a phenomenon also observed in the fennel trial for fennel cheese. The role of this compound in the formation of the distinctive cheese aroma remains a topic of debate. Some authors have proposed that this compound is associated with the flavour of mould-ripened cheeses (Fernández-García et al., 2004), while others have suggested that it contributes to a fruity flavour (Chiofalo et al., 2004).

Gene expression

The milk fat globules are a rich source of RNA, which effectively represents the metabolic status and activity of the MECs (milk epithelial cells). Indeed, the milk fat layer contains cytoplasmic components, primarily organelles and membranes, derived from MECs. These components are incorporated into the cytoplasm of fat globules through the mechanism of apocrine secretion by mammary epithelial cells (Brenaut et al., 2012). The technique of isolating RNA from milk fat globules has already been validated in the goat species (Brenaut et al., 2012) through a comparison of RNA from mammary tissue and milk fat, using microarray and qPCR. Furthermore, it is a non-invasive technique that yields high-quality RNA, which more accurately reflects the activity of epithelial living cells than RNA isolated from milk somatic cells.

The impact of various factors on gene expression in goats has been previously investigated, including nutritional aspects (Fougère and Bernard, 2019), genetic (Ollier et al., 2008), and

environmental or stress-related conditions (Hooper et al., 2020). Nevertheless, nutritional factors appear to exert a lesser influence on gene expression than genotype, even in studies where significant differences between groups are observed for other parameters (Bernard et al., 2022). It is important to note that there is not always a direct correlation between gene expression and protein translation. This is due to the presence of several post-transcriptional events that can influence protein synthesis (Bernard et al., 2022).

The metabolic pathway for de novo FA synthesis within the mammary gland is dependent on two key enzymes: *ACACA* and *FASN* are the key enzymes involved in the synthesis of new fatty acids, while *LPL* plays a role in the uptake of fatty acids from the plasma. Subsequently, these fatty acids may undergo desaturation by *SCD*, resulting in the synthesis of cis-9 unsaturated fatty acids.

No significant differences were observed in the expression of *ACACA*, *FASN* and *LPL1* among the experimental groups. While *ACACA* and *FASN* are involved in the synthesis of milk fatty acids, *LPL* has also been correlated with milk flavour (Deeth, 2006). In contrast to the other lipogenic genes, which are not significantly influenced by dietary factors, *SCD* is generally more regulated by unsaturated FA (UFA) supplementation in the diet, specifically on subcutaneous adipose tissue (Bernard et al., 2005b).

The relationship between *SCD* expression and milk quality has been the subject of extensive study. Stearoyl-CoA desaturase is responsible for the synthesis of approximately more than 75% of total CLA in milk (Chilliard et al., 2006). Among the various factors affecting *SCD* expression, the stage of lactation is a topic of ongoing debate. Indeed, while some authors have reported a >40-fold up-regulation of *SCD* at peak lactation (Bionaz and Loor, 2008), others have observed no differences (Janmeda et al., 2017) in dairy cows. Nutritional factors have been demonstrated to affect *SCD* expression, with specific nutrients, notably lipids, having a particularly marked impact. In the study conducted by Tudisco et al. (2019a), the supplementation of the diet with linseed resulted in a reduction in the activity index of *SCD*. This result is confirmed by the findings of Tsiplakou et al. (2009) and Bernard et al. (2009a), who observed reduced C14:1/C14:0 ratios in the linseed oil-treated group. However, no differences were noted at the mRNA level of *SCD*, similar to the observations made in the present study. The milk of the thistle and anise group exhibited a lower value of C14:1/C14:0, yet no significant differences were observed in the mRNA level of *SCD*. Accordingly, the discrepancies observed in milk FA may be attributed to post-transcription regulation, different substrate availability, or alternative metabolic pathways.

LEP and *LEPR* were selected for their involvement in energy metabolism, specifically in feed intake and the distribution of nutrients among tissues (El-Shorbagy et al., 2022), as well as for their regulatory role in lactogenesis (Avondo et al., 2019). This is because polyphenol-rich additives have been demonstrated to increase feed intake and efficiency (Fahim et al., 2022).
In response to stress conditions, these genes decrease their expression and induce a reduction in production performance in livestock animals (Angel et al., 2018). Nevertheless, they appear to be relatively unaffected by nutritional factors in the short and medium term, as evidenced by the findings of Palmioli et al. (2023) and our own observations.
The glucose metabolism in the mammary tissue is controlled by several genes, including *GLUT1*, the nutritional regulation of which is poorly documented. The impact of green anise supplementation on goat diets has been previously investigated by Iftikhar et al. (2017). Their findings indicated that green anise supplementation resulted in elevated blood glucose levels and enhanced milk production, feed intake, and body weight. GLUT1 is the most abundant glucose transporter in mammary tissue (Bionaz and Loor, 2011), and thus it was hypothesised that its expression was linked to herb supplementation. Although a higher mRNA level was observed in both supplemented groups, the differences were not considered significant. However, this gene does not appear to be affected by different nutritional conditions, as reported by Tsiplakou et al. (2016) on goats. In contrast, different results were observed in sheep (Tsiplakou et al., 2015).
The anti-inflammatory properties of green anise and milk thistle, as observed in medicinal herbs, have been the subject of investigation through the expression of *TLR4*, which activates nuclear factor (NF)-κB – a principal regulator of a vast number of genes associated with inflammatory processes (Chang et al., 2015). Liu et al. (2015) demonstrated that high grain feeding affected the mRNA levels of *TLRs* and modified the bacterial community of ruminal epithelium in male goats, thereby highlighting correlations between microbial community and *TLRs* expression. The present study did not identify any significant differences in *TLR4* mRNA levels, despite the non-supplemented group exhibiting higher values. As observed by Shah et al. (2022), by supplementing goats' diet with selenium, the use of feeding additives may reduce the mRNA levels of *TLR4*.
Silymarin has been demonstrated to function as an efficacious antioxidant, scavenger for ROS and inhibitor against lipid oxidation. Consequently, it may protect animal cells against ROS malfunctions comprising free radicals such as superoxide radical, hydroxyl radical, and hydrogen peroxide (Kshirsagar et al., 2013).

The results demonstrated a significant reduction in *SOD* mRNA levels in the milk thistle-supplemented group, which contrasts with the findings of Ghanem et al. (2022). In the present trial, the supplementation of herbs resulted in elevated antioxidant activity in the cheese of the treated groups, whereas a diminished expression was observed in the mRNA level of *SOD*. The work of Ghanem et al. (2022) was conducted on blood samples from periparturient goats, which may account for the discrepancies observed in our results. Furthermore, it can be postulated that the expression of *SOD* may operate in a manner consistent with a negative feedback mechanism. Chang et al. (1997) observed that the mRNA level of *SOD* in glial cells increased in response to a reduction in SOD activity due to hypoxic stress. In light of these findings, it can be hypothesized that the administration of antioxidant supplements may have resulted in a reduction in the expression of genes responsible for antioxidant activity.

Chapter VI

Fennel seed powder in the diet of dairy buffalo affects milk VOCs profile, antioxidant activity and SCD mRNA level

Experimental design

The trial was performed at Prete Carolina livestock farm (Pontinia, LT; 41°24'26.2"N 13°08'33.8"E) on 20 dairy buffaloes (river buffaloes). Twenty animals were selected homogenous for number of parity (2.6 ± 1,7), days in milk (DIM: 41.1 ± 7.17), BCS (6.8 ± 0.2) and daily milk yield (DMY: 13.6 ± 1.8 kg/head/day) and were randomly allocated into two groups (CON: control; FEN: supplemented with fennel). All animals were fed individually with unifeed twice a day composed by 4 kg of polyphite hay (40% ryegrass, 30% oat, 30% clover), 2 kg of alfalfa hay, 20 kg of corn silage and 6,5 kg of concentrate (corn meal 27.2%; barley 27.2%; soy f.e. 22.7%; soy flakes 15%; cotton seeds 7.5%). Unifeed chemical composition was determined according to AOAC (2012) and characterized by 14.32% of crude protein, 2.75% of ether extract, 41.32% of NDF, 37.20% of ADF, 4.57% of ADL, 22.69% of starch, 7.03% of ash and 0.9 UFL/Kg DM. In addition, the group FEN received 100 g of fennel seeds powder which was mixed in the unifeed for 4 weeks. FSP was analysed for chemical composition according to AOAC (2012) and characterized by 13.06% of crude protein, 7.85% of ether extract and 11.65% of crude fiber. Additionally, FSP was characterized for VOCs composition and antioxidant activity (VOCs %: Limonene 13.2; Fenchone 1.9; Estragole 4.1; trans-Anethole 74.8; TPC, mg GAE/g: 4.03; ABTS, mg/g TE: 6.65; DPPH, EC50 µg/ml: 11.58) and FA profile [∑ SFA: 11.98 % (C11:0 = 5.78%; C16:0 = 4.97%); ∑ MUFA: 77.65% (C18:1n12 = 76.89%); ∑ PUFA: 11.03% (C18:2n6 = 9.87%)] as described in chapter IV.

Samples collection

Feed samples were collected at the beginning and at the end of the trial whereas individual milk yield was daily measured and individual milk samples were collected consecutively for the last two days of the trial.

Body condition score of the buffaloes were performed fortnightly by using a procedure on 9 points scale (Matera et al., 2021).

Analysis

The analysis on feed and milk were the same conducted in the trial on fennels seeds on dairy goats described in chapter IV. Gene expression analysis was performed as described in the chapter V. The mean value of RNA integrity (RIN) was 3.9 (± 1.6), which is consistent with the results of other studies on milk fat compared to other matrices (Li et al., 2016). The candidate genes (*ACACA, LPL, NRF2, SCD1, SOD*) were investigated and normalized by adopting Luciferase as reference gene. Gene primers for buffaloes are reported in table 1.

Furthermore, the antioxidant enzymes present in the milk samples were analysed. Superoxide dismutase (SOD) activity was assayed in milk by enzymatic colorimetric modified method of McCord and Fridovich (1969). Catalase activity was determined in accordance with the procedure described by Beers and Sizer (1952). Glutathione peroxidase (GPx) was determined in milk by enzymatic colorimetric method using commercial diagnostic kit according to Forstrom and Tappel (1979). Each analysis was performed in triplicate.

Table 1. Primer sequences and conditions used for real-time qPCR.

Gene symbol	Encoded protein	Accession no[1]	Nucleotide sequence (5' – 3')[2]	Product size (bp)	Source
ACACA	Acetil Co-A carboxylase	NM_174224	F: GAGTTCCTCCTTCCCATCTACCA R: GGTGCGTGAAGTCTTCCAATC	123	Janmeda et al. 2017
LPL	Lipoprotein Lipase	DQ997818.2	F: TTCAGAGGCTATTACTGGAAATCC R: ATGTCAATCACAGCATTCATTCTACT	73	Janmeda et al. 2017
NRF2	Nuclear eritroid factor 2	XM_006051427.4	F: ATGACAAGCTGGCTGAGACT R: GTTCACTGTCAACTGGCTGG	197	Grewal et al. 2022
SCD1	Stearoyl-CoA Desaturase	NM_001290915	F: CGTGCCGTGGTATCTGTGG R: AAAGGTGTGGTGGTAGTTGTGG	217	Fan et al. 2021
SOD	Super oxide dismutase	NM_001290973.1	F: GAGAGGCATGTTGGAGACCT R: TCTGCCCAAGTCATCTGGTT	153	Khalil et al. 2021
PRLR	Prolactin receptor	NM_001039726	F: CATGGATACTGGAGTGAGTGGA R: TGTCCTTCACTGGGAAGTCAT	245	Safayi et al. 2010
Luciferase	Luciferase	-	F: AGA GAT ACG CCC TGG TTC CT R: ATAAATAACGCGCCCAACAC	202	Bui et al. 2009

[1]Accession number of the sequence used to design primers.
[2]Sequences: F = forward primer; R = reverse primer.

The mozzarella cheese yield (MCY) was calculated by the formula of Altiero et al. (1989)

$$MCY\ (kg) = [3.5\ (\%\ protein) + 1.23\ (\%\ fat) - 0.88] / 100$$

Statistical analysis

Data from were analysed by the one-way ANOVA with SPSS IBM software (V29.0.1.0). Differences were considered statistically significant at $P < 0.05$.

Results

In table 2 the results of milk yield and chemical composition are disclosed. BCS, milk yield and composition were not affected by the treatment. Fat, lactose and protein yield were determined as g/day and were not different between the groups.

Table 2. Milk yield and chemical composition.

Item	CON	FEN	SEM	*P* value
BCS	6.90	6.80	0.146	0.784
DMY (kg)	13.30	13.77	0.645	0.726
Fat (%)	7.24	7.45	0.138	0.465
Lactose (%)	5.41	5.34	0.065	0.582
Protein (%)	3.87	3.88	0.032	0.885
Fat (g/day)	953.05	1016.82	39.343	0.433
Lactose (g/day)	718.85	730.05	31.269	0.863
Protein (g/day)	516.61	536.03	26.534	0.725
Mozzarella yield	0.216	0.219	0.001	0.458

DMY: daily milk yield; SEM: standard error of the mean.

In table 3 milk FA profile is presented. Among short chain FA (SCFA) group FEN had significantly lower content of C12:0 ($P < 0.05$) and higher content of C14:1, C16:0 and C16:1. C18 isomers, in particular C18:1 trans 9 and C18:1 trans 11 (TVA) were higher in group FEN whereas very long chain fatty acids including C20:1, C22:6 and C20:5 were higher in group CON and only C20:2 resulted higher in group FEN.

Table 3. Milk FA profile expressed as g/100g of milk fat.

Item	CON	FEN	SEM	P value
C4:0	3.49	3.21	0.166	0.424
C6:0	1.59	1.61	0.056	0.876
C8:0	1.84	1.27	0.153	0.057
C10:0	2.10	1.99	0.100	0.593
C11:0	0.13	0.10	0.011	0.191
C12:0	3.98	2.99	0.207	0.013
C13:0	0.09	0.05	0.012	0.063
C14:0	13.06	12.34	0.266	0.172
C14:1	0.27	0.33	0.014	0.043
C15:0	0.82	0.82	0.051	0.973
C16:0	33.85	36.40	0.654	0.042
C16:1	0.92	1.50	0.123	0.014
C17:0	0.23	0.17	0.017	0.056
C17:1	0.03	0.02	0.003	0.003
C18:1 c6	0.02	0.02	0.002	0.487
C18:0	12.03	11.49	0.279	0.354
C18:1 t9	0.44	0.55	0.022	0.013
C18:1 t11	1.64	1.90	0.051	0.010
C18:1 c9	19.33	19.08	0.213	0.553
C18:1 c11	0.31	0.32	0.018	0.836
C18:1 c12	0.07	0.08	0.009	0.540
C18:2 t6	0.13	0.11	0.009	0.324
C18:2 c6	1.67	1.70	0.038	0.672
C20:0	0.13	0.13	0.006	0.947
C20:1	0.12	0.06	0.011	0.005
C18:3 n3	0.08	0.09	0.004	0.323
C18:2cis9trans11	0.27	0.21	0.015	0.052
C18:2trans10cis12	0.15	0.14	0.007	0.281
C20:2 n6	0.01	0.02	0.001	0.048
C22:0	0.06	0.06	0.004	0.752
C20:3 n6	0.02	0.01	0.002	0.480
C22:1	0.03	0.03	0.003	0.262
C20:4 n6	0.07	0.07	0.003	0.981
C22:2 n6	0.01	0.01	0.002	0.804
C24:0	0.02	0.02	0.002	0.638
C20:5 n3	0.03	0.02	0.004	0.015
C24:1	0.03	0.02	0.006	0.238
C22:4 n6	0.03	0.03	0.005	0.617
C22:5 n3	0.01	0.01	0.002	0.188
C22:6 n3	0.02	0.01	0.003	0.011

SEM: standard error of the mean.

In table 4 the main FA classes, ratios and indexes are reported. De novo and mixed FA were higher in group FEN. Concerning the indexes of desaturation C14:1/C14:0 and C16:1/C16:0 was higher in group FEN while the index C18:2c9t11/C18:1t11 resulted higher in group CON.

Table 4. Milk FA classes and ratios.

Item	CON	FEN	SEM	P value
SFA	73.491	72.782	0.2781	0.209
MUFA	23.262	23.941	0.2342	0.152
PUFA	2.545	2.461	0.0531	0.468
omega 6	1.948	1.960	0.0430	0.832
omega 3	0.151	0.132	0.0075	0.080
CLA	0.430	0.360	0.0195	0.051
PUFA/SFA	0.032	0.032	0.0011	0.650
omega 6/omega 3	12.991	15.072	0.6390	0.105
LA/ALA	19.270	17.916	0.5748	0.249
AA/EPA	2.785	4.215	0.3616	0.044
De novo	27.433	24.752	0.5974	0.020
Mixed	34.771	37.970	0.7294	0.024
Preformed	37.080	36.411	0.4532	0.501
C14:1/C14:0	0.021	0.027	0.0010	0.013
C16:1/C16:0	0.026	0.041	0.0032	0.025
C18:1/C18:0	0.001	0.002	0.0001	0.323
C18:2c9t11/C18:1t11	0.168	0.113	0.0100	0.004

De novo FA: (C4 -C16); Mixed FA: (C16:0-C16:1); Preformed FA: (> C16); SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; CLA: conjugated linoleic acids; LA: linoleic acid; ALA: alpha linolenic acid; AA: arachidonic acid; EPA: eicosapentaenoic acid; SEM: standard error of the mean.

Table 5 shows the results on antioxidant activity and cholesterol content of milk. Group FEN was characterized by a lower cholesterol content ($P < 0.001$) and higher content of the antioxidant parameters considered including SOD, CAT, GPx, TAC, DPPH ($P < 0.05$ - $P < 0.001$) and also a higher content of TPC.

Table 5. Milk Antioxidant activity and cholesterol content.

Item	CON	FEN	SEM	P-value
Cholesterol (mg/100g milk fat)	277.65	248.40	3.558	0.000
SOD, % inib. rate	76.41	93.60	1.999	<0.001
CAT, µmol/min/ml	229.65	245.70	2.208	<0.001
GPx, mmol/min/ml	0.38	0.40	0.005	0.020
TAC, nmol/µl	97.04	111.53	1.678	<0.001
TPC, mg GAE/l	77.01	81.52	0.537	<0.001
DPPH, % scav. act.	36.93	40.09	0.401	<0.001

SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; TAC: total antioxidant capacity; TPC: total phenolic content ; DPPH: 2,2-diphenyl-1-picrylhydrazyl; SEM: standard error of the mean.

In table 6 VOCs profile are presented. All the items detected were affected by the treatment. The levels of acetic acid, butanoic acid, hexanoic acid, nonanoic acid, tetradecanoic acid, 2-heptanone and 2-nonanone were significantly lower ($P < 0.001$) in the milk of group FEN compared to group CON, whereas decanoic acid, dodecanoic acid, limonene, fenchone, estragole and anethole were significantly higher ($P < 0.001$) in group FEN. In particular limonene was only detected in the milk of group FEN.

Table 6. VOCs in the milk of the two groups.

Item (%)	CON	FEN	SEM	P-value
Acetic acid	10.487	8.498	0.2713	<0.001
Butanoic acid	11.005	6.859	0.4811	<0.001
Hexanoic acid	16.581	10.274	0.7403	<0.001
Nonanoic acid	9.042	6.770	0.2732	<0.001
Decanoic acid	15.047	20.010	0.5938	<0.001
Dodecanoic acid	8.261	9.293	0.2433	0.030
Tetradecanoic acid	8.167	7.223	0.1466	<0.001
2,3-Butanediol	nd	nd	-	-
2-Butanol	nd	nd	-	-
3-methyl 1-butanol	nd	nd	-	-
Phenylethyl alcohol	nd	nd	-	-
Ethyl hexanoate	0.015	0.010	0.0011	0.025
2-Heptanone	13.065	10.024	0.4272	<0.001
Ethyl octanoate	nd	nd	-	-
Ethyl decanoate	nd	nd	-	-
2-Nonanone	7.953	6.765	0.1471	<0.001
Limonene	nd	3.277	0.1133	<0.001
Fenchone	0.199	2.886	0.3130	<0.001
Estragole	0.155	4.238	0.4726	<0.001
Anethole	0.025	3.875	0.4430	<0.001

SEM: standard error of the means.

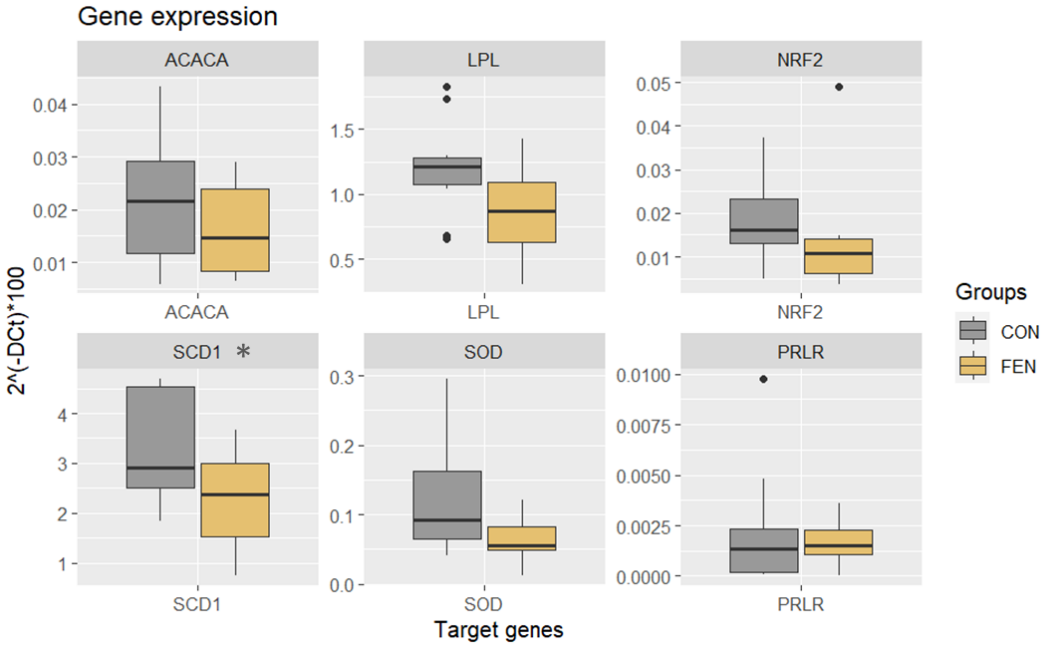
In table 7 and figure 1 the mRNA levels of candidate genes are presented. Group FEN had lower (P<0.05) mRNA levels of *SCD1* than group CON. Similarly, group FEN exhibited lower mRNA values for *LPL* and *SOD*, even if not significantly (P = 0.05). *ACACA*, *NRF2* and *PRLR* resulted not affected by the treatment.

Table 7. Candidate gene expression.

Target genes	CON	FEN	SEM	P value
ACACA	0.0213	0.0161	0.00240	0.290
LPL	1.2080	0.8646	0.09181	0.059
NRF2	0.0187	0.0135	0.00263	0.337
SCD1	3.3143	2.2421	0.26055	0.036
SOD	0.1215	0.0651	0.01473	0.053
PRLR	0.0022	0.0016	0.00049	0.566

SEM: standard error of the mean.

Figure 1. Candidate gene expression boxplot. Candidate genes with (*) are statistically different (P < 0.05) between groups.



Discussion

Milk yield and FA profile

The primary objective of buffalo milk production is the manufacture of mozzarella cheese, a fresh PDO Italian cheese (Lotito et al., 2023). The distinctive attributes of buffalo milk are of particular significance in the production of mozzarella, primarily in relation to its fat and protein content (Sameen et al., 2008). The supplementation of fennel seed powder did not affect the protein and fat content of the milk, and thus did not negatively impact the yield of cheese production.

The milk yield remained consistent between the groups, in contrast to the findings of the fennel trial on grazing dairy goats (Chapter IV) and the observations of Fahim et al. (2021), who noted an improvement in milk yield and composition in dairy buffaloes supplemented with 75 g/head/day of fennel. As previously stated, *Foeniculum vulgare Mill.* is regarded as a galactagogue agent and is commonly utilised in women experiencing breastfeeding difficulties (Facchinetti et al., 2012). However, the precise mechanism of action, which includes a role in the release of PRL levels, remains a topic of contention in ruminants. There is evidence to suggest that it functions as an orexigenic agent, and that it improves feed efficiency (Fahim et al., 2021). In the present study, the animals in the stable were not provided with the opportunity to consume more than the amount of feed that was administered to them. This is in contrast to the conditions observed in the grazing trial. In this instance, even if fennel had exerted orexigenic effects, the animals were not afforded the opportunity to consume more food. Furthermore, a potential limitation of this study is the quantity of fennel seed powder administered, which may have been insufficient for this species to elicit a significant increase in production.

The cholesterol content was lower in the group that had been supplemented. Buffalo milk is typically characterised by a lower cholesterol content than cow milk (Zicarelli et al., 2004a, 2004b). The cholesterol content of animal-derived food could represent a significant concern for consumers, given the potential impact on human health. Indeed, cholesterol oxidation products (COPs) may be generated during harsh storage and have been demonstrated to represent a toxic concern for human health (Sieber, 2005). Consequently, the development of strategies to reduce its content may represent a key objective for nutritionists in order to obtain healthier animal-derived foods. It has been demonstrated that *Foeniculum vulgare Mill.* was able to reduce total serum cholesterol content in rats fed a high-fat diet and that it improved the HDL/LDL ratio, thereby acting as a protective agent against cardiovascular disease risk (Rezq, 2012). This result suggests that the cholesterol-lowering activity of fennel may be related to a

rapid catabolism of LDL, as demonstrated by Guimaraes et al. (2000). Other authors (Cao and Prior, 1998) have proposed that the hypolipidaemic activity of fennel is attributable to the presence of phenolic compounds. Indeed, anethole (trans-anethole), the primary compound of fennel, has been demonstrated to possess notable antioxidant activity. The presence of trans-anethole and flavonoids, which also act as antioxidant agents, may be associated with a reduction in total lipids (TL), total cholesterol (TC), triglycerides (TG) and low-density lipoprotein cholesterol (LDL-c) levels. Moreover, flavonoids have been demonstrated to increase HDL concentration and decrease LDL and VLDL levels in hypercholesteremic rats (Patel et al., 2009).

With regard to the composition of milk fatty acids, buffalo milk is typically characterised by an average total SFA and UFA content of 71.7% and 28.3%, respectively (Abd El-Salam et al., 2011). This aligns with our findings. Specifically, buffalo milk contains higher levels of C16:0 and C12:0, and lower levels of MUFA than cow's milk fat under similar conditions (Talpur, 2007). With regard to the total content of CLA, buffalo milk contains between 0.51 and 1.06 grams per 100 grams of milk fat (Varricchio et al., 2007; Tudisco et al., 2013), which is slightly lower than that observed in cow and goat milk under similar feeding conditions. The trial yielded no discernible differences in SFA, MUFA, PUFA, and total CLA content, a finding that contrasts with the results reported by Fahim et al. (2021). The addition of fennel seed powder resulted in an increase in the content of milk C18:1 and C18:1t11, both intermediate compounds of rumen biohydrogenation. It has been demonstrated that polyphenol-rich additives are capable of protecting FAs from complete biohydrogenation. Furthermore, the group treated with fennel seed powder exhibited a reduction in the de novo fatty acids and an increase in mixed fatty acids (C16:0, C16:1). De novo fatty acids are primarily derived from new synthesis in the mammary gland. With regard to preformed FAs, C16:0 is also released by adipose tissue during lipid mobilisation, which typically occurs to a greater extent during early lactation (Chilliard et al., 2006).

Milk antioxidant activity and VOCs composition

The development of novel strategies to enhance the antioxidant capacity of animal-derived foods has emerged as a prominent area of research in recent years. This is driven by the potential to extend the shelf life, improve the nutritional profile and enhance the beneficial properties of these foods without the use of synthetic additives or preservatives (Malik et al., 2020). Over the past two decades, research on the administration of polyphenols to ruminants has seen a significant increase, with studies more than doubling in number (Serra et al., 2021).

The current study demonstrates that the supplementation of the diet with fennel seed powder, which is rich in polyphenols, resulted in an increase in milk TPC. The presence of polyphenols in milk has been shown to be directly correlated with the quantity present in the animal diet (Kuhnen et al., 2014). This suggests that these compounds underwent minor modifications when metabolised by animals, thereby providing the potential for enriching dairy products with bioactive molecules fed directly to animals, which could have positive implications for product stability (Giorgio et al., 2019) and consumer health (Dupas et al., 2020). Polyphenols have been demonstrated to prevent the oxidation of PUFA (Lindmark-Månsson and Åkesson, 2000) and have been proven to be more effective as antioxidants than vitamins C and E (Kim et al., 2002). The milk of the treated group exhibited a higher antioxidant capacity and scavenging activity than that of the control group.

SOD, CAT and GPx are regarded as the primary enzymatic agents involved in antioxidant repair and were observed to be present at elevated levels in the milk of supplemented group. These enzymatic systems are present in blood and milk (Lindmark-Månsson and Åkesson, 2000) and are known to be influenced by a number of factors, including diet and management. They are also linked to the stress response. In general, when considering antioxidant characteristics, buffalo milk has a higher antioxidant capacity compared to cow milk, due to its higher concentration of bioactive compounds that can contribute to a healthier diet (Khan et al., 2017).

The volatile compounds of Buffalo milk and mozzarella have been the subject of comparatively little study, in contrast to those of cow's milk products. One of the principal reasons for this is that cheese aroma is primarily developed through a series of chemical and biochemical processes that are related to the production techniques employed and the activity of microorganisms during the maturation process (McSweeney and Sousa, 2000). However, the majority of this data has been obtained from studies on aged cheeses, which exhibit significant differences from fresh milk. It is notable that buffalo milk and mozzarella exhibit a distinct VOC profile when compared to the dairy products from other species. As observed by Sacchi et al. (2020), the aroma of raw buffalo milk is markedly distinct from that of other ruminants, due to the presence of specific VOCs, including 1-octen-3-one, nonanal, indole, 3-hydroxy-2-butanone, 3-methyl-2-buten-1-ol, 2-octanone, 2-hydroxy-3-pentanone, and heptanal, which are the primary VOCs responsible for the characteristic odor of buffalo milk. These native milk VOCs are primarily derived from the animal's diet, with some present in the feed and others undergoing transformation during digestion, subsequent absorption and transfer directly into the milk through the rumen or respiratory system (Honkanen et al., 1964). Although mozzarella

does not undergo an ageing process, its flavour is primarily derived from the interaction between the native components of the milk, microbial cultures, rennet, cheese-making techniques and the enzymes produced by secondary microflora.

In comparison to goat's milk, buffalo milk typically exhibits a higher concentration of acidic compounds, with the exception of decanoic and dodecanoic acids, which are also present in notable quantities in goat's milk. Nevertheless, acetic acid was identified exclusively in buffalo milk, in considerable quantities. According to Sacchi et al. (2020), this outcome may be associated with silage feeding, given that acetic acid is a primary byproduct of carbohydrate fermentation during the ensiling process. Nevertheless, there is a paucity of data concerning the transfer of acids from silage to milk and cheese.

With regard to acid compounds, in the trial involving grazing goats supplemented with fennel, the treatment only affected the levels of decanoic, nonanoic, and dodecanoic acids, while more significant differences were observed in this study. The ability to graze likely provided a greater variability of volatile compounds in their diet, thereby reducing the effects of the treatment.

It is also noteworthy that no alcohols, such as 2,3-butanediol, 2-butanol, 3-methyl-1-butanol, or phenylethyl alcohol, were detected in buffalo milk, as also reported by Sacchi et al. (2020). These compounds were not affected by the treatment in the goat study. Furthermore, the concentration of 2-heptanone was observed to be higher in the control group. In the study by Natrella et al. (2020), this compound was found to be present in higher concentrations in animals that were stall-fed in comparison to those that were allowed to graze.

In addition, as expected, the principal volatile compounds derived from fennel were transferred into buffalo milk, as was also observed in the goat study.

The impact of these aromas on the sensory perceptions associated with the tasting of these products requires further investigation. Furthermore, additional research is necessary to evaluate the presence of these compounds in mozzarella, to evaluate the effects of the cheesemaking process on the final product and to determine their sensory impact through panel testing.

Gene expression

The number of studies on dairy buffaloes is relatively limited in comparison to those on dairy cows, particularly in relation to gene expression investigations. Accordingly, a limited set of genes has been selected for this trial, for which primers have already been validated by other authors (Table 1). Nevertheless, the use of MFG (milk fat globule) as a source of RNA has already been corroborated in the buffalo species by Chen et al. (2016) and Sharma et al. (2018).

In general, some genes exhibited higher expression levels compared to other ruminants. For instance, the mRNA levels of *SCD1* were up to sixfold higher than those observed in goats, while the *LPL1* mRNA levels were up to fivefold higher. Indeed, Li et al. (2020) conducted a comparative study of the lipogenic genes of dairy buffaloes and Holstein cows using RNA extracted from MFG. Their findings revealed that dairy buffaloes exhibited higher mRNA levels of lipogenic genes, including *ACACA*, *FASN* and *SCD*, compared to cows.

Both *ACACA* and *LPL* are genes involved in the de novo synthesis of FA in mammary tissue (Bionaz and Loor, 2008). *LPL* is specifically involved in the breakdown of TAG (triacylglycerol) in plasma and in the uptake of FA in mammary tissue (Zhao et al., 2014), whereas *ACACA* plays a key role in the synthesis of fatty acids, facilitating the conversion of acetyl-CoA into malonyl-CoA (Badaoui et al., 2007). The results of gene expression are in accordance with the findings of a lower content of de novo FA in milk of the FEN group.

The study of *SCD* in the buffalo species has already been conducted by Yadav et al. (2015) on Murrah buffaloes across the lactation period. The authors demonstrated that the levels of *SCD* mRNA are influenced by the stage of lactation, with higher levels observed at the peak of lactation. Nevertheless, there is a paucity of research examining the correlation between nutritional factors and *SCD* in the buffalo species. The present study demonstrated that *SCD* mRNA levels were significantly affected by the treatment, which contrasts with the findings of our previous study on buffalo bulls (Iommelli et al., 2021), in which *SCD* expression was found to be unaffected by dietary treatment in muscle-adipose tissue. As stated by Bernard et al. (2013), the activity and expression of *SCD* vary among different ruminant species in response to dietary treatments. In goats, the composition of the diet, defined as the forage:concentrate ratio, and the addition of lipids may result in increased SCD activity but a reduction in the expression of the gene. In the present trial, the supplementation of FSP in the diet resulted in an increase in the C14:1/C14:0 index while concurrently reducing the mRNA levels of *SCD*. As documented by numerous researchers (Bernard et al., 2005b, 2009a, b; Ollier et al., 2009), the correlation between *SCD1* mRNA responses and enzyme activity is relatively weak, suggesting the potential involvement of post-translational regulatory mechanisms.

The supplementation with FSP resulted in a significant increase in antioxidant activity of milk, as evidenced by elevated levels of SOD, CAT, TAC and DPPH activity. However, the mRNA levels of *SOD* were observed to be lower in comparison to the control group, even if not significantly. As was also observed in the trial on goats supplemented with milk thistle and green anise, the integration of the diet with medicinal herbs was found to enhance the antioxidant activity in dairy products, and this was linked to a reduction in *SOD* expression.

However, Grewal et al. (2022) demonstrated that the treatment of buffalo mammary epithelial cells (MECs) with curcumin induced an upregulation of the expression of antioxidant genes, including SOD, CAT and GPx. However, it should be noted that the aforementioned study was conducted on MECS in a hyperthermia-induced oxidative stress model, which may explain the observed differences compared to our study. It is possible that the response in gene expression may vary in healthy tissues and in those that have been subjected to stress.

In contrast, the other candidate gene involved in the antioxidant response, namely *NRF2*, did not exhibit a significant response to the treatment. *NRF2* is a principal antioxidant molecule that is vital for providing cellular protection against a range of oxidants. The knockout of the *NRF2* gene in a murine model resulted in a heightened susceptibility to oxidative damage, chemical toxicity, and a greater severity of disease (Kensler et al., 2007). Furthermore, the pharmacological enhancement of *NRF2* activity has been demonstrated to substantially mitigate oxidative damage in animal models (Talalay et al., 2003).

Finally, *PRLR* expression levels were investigated, given that fennel seeds have been demonstrated to exhibit galactagogue properties as a result of their activity in modulating PRL levels. The expression levels of *PRLR* were studied and no significant differences were detected between the groups. The action of PRL is mediated by its receptor, the prolactin receptor, which is encoded by the *PRLR* gene. This receptor has been correlated with milk productive traits in buffalo species and in other ruminants (Cosenza et al., 2018).

Chapter VII

Fennel seed powder supplementation in dairy goats: investigation on milk productive traits, FA profile and gene expression

Experimental design

The trial was performed at Azienda Zootecnica Antonio Amato located in Casaletto Spartano (SA, Italy; 832 m a.s.l., 40°09' N; 15°37'E) on 20 multiparous lactating Murciana goats. Twenty animals, homogeneous for parity (3^{rd}), days in milk (DIM: 60 ± 7,2), body weight (BW = 50 kg ± 2.0 kg) and milk yield (1922 ± 210 g/day), were randomly allocated into two groups (CON: control and FEN: fennel). All the animals were housed in stall and received 1 kg of alfalfa hay and 700 g/head/day of corn meal in individual pen; in addition, for 4 weeks group FEN received 15 g/head/day of fennel seeds powder (FSP) (*Foeniculum vulgaris*) which was mixed into the concentrate before been offered to the animals. FSP was characterized for VOCs profile and antioxidant activity (VOCs %: Limonene 13.2; Fenchone 1.9; Estragole 4.1; trans-Anethole 74.8; TPC, mg GAE/g: 4.03; ABTS, mg/g TE: 6.65; DPPH, EC50 µg/ml: 11.58) All the animals had free access to fresh water.

Samples collection

Feed samples were collected at the beginning and at the end of the trial whereas individual milk yield was daily measured and samples were collected consecutively for the last two day of the trial.

Analysis

The analysis on feed and milk were the same conducted in the trial on fennels seeds on dairy goats described in chapter IV. Gene expression analysis were performed as described in chapter V. The mean value of RNA integrity (RIN) was 3.7 (± 0.7), which is consistent with the results of other studies on milk fat compared to other matrices (Li et al., 2016). The candidate genes (*LPL1, NRF2, SCD1, SOD, TLR4, PRLR*) were investigated and normalized by adopting Luciferase as reference gene.

Statistical analysis

Data from were analysed by the one-way ANOVA with SPSS IBM software (V29.0.1.0). Differences were considered statistically significant at $P < 0.05$. Gene expression analysis was performed as described in the trial on thistle and anise in dairy goats. The candidate genes

(*LPL1, NRF2, SCD1, SOD, TLR4, PRLR*) were investigated and normalized by adopting Luciferase as reference gene. Gene primers are reported in table 1.

Table 1. Primer sequences and conditions used for real-time qPCR.

Gene symbol	Encoded protein	Accession no[1]	Nucleotide sequence (5' – 3')[2]	Product size (bp)	Source
LPL1	Lipoprotein Lipase	DQ997818.2	F: TTCAGAGGCTATTACTGGAAATCC R: ATGTCAATCACAGCATTCATTCTACT	235	Bernard et al. 2005a
NRF2	Nuclear eritroid factor 2	NM_001314327.1	F: CCAACTACTCCCAGGCAGCCC R: AGCAGTGGCTACCTGAACG	227	Wang et al. 2021
SCD1	Stearoyl-CoA Desaturase	NM_173959	F: TGCTGACAACTTATCTGGATGC R: AAGGAATCCTGCAAACAGCTA	179	Bernard et al. 2005a
SOD	Super oxide dismutase	AB201469.1	F: AGGCAAAGGGAGATAAAGTCGTCG R: TGCACTGGTACAGCCTTGTGTATTG	113	He et al. 2012
TLR4	Toll Like Receptor 4	JF825527	F: CTTGCGTCCAGGTTGTTCCTAA R: CTGGGAACCTGGAGAAGTTATG	153	Paul et al. 2015
PRLR	Prolactin receptor	NM_001039726	F: CATGGATACTGGAGTGAGTGGA R: TGTCCTTCACTGGGAAGTCAT	245	Safayi et al. 2010
Luciferase	Luciferase	-	F: AGA GAT ACG CCC TGG TTC CT R: ATA AAT AAC GCG CCC AAC AC	202	Bui et al. 2009

[1]Accession number of the sequence used to design primers.
[2]Sequences: F = forward primer; R = reverse primer. P = probe.

Results

No concentrate refusals were detected. Alfalfa hay had good content in CP and nutritional value. FSP as also observed in chapter IV, was characterized by a high lipid content (Table 2). Corn meal and alfalfa hay had the highest amount of PUFA. Specifically, corn meal was characterized by the highest amount of C18:2n6 (linoleic acid) whereas alfalfa hay had the highest amount of C18:3n3 (linolenic acid). The key FA of FPS were C18:1n12, characteristic of the family apiaceae and linoleic acid.

Table 2. Feed chemical composition (g/KgDM) and FA profile.

Item	Corn meal	Alfalfa hay	FSP
CP	89.81 ± 1.6	189.70 ± 1.19	130.11 ± 0.7
EE	29.72 ± 0.4	18.52 ± 0.3	82.75 ± 0.18
ash	11.62 ± 0.11	98.61 ± 0.83	13.81 ± 0.81
CF	29.61 ± 0.11	288.86 ± 0.06	111.90 ± 1.5
NDF	-	430.22 ± 0.89	-
ADF	-	350.31 ± 0.25	-
ADL	-	99.01 ± 1.13	-
UFL/KgDM	1.02	0.77	0.80
FA expressed as g/100 g of milk fat			
C11:0	nd	nd	5.78
C12:0	nd	nd	0.01
C14:0	nd	6.3	nd
C16:0	12.08	22.45	4.22
C18:0	2.79	2.65	2.04
∑ SFA	14.87	31.4	12.87
C16:1	0.04	nd	0.20
C18:1n9	28.03	6.53	0.31
C18:1n12	nd	nd	76.23
C20:1n9	nd	nd	0.12
∑ MUFA	28.07	6.53	76.34
C18:2n6	54.63	19.56	9.84
C18:3n3	2.41	42.48	0.25
C20:5n3	nd	nd	0.04
C22:6n3	nd	nd	0.02
∑ PUFA	57.04	62.04	10.46

DM: dry matter; CP: crude protein; CF: crude fiber; EE: ether extract; NDF: neutral detergent fiber ; ADF: acid detergent fiber; ADL: acid detergent lignin; UFL: feed unit for lactation; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

In table 3 milk yield and chemical composition are presented. Body weight didn't change between the groups and no feed refusal were detected. Group F had higher milk yield even if it was not statistically significant ($P > 0.05$). No differences were observed for fat and lactose content whereas protein content resulted significantly lower in the supplemented group ($P <$

0.05). Fat, lactose and protein yield were measured as g/day and were not affected by the treatment ($P > 0.05$).

Table 3. Body weight, milk yield and chemical composition.

Item	CON	FEN	SEM	P-value
BW (Kg)	49.80	50.20	0.234	0.657
DMY (g)	1553	1697.50	131.959	0.598
Fat (%)	4.02	3.76	0.083	0.125
Lactose (%)	4.18	4.25	0.072	0.668
Protein (%)	3.95	3.26	0.108	<0.001
Fat (g/day)	62.25	62.91	4.884	0.948
Lactose (g/day)	64.27	71.61	5.302	0.503
Protein (g/day)	61.21	56.72	5.066	0.670

BW: body weight; DMY: daily milk yield; SEM: standard error of the mean.

Milk FA profile of the two groups is disclosed in table 4. Only few differences were detected between groups. Specifically, among short chain FA, C8:0, C10:0 and C12:0 resulted significantly lower ($P < 0.05$) in group treated and regarding long chain FA only C22:6 differed between groups, being higher ($P < 0.05$) in group FEN.

Table 4. Milk FA profile expressed as g/100g of milk fat.

Item	CON	FEN	SEM	P-value
C4:0	2.980	3.178	0.2136	0.658
C6:0	1.677	1.634	0.1057	0.847
C8:0	3.117	2.612	0.0933	0.003
C10:0	12.447	10.372	0.3292	0.000
C11:0	0.348	0.272	0.0220	0.083
C12:0	5.459	4.473	0.1969	0.008
C13:0	0.073	0.060	0.0056	0.266
C14:0	10.967	10.923	0.2209	0.924
C14:1	0.222	0.225	0.0152	0.909
C15:0	0.872	0.847	0.0425	0.771
C16:0	31.161	31.387	0.6322	0.864
C16:1	0.733	0.826	0.0501	0.368
C17:0	0.465	0.398	0.0247	0.184
C17:1	0.054	0.027	0.0139	0.359
C18:1 c6	0.028	0.026	0.0038	0.795
C18:0	8.070	9.463	0.4931	0.164
C18:1 t9	0.403	0.494	0.0307	0.143
C18:1 t11	nd	0.587	0.0821	0.000
C18:1 c9	17.323	18.092	0.4900	0.449
C18:1 c11	0.204	0.227	0.0133	0.406
C18:2 t6	0.145	0.135	0.0105	0.654
C18:2 c6	1.350	1.548	0.0614	0.109
C20:0	0.229	0.247	0.0103	0.408
C20:1	0.042	0.045	0.0032	0.695
C18:3 n3	0.300	0.341	0.0180	0.262
C18:2cis9trans11	0.164	0.121	0.0148	0.148
C18:2trans10cis12	0.144	0.170	0.0143	0.378
C20:2 n6	0.009	0.010	0.0012	0.711
C22:0	0.108	0.118	0.0072	0.487
C22:1	0.017	0.019	0.0014	0.629
C20:4 n6	0.119	0.121	0.0062	0.846
C22:2 n6	0.022	0.013	0.0024	0.051
C24:0	0.040	0.035	0.0034	0.453
C20:5 n3	0.035	0.038	0.0029	0.702
C24:1	0.023	0.034	0.0054	0.312
C22:4 n6	0.010	0.011	0.0027	0.969
C22:5 n3	0.028	0.029	0.0020	0.967
C22:6 n3	0.035	0.052	0.0030	0.002

SEM: standard error of the mean.

In table 5 the main FA classes, ratios and indices are depicted. No differences were detected between groups for SFA, MUFA and PUFA content whereas de novo FA (C4:0 to C16:0) resulted significantly lower in group FEN.

Table 5. Main milk FA classes and ratios.

Item	CON	FEN	SEM	P- value
SFA	78.020	76.025	0.6104	0.104
MUFA	19.053	20.614	0.5541	0.165
PUFA	2.368	2.594	0.0928	0.234
omega 6	1.658	1.841	0.0701	0.202
omega 3	0.400	0.461	0.0190	0.112
CLA	0.309	0.292	0.0110	0.444
PUFA/SFA	0.030	0.034	0.0014	0.191
omega 6/omega 3	4.188	4.031	0.1083	0.487
LA/ALA	4.646	4.661	0.1811	0.969
AA/EPA	3.416	4.811	0.7747	0.384
De novo	38.160	34.591	0.6602	0.004
Mixed	31.891	32.210	0.6356	0.81
Preformed	29.384	32.414	0.9123	0.099
C14:1/C14:0	0.020	0.020	0.0016	0.907
C16:1/C16:0	0.023	0.026	0.0016	0.452
C18:1/C18:0	0.003	0.002	0.0003	0.582

De novo FA: (C4 -C16); Mixed FA: (C16:0-C16:1); Preformed FA: (> C16); SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; CLA: conjugated linoleic acids; LA: linoleic acid; ALA: alpha linolenic acid; AA: arachidonic acid; EPA: eicosapentaenoic acid; SEM: standard error of the mean.

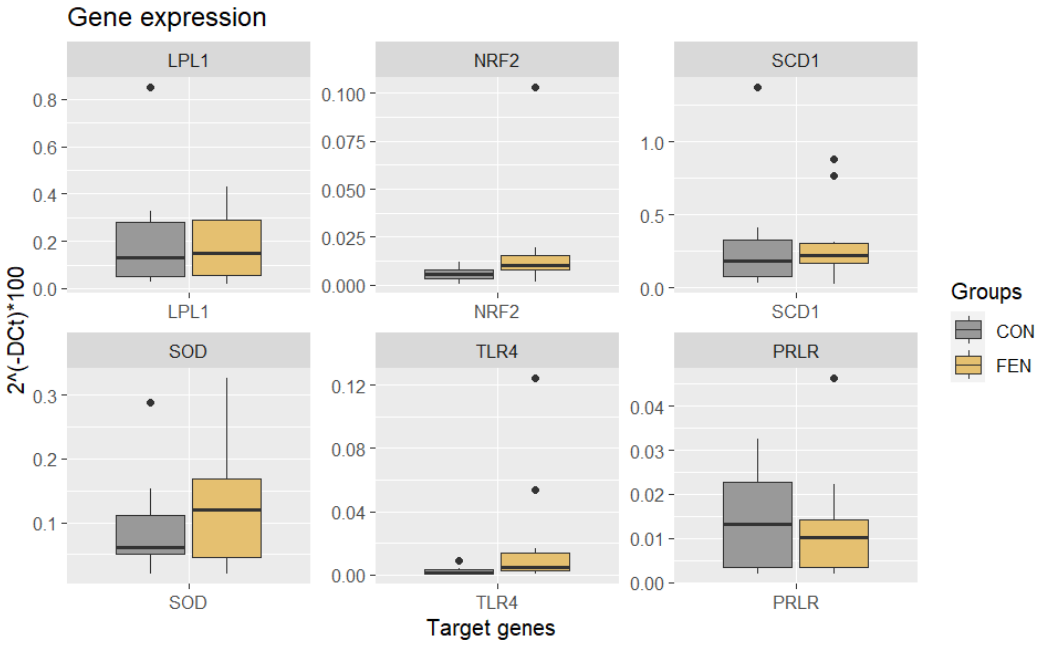
In table 6 the mRNA levels of the candidate genes for the two groups are presented. The expression of the candidate genes was not influenced by the treatment. The mRNA levels were found to be the highest for *SCD1*, followed by *LPL* and *SOD*. In contrast, the lowest values were observed for *PRLR*, *NRF2* and *TLR4*.

Table 6. Candidate gene expression.

Target genes	CON	FEN	SEM	P-value
LPL1	0.2113	0.1823	0.04531	0.758
NRF2	0.0058	0.0195	0.00490	0.171
SCD1	0.3046	0.3098	0.07561	0.974
SOD	0.0937	0.1233	0.01925	0.455
TLR4	0.0024	0.0218	0.00652	0.140
PRLR	0.0142	0.0129	0.00273	0.816

SEM: standard error of the mean.

Figure 1. Candidate gene expression boxplot.



Discussion

Diet

The chemical composition of alfalfa hay displayed a high crude protein and NDF content, as reported by Tudisco et al. (2019a). Additionally, the hay exhibited a considerable concentration of n3 PUFA. Indeed, the administration of n3 PUFAs has been demonstrated to increase the levels of beneficial FA in food of animal origin, thereby exerting favourable effects on human health (Cavaliere et al., 2018). The results demonstrated that fennel seeds were rich in lipids, in accordance with the observations of Unal et al. (2013). They represent a significant source of bioactive compounds, which serve to enhance their therapeutic properties (Díaz-Maroto et al., 2006). The fennel fatty acid profile was found to be characterised by a high content of MUFA, primarily represented by petroselinic acid, and a relatively low content of PUFA and SFA, as reported by Najdoska-Bogdanov et al. (2015). Petroselinic acid (C18:1n12) is a distinctive constituent of the Apiaceae plant family, exhibiting particularly high concentrations in fennel oil. A further distinctive feature of the fennel FA profile is the presence of C11:0, which has been demonstrated to possess antimicrobial activity, particularly against bacteria responsible for alimentary diseases, in the form of a monoacylglycerol preparation (Doležalová et al., 2010).

Milk yield, chemical composition and fatty acid profile

The efficacy of fennel seeds as a galactagogues feeding supplement in several animal species has already been demonstrated (Moosavi-Zadeh et al., 2023; El-Hendawy et al., 2018; Fahim et al., 2022) with results showing an improvement of performances in terms of milk yield and chemical composition. Indeed, fennel seeds are a rich source of trans-anethole, a compound that functions as an antagonist of dopamine and is therefore capable of increasing prolactin (PRL) release. Furthermore, it has been demonstrated that fennel seeds improve feed efficiency and feed intake in calves and dairy cows (Kargar et al., 2021; Moosavi-Zadeh et al., 2023). In our previous work (Chapter IV), a significant increase in milk yield was recorded in grazing goats supplemented with 15 g/head/day of whole fennel seeds. In contrast, the present trial did not yield significant differences in milk production, a finding that is also observed in chapter VI on dairy buffaloes supplemented with FSP.

Furthermore, a reduction in milk protein content was observed in the group that had been supplemented, which contrasts with the findings of El-Hendawy et al. (2018) on Damiati goats.

With regard to milk fatty acid (FA) composition, the group receiving FSP supplementation exhibited significantly reduced de novo FA content, particularly in C8:0 (caprylic), C10:0 (capric), and C12:0 (lauric acid) FA.

Medium-chain fatty acids constitute a family of saturated fatty acid esters of glycerol, comprising caprylic acid (C8:0), capric acid (C10:0) and lauric acid (C12:0). They are naturally present in foodstuffs and are particularly abundant in goat milk (Kim and Rhee, 2016). These fatty acids have been demonstrated to possess antimicrobial properties, specifically against various foodborne pathogens, including *Campylobacter jejuni*, *Cronobacter spp.*, *Helicobacter pylori* and *S. aureus* (Kim and Rhee, 2016). Additionally, capric and caprylic acids are primarily responsible for the distinctive aroma of goat milk (Lo Presti et al., 2023). The FA were not affected by the trial involving grazing goats with fennel seed supplementation, and they were found in greater quantities in goats housed in stalls. In the present trial, C22:6 (docosahexaenoic acid, DHA) was also affected by the treatment, exhibiting higher levels in group FEN. This FA has significant medical implications, as its dietary presence has been linked to the prevention of numerous human afflictions, including cancer and heart disease (Stillwell & Wassall, 2003).

Gene expression

Nutrition represents one of the most significant environmental factors influencing the interaction between genetics and the environment. Advances in molecular biology and genetics have facilitated research on diet and on the relationship between diet and gene expression. For example, polyunsaturated fatty acids (PUFA) have been observed to suppress the expression of the *FASN* (Simopoulos, 2020). In addition to nutrients, non-nutritive dietary phytochemicals, such as phenolic compounds, which are present in FSP, have also being investigated for their effects on various aspects of animal metabolism. Nutrigenomics has the potential to facilitate the development of novel foods designed to enhance health and manage chronic diseases in accordance with individual genotypes. Nevertheless, the regulation of gene expression in response to dietary changes occurs at multiple levels, including transcription (via transcription factors), post-transcription (such as mRNA stability), translation (e.g., initiation or microRNA activity), and post-translation (e.g., enzyme turnover or activity regulation) (Bernard et al., 2013). However, it is frequently unclear whether the regulatory agents are the nutrients themselves, their metabolites, or the hormones produced in response to dietary shifts. Furthermore, the majority of studies are unable to identify the precise level of regulation

involved, as measurements of mRNA, protein levels, and enzyme activity are seldom taken concurrently.

No significant differences were observed in the expression of lipogenic genes (*LPL1*, *SCD1*) between the experimental groups. In contrast to other lipogenic genes, which are less influenced by dietary factors, *SCD* is typically more responsive to dietary UFA supplementation, particularly in subcutaneous adipose tissue (Bernard et al., 2005a).

The relationship between *SCD* expression and milk quality has been the subject of extensive research. Stearoyl-CoA desaturase is responsible for the production of over 75% of the total CLA in milk (Chilliard et al., 2006). Nutritional factors, particularly those containing lipids, have been demonstrated to exert an influence on *SCD* expression. Tudisco et al. (2019) demonstrated that linseed supplementation reduced *SCD* activity, a result that was supported by Tsiplakou et al. (2008) and Bernard et al. (2009). Nevertheless, the altered activity of SCD was not correlated with changes in *SCD* mRNA levels, as observed in the present study.

PRLR expression levels were examined due to the galactagogue properties of fennel seeds, which are known to influence prolactin (PRL) levels. Despite this, no significant differences in *PRLR* expression were found between the experimental groups.

The anti-inflammatory properties of fennel, which are commonly observed in medicinal herbs, have been investigated by examining the expression of Toll-like receptor 4 (*TLR4*), which activates nuclear factor (NF)-κB, a key regulator of numerous genes involved in inflammatory responses (Chang et al., 2015). Liu et al. (2015) demonstrated that high-grain feeding influenced *TLR* mRNA levels and altered the bacterial composition of the ruminal epithelium in male goats, thereby establishing a connection between the microbial community and *TLR* expression. The current study revealed no significant differences in *TLR4* mRNA levels.

The treatment did not affect the expression of genes involved in antioxidant activity, including *SOD* and *NRF2*. In contrast with the findings of the preceding trials, the dietary treatment did not affect the levels of mRNA of *SOD*. As observed in the trial on grazing goats (Chapter IV), the supplementation with fennel seeds was found to increase the antioxidant activity of cheese, as measured by TAC and DPPH. This finding may be attributed to the relatively brief supplementation period, which contrasts with the aforementioned trial. Accordingly, further investigation is required to ascertain the optimal time of administration and dosage in order to evaluate the impact on mRNA levels of candidate genes.

Chapter VIII

Global discussion and conclusion

The experimental trials conducted during this PhD have demonstrated the potential of incorporating medicinal herbs into the diet of dairy ruminants, although certain considerations must be taken into account. The administration of selected plants, which have been identified for their galactagogue and antioxidant properties, has been demonstrated to effectively promote milk production and enhance antioxidant levels. In all trials conducted, groups treated with these herbs exhibited an increase in milk yield, although not always statistically significant.

It is particularly noteworthy that the galactagogue effect was observed despite the absence of any discernible alteration in the chemical composition of the milk in the majority of trials.

This indicates that the elevated production was not attributable to a dilution effect, but rather that the milk's original components remained intact. It is of particular significance for species such as goats and buffaloes, whose milk is predominantly used for the manufacture of cheese and other dairy products, where the fat and protein content are of paramount importance for quality. Furthermore, in the context of goat production, the capacity to enhance yields is particularly relevant. In southern Italy, a significant proportion of goat farms are based on pasture-feeding systems and the use of local breeds. While these practices assist in maintaining the quality of the products, they frequently result in a reduction in yield. In this context, the utilisation of natural resources, such as medicinal herbs, enables an increase in milk production without compromising the nutritional properties of the final products. In the goat trials, the decision was taken to test the same plant (fennel seeds) in both the pasture and the barn. This was done to account for the possible interaction of the supplement with the plants present in the pasture which could result in disparate outcomes, as evidenced by the comparison of the same supplement in the barn. Unfortunately, due to the constraints of the project timeline, it was not feasible to conduct a comparison with the other plants that were tested as part of this project.

Additionally, future studies should investigate the potential benefits of varying the dosage and administration schedule of these supplements, with the aim of enhancing their efficacy. Indeed, the dosage was selected on the basis of previous studies involving the supplementation of whole medicinal plants in the diet of lactating ruminants, as was done in the trials conducted in this project. Two factors precluded the possibility of increasing the dosage. The first factor is palatability. The incorporation of these products into the diet necessitated varying periods of adaptation, contingent on the product in question, for the animals to accept the novel ingredient. It can be reasonably assumed that larger doses would not be accepted by the animals. The second reason is that both companies selected for these trials are private companies that sell

their derived products. Therefore, there was a risk for the company that a high proportion of unwanted flavourings would pass into the products.

In addition, the products resulting from these trials demonstrated an increase in antioxidant activity, which is a pivotal factor in enhancing product stability over time, consumer appeal and nutritional value. The capacity to naturally enhance dairy products with antioxidants provides additional protection against oxidative processes, particularly lipid peroxidation, which has the potential to impair the nutritional value and alter the flavour of dairy products. Consumers are becoming increasingly aware of the production methods employed in the manufacture of animal products and are consequently demonstrating a preference for natural products over those containing synthetic additives.

In this regard, one limitation of the study was that oxidative damage of dairy products was not measured. The values of antioxidant activity and oxidative damage are able to provide a more accurate view of the product's true antioxidant potential, without this being influenced by the chemical and physical characteristics of the food in question.

With regard to the mechanisms underlying the observed differences in the experimental trials, gene expression analyses provided some insights. For example, in two of the trials, the gene *SOD* exhibited a sort of negative feedback mechanism in response to the inclusion of medicinal herbs in the diet of healthy subjects. Furthermore, it was demonstrated that while the delta-9 desaturase indexes increased, there was no corresponding change in the expression of the *SCD* gene, as previously reported in other studies. Nevertheless, no additional notable distinctions in gene expression were identified between the treated and control groups. This outcome may be attributed to a number of potential explanatory factors identified throughout the course of this project.

Firstly, it should be noted that a smaller number of validated primers are available for goats and buffalo species, in comparison to those available for cattle. Consequently, the genes reported in this study are those for which the selected primers were found to be effective. Moreover, as these two species have been less extensively studied than cattle, a considerable number of the polymorphisms that could potentially be responsible for alterations in gene expression has yet to be identified.

It is conceivable that more pronounced differences in gene expression may be observed in response to more significant dietary alterations that affect the animals' metabolism more markedly and over a longer period. Further studies may wish to examine the effects of higher doses of medicinal herbs or longer administration periods.

Moreover, the impact of these medicinal plants on the animals' overall well-being merits investigation. The physiological demands of lactation result in a high energy expense, which in turn leads to an increased production of ROS and greater stress, particularly in intensive farming systems. Among the numerous strategies that may be employed to enhance animal welfare, the utilisation of medicinal herbs could be advantageous due to their potent antioxidant, anti-inflammatory and antimicrobial properties, which may confer preventive benefits as well.

A further area that requires additional consideration is the alteration in the aromatic profile of the products obtained through the incorporation of medicinal herbs into the diet of the animals. In all trials, the aromatic characteristics of the products differed between the treated and control groups. Although the molecules in question have been previously studied and characterised in sensory terms by other authors, their combination may result in a unique flavour profile in the final product. It is therefore recommended that this aspect be further investigated through sensory evaluations with panel or consumer tests in order to determine whether the use of medicinal herbs has an adverse effect on the flavour acceptability of the final product.

Lastly, but no less importantly, economic considerations must also be taken into account. The decision to use locally sourced materials that had not undergone any form of processing or treatment was made with the dual objective of enhancing the intrinsic value of the region's natural resources and reducing the environmental and economic costs associated with their extraction and subsequent transportation. Nevertheless, the incorporation of such dietary components entails an additional financial burden on animal feed, which must be meticulously assessed in view of the observed enhancement in productivity. Furthermore, this analysis should consider the increased nutritional value derived from the higher antioxidant activity and the potential for an extended shelf life, which could justify a higher product price.

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