UNIVERSITÀ DEGLI STUDI DI SASSARI

CORSO DI DOTTORATO DI RICERCA

Scienze Agrarie

Curriculum Scienze e Tecnologie Zootecniche

Ciclo XXXVII

STUDY OF THE NUTRITIONAL CAUSES OF MILK FAT DEPRESSION IN GRAZING SHEEP: THE CASE OF WATER-SOLUBLE CARBOHYDRATES OVERLOAD FROM GRASSES

dr. Maria Angela Porcu

Direttore della Scuola	prof. Severino Zara
Referente di Indirizzo	prof. Corrado Dimauro
Docente Guida	prof. Antonello Cannas

Anno Accademico 2023-2024

To my Family

Acknowledgements

I would like to sincerely thank my PhD supervisor, Professor Cannas, for all the knowledge shared with such passion and enthusiasm throughout this enriching experience. Thank you for every opportunity given, and for all the support and encouragement I have received.

Thanks to the nutrition research group and to all the colleagues and technicians of the "Scienze Zootecniche" unit of the Department of Agriculture at the University of Sassari, for their help and support.

I am grateful to the technicians of the experimental farm in Ottava and to all the thesis students for their help and for sharing the experimental trials we conducted together, always with curiosity and joy.

Thanks to the nutrition technicians of the Cargill group for their support during the experimental trials and for allowing me, together with the breeders of the studied farms, to study the topic of my research directly in the field.

Index

CHAPTER 1

General introduction

Introduction

Dairy sheep farm systems in Sardinia (Italy) are semi-extensive, characterized by the use of natural or artificial pastures that have a seasonal production pattern (Molle et al., 2008). The season influences directly the quantity and quality of pastures, which affects milk yield and chemical composition of milk (Pulina et al., 2021). During late winter and early spring, the pastures are in their vegetative stage, coinciding with the high quality of the plant and high nutritional requirements and production levels of lactating ewes. Thus, there is a larger use of pasture in ewe's diet during these seasons, which can lead to nutritional unbalances related to grazing, which can negatively affect milk yield and composition, especially milk fat content, particularly sensible to nutritional changes (Atalay, 2019). In fact, during these months, herbage is very nutrient-dense, with low NDF and physically effective NDF (peNDF), and high CP or high NFC and/or WSC, and this makes it easy to encounter nutritional problems due to the difficulty of balancing this feed, exacerbated by the difficulty to estimate the right intake at pasture. The most commons nutritional issues related to grazing include excess of dietary protein leading to elevated milk urea levels (Cannas et al., 1998; Cannas, 2004; Molle et al., 2008) or, more recently, WSC overload. In the last decades, a decrease in milk fat content has been observed on sheep farms in Sardinia, often accompanied by a frequent inversion of the fat to protein ratio, leading to lower cheese yield during late winter and early spring. This represents a significant issue, considering that the main destiny of sheep's milk production in Sardinia is its transformation into cheese. In addition to factors related to genetic selection focused on production rather than milk composition and cheese yield, and the physiological stage of lactation, another cause of lower milk fat content is ruminal acidosis. The

ruminal acidosis (subclinical or chronic) is a syndrome related to a rumen fermentative disorder that involves a decrease in ruminal pH (Hall, 2002) due to an excess of non-structural carbohydrates (NSC), a fibre deficiency in the diet (Li et al., 2014), inadequate physical effective NDF (peNDF) levels and particle size (Kara et al., 2020), and low forage to concentrate ratio (Mele et al., 2006; Kara et al., 2020). Ruminal acidosis can lead to decreased milk production, digestive disorders, laminitis (Hall, 2002) and it is a cause of decreased milk fat content (Kooman et al., 2018; Kitkas et al., 2019; Atalay, 2019). Recently in Sardinia, it has been observed that dairy sheep grazing on grasses rich in water soluble carbohydrates and low in protein and fibre can experience subacute ruminal acidosis (SARA) (Molle et al., 2022) and a reduction in milk fat content due to WSC overload (Satta et al., 2023). This chemical composition is typical of cool season grasses (C3) during late winter and early spring months in Mediterranean regions, during years characterized by low temperatures, especially with night frosts, and sunny days. In Sardinia, the increasing use of modern annual ryegrass varieties, especially tetraploid types, as pasture for dairy sheep can contribute to this issue. In fact, these varieties tend to accumulate high concentrations of WSC during years characterized by the weather conditions described above, as they are selected for ensiling, and success of this conservation method often depends on the amount of sugars, as a high sugar content favors acid production by lactic bacteria (Van Soest, 1982; Jafari, 2012). Thus, these weather conditions, frequent in Mediterranean regions where most dairy sheep are bred, combined with the utilization of these new annual ryegrass varieties that may have very high WSC content (often >30% of DM), may be one of the causes of milk fat depression due to SARA in lactating ewes grazing on these pastures.

Water soluble carbohydrates

Sugars are a generic name that include different types of plants carbohydrates, ranging from monosaccharides, such as glucose and fructose, to molecules formed by their condensation, including disaccharides and oligosaccharides, or by their polymerization, such as fructans. These carbohydrates are present in all forage species, except for fructans, which are specific to C3 grasses. In plants, carbohydrates are transported from photosynthetic tissues (source) such as leaves, to non-photosynthetic tissues (sinks) such as stems and roots, in a process called carbohydrate partitioning (Dhungana and Braun, 2021). Sucrose, a disaccharide, is a primary synthesis photosynthesis product and the most abundant sugar in the plant sap. It is also the primary sugar loaded into the phloem, transported, and released into the storage tissues for utilization or converted into other carbohydrates forms for storage (Van Soest, 1982; Stein and Granot, 2019; Dhungana and Braun, 2021; Jakubowski and Flatt, 2023). In sink organs, it can be metabolized or transported to vacuoles, where it can be stored as sucrose, hydrolyzed into glucose and fructose, and stored as hexoses, or converted into fructans (Jakubowski and Flatt, 2023). Over the day, sucrose is the main component driving WSC increases (Johansen et al., 2022). Sugars are analyzed in feeds as water-soluble carbohydrates (WSC) or ethanol-soluble carbohydrates (ESC). The procedures for testing sugar content in a feed are numerous and lack standardization, even among laboratories, in terms of analytical methods used (Watts, 2004), making comparisons of data between studies difficult (Klevenhusen and Zebeli, 2021). Comparing chemical analyses for feed sugars conducted in different laboratories can be problematic, especially considering the highly variable terminology used to describe the same fractions of carbohydrates (Geor, 2009). The

methods used to assess ESC include the extraction with 80% ethanol used for solubilizing simple sugars and short chain fructans (Undersander, 2013; Kagan, 2022), while WSC are extracted only with water. Kagan et al. (2014), summarizes the methods to determine the WSC and ESC in colorimetric assay, enzymatic assay coupled with colorimetry, titration, and chromatography. The colorimetric anthrone method (e.g. Deriaz et al., 1961) is an historical method used to determine WSC content in forages, which is associated of their fermentability (Weiß et al., 2018). Simple sugars and fructans are usually detected using chromatography, principally by high performance liquid chromatography (HPLC), and reducing sugars (glucose and fructose) by redox/gravimetric methods (Weiß et al., 2018). Kagan (2022), in a review, reported the different quantitative methods to measure WSC and ESC, highlighting that the method used influences the values obtained. Kagan et al. (2014) and Kagan et al. (2022) reported that values of WSC and ESC were higher when analyzed by colorimetric assay than by chromatographical method. In fact, as regards WSC analyses, Kagan (2022), reported that with anthrone method, similar to phenol-sulfuric acid (e.g. Dubois et al., 1956), the WSC values obtained were higher than with ultra-performance liquid chromatography (UPLC). Goñi et al. (2024) developed and validated a near infrared reflectance spectroscopy (NIRS) calibration model to determine WSC content in stem of maize, canola and sorghum, using as reference the anthrone method. The NIRS resulted a reliable and convenient method compared to traditional determination of WSC, such as anthrone method that represents an expensive and time-consuming procedure. Chemically, WSC and ESC represent heterogeneous groups of carbohydrates (Klevenhusen and Zebeli, 2021). The WSC includes monosaccharides (glucose, fructose), disaccharides (sucrose),

oligosaccharides and polysaccharides (fructans) (Klevenhusen and Zebeli, 2021; Kagan, 2022). Glucose and fructose represent a lower percentage of total WSC concentration compared sucrose and fructans (Kagan, 2022). The ESC comprises the same sugars as WSC but include only a small portion of fructans, specifically short chain fructans with a degree of polymerization (DP) of up to around 20 fructose units (Kagan, 2022). In fact, the difference between WSC and ESC consist in the fructan fraction: ESC contains only low DP fructans, whereas WSC the entire amount of fructans (Klevenhusen and Zebeli, 2021), as ethanol partially extracts fructans, whereas water extracts them completely (Hall and Eastridge, 2014). Therefore, estimating fructans by calculating the difference from WSC and ESC is wrong, as it overestimates the value, because some fructans may be included in the ESC (Geor, 2009). Some feed laboratories define the ESC as "sugars" (Undersander, 2013) or "simple sugars". The sum of WSC and starch constitutes the non-structural carbohydrates (NSC) (Francis et al., 2002; McIntosh, 2006; Longland and Byrd, 2006, Siciliano et al., 2017; Ghajar, 2020; Kagan, 2022). The NSC is determined by water and alcohol extraction followed by enzymatic or colorimetric method to calculate the quantity of each carbohydrate include in this (Zhao et al. 2008 cited by Undersander, 2013). Another term used to define the sum of WSC and starch is total non-structural carbohydrates (TNC) (Hall and Eastridge, 2014; Jensen et al., 2014); as C3 grasses accumulate starch only in the seed, WSC, TNC and NSC are considered equivalent values (Smith and Prince, 2017). Simple sugars are composed mainly of glucose, fructose and sucrose, while fructans are a distinct class of complex carbohydrates and are the prevailing WSC component in C3 grasses (Jensen et al., 2014; Klevenhusen and Zebeli, 2021). A study by McGrath (1988) cited by Jafari (2012), reported that in

perennial ryegrass, fructans accounted for about 70% of WSC, and the rest consisted of sucrose, glucose and fructose. The fructans are composed of linear and branched polymers of D-Fructose but often contain a terminal glucose unit (Hall and Weimer, 2016). Depending on the number of fructose molecules, fructans can be divided in oligosaccharides (less than 10 monosaccharide units) or polysaccharides (more than 10 units). Plant fructans are different in molecular structure and weight, DP, and linkage type (Abeynayake et al., 2015). Sucrose is the precursor of fructans and is synthesized in cytoplasm, whereas the fructans are produced and stored in the vacuole of plants cells (Geor, 2010; Superchi et al., 2010; Klevenhusen and Zebeli, 2021), in different sites both in primary heterotrophic organs (leaf sheats, roots, shoots, stems, grains) and autotrophic organs (leaves and floral tissues) (Pollock and Cairns, 1991). Based on the chemical structure, fructans are divided into three main classes: inulin, levan or phlein, and graminan. Inulins are linear molecules linked by β (2,1) bonds, characterised by low molecular weight and with DP less than 10; levans or phlein have predominating β (2,6) linkages, while graminans, as in cereals, contain both β (2,1) and β (2,6) linkages, are highly branched and have higher DP (40-100) compared to inulin (Geor, 2010; Lafiandra et al., 2012; Hall and Weimer, 2016). The type of fructans vary among grass species (Geor, 2009), for example in terms of molecular size of fructans. Indeed, ryegrass fructans have a low molecular weight with a short chain length and DP of 30 to 40 units, whereas timothy (*Phleum pratense*) and orchardgrass (*Dactylis glomerata*) contain larger fructans, with DP of 100 units or more (Pollock and Cairns, 1991; Geor 2009; Geor, 2010). However, perennial ryegrass produces high levels of fructans as a mix of oligosaccharides and polysaccharides with different DP (Abeynayake et al., 2015). The fructans synthesis occurs when the

product of photosynthesis activity is an excess with respect to plant demand, and the sink organs reach a critical level of sucrose (Livingston et al., 2009). Based on the photosynthetic pathway, the forage grasses are divided into cool season (C3) and warm season (C4). Starch is the primary storage carbohydrate of the C4 grasses (Geor, 2010; Jensen et al., 2014) and legumes (Weinmann, 1961). Legumes and C4 grasses accumulate starch in seed and vegetative tissues (Geor, 2010), while C3 grasses accumulate starch in the seed only (Van Soest, 1982). Instead, fructans represent a primary storage carbohydrate in vegetative tissues (leaves and stems) of C3 grasses (Watts, 2004; Geor, 2009; Livingston et al., 2009). The principal C3 grasses, among the most used forage grasses in ruminant feeding are perennial ryegrass (*Lolium perenne*), annual ryegrass (*Lolium multiflorum*), small grain hays (oat, wheat, rye, triticale), orchardgrass (*Dactylis glomerata*), fescue (*Festuca* sp.), brome (*Bromus* sp.), and timothy (*Phleum* sp.) (Watts, 2004; Superchi et al., 2010). In the C3 grasses the storage reserve carbohydrates site are the stems, and their base is considered a storage organ (Watts, 2004), which contains the highest concentration of fructans, especially in the lower few centimeters of the plant (Striegel, 2008), even though often the apical parts have high WSC concentration in the afternoon because more exposed to light (Delegarde et al., 2000 cited by Watts 2008). Jafari (2012) citing a study of McGrath (1988), reported that WSC concentration was about 50% higher in stem compared to leaf. However, storages sites also include roots, rhizomes and lower leaves of grasses (Undersander, 2013). The accumulation of fructans occurs in the cell vacuoles of the leaves in C3 grasses (McIntosh, 2006; Geor, 2010; Jensen et al., 2014; Ghajar, 2020), and they are transported and stored in the vacuoles of the stems, for use when the plant needs energy (McIntosh, 2006). Instead, in the C4 grasses and legumes,

the storage site of starch are the leaf tissues (Watts, 2004; McIntosh, 2006; Undersander, 2013), where starch accumulates in the amyloplasts and chloroplasts of leaf cells (McIntosh, 2006; Geor, 2010; Jensen et al., 2014; Ghajar, 2020). The storage mechanism in the vacuoles of C3 grasses is not self-limiting, which can lead to a very high fructan content. Conversely, starch accumulation in C4 grasses and legumes presents a self-limiting mechanism, and at chloroplasts saturation starch production stops (McIntosh, 2006; Superchi et al., 2010; Geor 2010; Ghajar, 2020). Indeed, the accumulation of carbohydrates in C3 forages is generally higher than in C4 forages and legumes (Watts, 2004; Longland and Byrd, 2006; Ghayar, 2020) due to differences in photosynthesis pathways and the carbohydrate storage allocation mechanisms.

Factors that influence the water soluble carbohydrate content in grasses (Poaceae)

The WSC content is a result of highly dynamic processes in plants. In fact, the concentrations of storage carbohydrates in plants are constantly changing depending on the balance between photosynthesis, respiration and their utilization for growth and development (Longland and Byrd, 2006), which is a result from the complex interaction between the plant and environment. A larger number of factors influence the WSC content of grasses, such as: plant part considered, growth stage, species, cultivar, ploidy, time of day, temperature, light intensity, season, water availability, fertilization and management of the crop (Watts and Chatterton, 2004; Watts, 2010; Olszewska, 2021; Kagan, 2022). The variation of WSC levels in C3 grasses occurs both diurnally and seasonally (Niyigena et al., 2021). Daily fluctuations can coincide with energy storage patterns and their use (Geor, 2010). Photosynthesis capacity and the consequent production of sugars are directly correlated with light intensity and

duration (Watts, 2004). Delegarde et al., 2000 cited by Watts 2008, reported that on the evening the nonstructural carbohydrates content is higher than in the morning especially in the upper layers which are more exposed to solar radiation. Additionally, Watts (2008) found a strong correlation between levels of plant nonstructural carbohydrates in the afternoon and the solar radiation of the sampling day and the two previous days. Conversely, shade decreases the WSC concentration (Watts, 2004; Ciavarella et al., 2000; Longland and Byrd, 2006; Allsop et al., 2009; Watts, 2009). Additionally, cloudy conditions limit the photosynthesis activity and result in lower WSC content (Jafari, 2012; Kagan, 2022). The WSC content on sunny days tends to rise in the morning, reach the maximum in the afternoon, as plants through photosynthesis produce carbohydrates, and are reduced overnight because the plants consume WSC with respiration during the night (Geor, 2009; Ueda et al., 2016; Niyigena et al., 2021). The diurnal variation of WSC content represents the most important change in the chemical composition of plants throughout the day (Cajarville et al., 2015). The C3 grasses can accumulate large quantities of fructans during slow growth periods, when the products of photosynthesis exceed the request and uses of the plant (Livingston, 2009). This typically occurs when temperatures are low, but the days are sunny. During sunny days, the respiration rate decreases when temperatures are below 5 °C (Watts, 2004), and plant growth may decrease or block at freezing temperatures, while sugars continue to be synthesized and accumulated (Watts, 2005). Indeed, enzymes responsible for sugar respiration are very dependent on temperatures (Watts, 2008), decrease their activity below 5 °C and cease it only below freezing temperature, while respiration becomes more efficient at warm temperatures (Watts, 2010). During cool weather, there is an accumulation of large amounts of

carbohydrates storage (Watts, 2004), and the minimum night temperature was inversely related to the WSC content (Tabacco et al., 2004). A study showed that one of the most significant environmental factors which influence the variation of WSC content is the minimum average temperature of the three days preceding the harvest (Tabacco et al., 2004). The environmental conditions that restrict plant growth but not photosynthesis activity, result in increased amounts of NSC in forage (Jensen et al., 2014), because the use of carbohydrates is limited but the synthesis of these continues. In fact, stress conditions such as low temperatures, killing frosts, drought, and low soil fertility (Geor, 2009) result in accumulation of NSC in plants. Watts (2004) reported that grass on stress conditions may contain up to 35% of the DM of NSC (Watts, 2004). In contrast, favorable conditions for plant growth and development result in low NSC content, as sugars are utilized by the plant to produce fibre, protein, and energy. For example, increasing amounts of nitrogen applied to soil were inversely related to WSC content of plants (Watts, 2010). After defoliation (by grazing or cutting) pastures have a lower level of WSC, possibly because utilization of WSC increases for regrowth (Siciliano et al., 2017; Kagan, 2022); in fact, the stem of mature grasses may contain higher content of fructans compared to regularly grazed or cut pasture grasses (Harris et al., 2006). For the same reasons, at passage from vegetative to reproductive stage the WSC levels tend to be high (Harris et al., 2006). Plant maturity involves a reduction in the leaves to stems ratio, which results in an increase in WSC with advanced plant maturity (Jafari, 2012). However, environmental factors can influence the soluble carbohydrates content more than the stage of growth (Watts and Chatterton, 2004). Regarding the variation of WSC levels during the seasons, they are generally lower in autumn pastures than in spring pastures (Beever et al., 1978; Maas et al., 2001; Jafari,

2012), due to reduced solar radiation, lower sunlight hours and, usually, high night temperature that favors respiration of sugars during the night in autumn (Jafari, 2012). A study conducted in New Zealand, characterized by temperate climate, by Sun et al. (2023), showed that ryegrasses grown during winter had lower CP and fibre but higher NSC concentrations, including soluble sugars and starch, than those grown in late autumn. Another study, conducted in Mediterranean conditions (Sardinia, Italy), showed that the average content of WSC was higher in winter and early spring compared to autumn grass samples (21.5 vs. 3.5 % DM), reaching maximum values of 36.2 % and 6.4 % DM, respectively. Conversely, the average content of CP and the fraction of soluble protein were higher in autumn compared to winter and early spring (27.7 vs. 15.8 and 14.2 vs. 8.3 % DM, respectively), indicating a negative correlation between pasture WSC and CP content ($P < 0.001$; Satta et al., 2023).

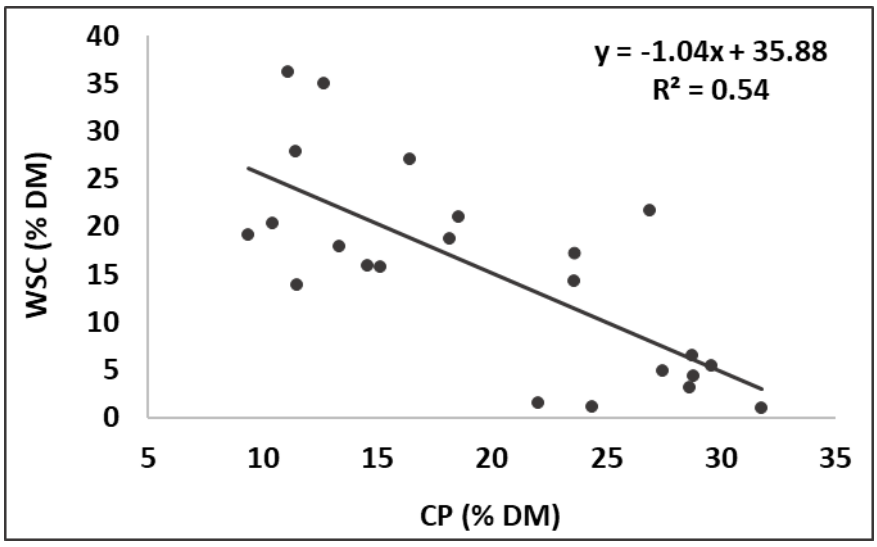

Figure 1. Regression analyses between WSC content (% DM) and CP content (% DM) in pasture samples collected in Sardinia (Italy) ($P < 0.001$; Satta et al., 2023).

Cool weather is a fundamental condition for achieving high contents of WSC in a plant, leading to differences between Mediterranean and Nordic regions regarding which season corresponds to the weather conditions that promote WSC accumulation. McIntosh reported a personal communication by Longland et al. (year not reported), who in a study of various ryegrass cultivations at different sites in five Northern European countries, observed that the highest concentrations of WSC, 385 g/kg DM, occurred in site with cooler temperature. The WSC content varies among different forage families and species, and legumes, such as clovers, are typically higher in CP and lower in WSC and NDF concentration than C3 grasses, and do not contain fructans. However, legumes usually accumulate starch at higher concentrations than C3 grasses (Kagan et al., 2020). A significantly positive relationship has been demonstrated between the percentage of grasses in the pasture and the WSC content, in a study conducted in Sardinia (Italy) (Figure 2; Satta et al., 2023).

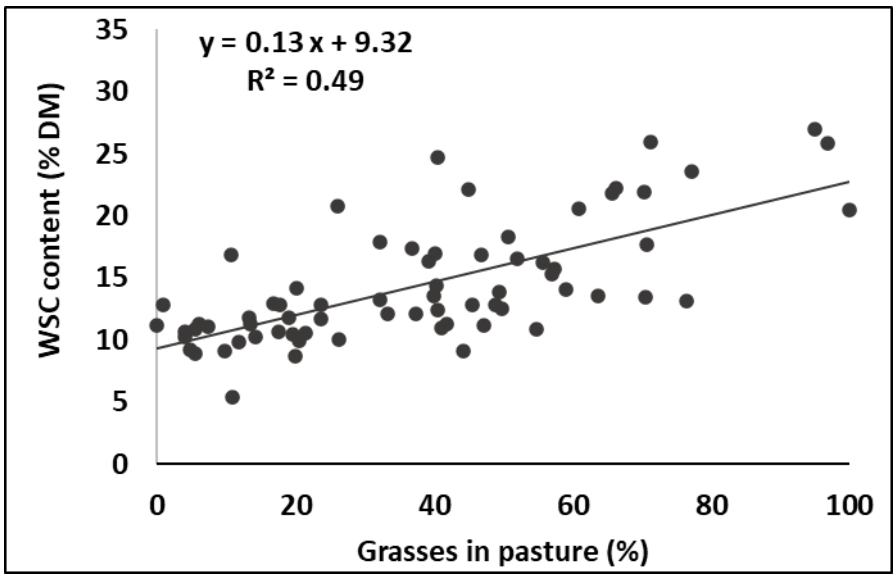

Figure 2. Regression analyses between percentage of grasses in pastures (Sardinia, Italy) and their WSC content (% DM) ($P < 0.001$; Satta et al., 2023).

As mentioned before, different species can vary significantly in their composition and concentration of WSC or fructans, and even cultivars within the same species may be different in the content of accumulated WSC (Kagan, 2022). Additionally, the ploidy of a plant also influences WSC concentration, with tetraploid cultivars typically having higher concentrations than diploid cultivars (Olszewska, 2021; Kagan, 2022; Rech et al., 2022). Nonstructural carbohydrate levels are very high in modern forage varieties, particularly in ryegrasses of tetraploid type (Duggan et al., 2022). Indeed, a study conducted in Denmark compared different C3 grasses species including timothy (*Phleum pratense*), tall fescue (*Festuca arundinacea* Schreb), orchard grass (*Dactylis glomerata* L.), meadow fescue (*Festuca pratensis* Huds.), diploids and tetraploids types of perennial ryegrass, and lucerne, showed that both in August and October, the tetraploid ryegrass exhibited the highest WSC content (Johansen et al., 2022). Varieties of perennial ryegrass called 'high sugars' have been genetically selected for high WSC content trait, but the extent and consistency with which this trait is expressed is strongly related to the environmental conditions (interaction genotype x environment), and the low temperatures are necessary for expression of this genetic trait (Parsons et al., 2004; Turner et al., 2014; Rivero et al., 2019). Scientific literature on high sugar annual ryegrass varieties is limited, and the ability of these varieties to accumulate WSC has been tested in only a limited number of studies than perennial ryegrass (Hopkins et al., 2002; Alende 2016). However, in Mediterranean areas such as in Sardinia, annual ryegrass, also known as Italian ryegrass (*Lolium multiflorum*), is one of the most commonly used grasses as pasture and for hay and baled silage production. A recent study conducted in New Zealand by Sun et al. (2023) confronted nine ryegrass cultivars, among which perennial, hybrid, and Italian types, and found

that the Italian ryegrass (*Lolium multiflorum*) had the highest levels of soluble nutrients, soluble sugars, and starch; indicating that the choice of ryegrass cultivar is very important, as it can significantly affect the chemical composition, particularly the WSC content (Kagan, 2022; Sun et al., 2023). The conservation methods of fresh forage involve the consumption of plant sugars during both haymaking and ensiling for hay and baled silage production, respectively. In fact, the conservation of forage results in losses of water and nutrients, especially sugars, due to respiration processes occurring post-cutting and post-harvest, as consequence of fermentation and surface deterioration (Wilkinson, 1981). Müller et al. (2016) showed that silage had lower WSC content than hay, due to WSC fermentation by lactic acid bacteria, which produce lactic acid during ensiling. In fact, having sufficiently high WSC concentration is a necessary condition for good fermentation by lactic acid bacteria and preservation of silage (Jafari, 2012). Hay has low levels of sugar than fresh grass due to respiration that occurs during cutting and drying processes (Van Soest, 1982; Watts and Chatterton, 2004). During hay production, the higher sugars losses occur during the first week of storage after harvesting, and one month after storage the sugar content is stabilized (Müller et al. 2016). However, the WSC content of fresh forage influences the WSC hay content more than drying time between cutting and hay baling (Deroche et al., 2022). Therefore, the nutrient value of forage from temperate pasture is commonly higher when fresh forage is considered than when it is conserved as hay or silage (Glenn, 1994 cited by Rearte, 2005), which are both subjected to nutrient losses.

Water soluble carbohydrates in equine nutrition: the pasture-associated laminitis

The most extensive and detailed literature on WSC from a nutritional point of view is related to equine nutrition. In fact, WSC and particularly fructans, are widely studied in the field of equine nutrition, due to their involvement in the onset of pasture-associated laminitis. Laminitis is a preventable disease that affects the laminae within the hoof, leading to decreased blood flow to this area, resulting in their inflammation, death of tissue, and in critical cases, displacement of the coffin bone from the hoof wall (Ivey, 2017; Duggan, 2022). The nutritional factors involved in the onset of laminitis, including excess of grain and lush pasture overgrazing (Milinovich et al., 2006), cause primarily intestinal acidosis that precedes laminitis. Studies in the literature have shown that majority of cases of laminitis occur in horses and ponies grazing on lush pasture (Anon, 2000, cited by Van Eps and Pollitt, (2006)), which led to the term "pasture-associated laminitis" (Geor, 2009). Indeed, over consumption of pasture with high levels of WSC was linked to the development of this pathological condition (Schmidt et al., 2023). The fructans, a very complex class of carbohydrates, are the predominant carbohydrate among the WSC on C3 grasses widely used in horse pastures in both Nordic and Mediterranean areas. The fructans, although they are considered soluble carbohydrates, may not be completely digested in the equine foregut like simple sugars and starch, but they are fermented in the hindgut as well as fibrous structural carbohydrates (Striegel, 2008). Equine does not produce enzymes that hydrolyzes fructans in foregut, but in the hindgut is used as microbial substrate (Murray, 2021). However, fructans are rapidly degraded in the hindgut (Undersander, 2013), as are soluble carbohydrates, and this can cause intestinal disorders that can trigger a series of changes that causes laminitis (Geor, 2009). Thus, fructans are

fermented in hindgut as fibre but their degradation is fast, as soluble carbohydrates. The rapid fermentation of large amounts of WSC, as well as of starch, may results in elevated production of volatile organic acids and lactate, which causes a lowering of the intestinal pH, leading to hindgut acidosis and associated metabolic disorders, like laminitis and colic (Longland and Byrd, 2006; McIntosh, 2006; Geor, 2010; Undersander, 2013), similar to those caused by excess starch (Van Eps and Pollitt, 2006). The lowering of intestinal pH causes a shift in the microbial population from a predominance of Gram-negative to Gram-positive bacteria (Milinovich et al., 2006). A study conducted by Milinovich et al. (2006), suggested that streptococci from the Streptococcus bovis/equinus complex may contribute to the changes prior to the onset of horse's laminitis. All these alterations of the intestinal environment involve the production and absorption of substances, such as vasoactive amines, exotoxins and endotoxins, which lead to a systemic inflammatory response, triggering the development of laminitis (Geor, 2009). Bailey et al. (2003), cited by Thoefner et al. (2014), showed that Streptococcus bovis and five Lactobacilli spp. are involved in the production of vasoactive amines, that are suggested as causal factors of laminitis (Thoefner et al., 2014). Van Eps and Pollitt (2006) tested a model of laminitis induction using oligofructose and suggested the existance of a link between pasture fructans, the proliferation of Gram-positive bacteria, histopathological changes in lamellar, and laminitis, confirming that the clinical and metabolic effects of fructan overload are like those of starch overload. The biochemical structure of fructans influences their fermentation rate (Geor, 2010) and consequently their ability to cause laminitis. Short chain fructans are likely to be fermented more quickly by hindgut bacteria, posing a greater risk for laminitis (Kagan et al., 2018). For example, the

fructans of cocksfoot and timothy have a higher molecular weight and longer chain length than ryegrass fructans and are fermented more slowly in the hindgut (Harris et al., 2006; Geor, 2010) compared to ryegrass, which can be riskier for the onset of pasture-associated laminitis (Geor, 2010). In horses, it was found a significant positive association between hours of sunshine and incidence of laminitis, due to the higher intake of soluble carbohydrates during periods with high plant photosynthesis processes and carbohydrate accumulation (Geor, 2009). Confirming this, a study conducted on mares grazing in spring showed that the serum insulin concentrations followed the same circadian pattern of the NSC content of grass, reaching a lowest value in the morning and highest in the afternoon (Geor, 2009). In general, in temperate regions during late spring characterized by sunny days and cool nights, conditions that cause very high levels of soluble carbohydrates, the incidence of laminitis in grazing horses is higher (Harris et al., 2006; Watts, 2010).

Water soluble carbohydrates in ruminant nutrition: effects on intake, rumen function, milk synthesis and animal health

Studies in ruminants on the effects of WSC, particularly fructans, in grasses, are especially limited on sheep. Research on WSC in ruminant nutrition mainly concerns their effects on palatability, intake, and production, but there is a lack of studies on the impact of WSC overload on metabolism and milk composition, especially on sheep. In fact, there is limited knowledge regarding the effect of the content of sugars of grazed grasses on the total daily supply of NSC in ewes and on the associated risks of sub-acidosis, especially when ewes receive starch-rich supplements (Cannas et al., 2023). The WSC content is not a commonly used parameter in the rationing and

formulation of ruminant diets, especially for dairy ewes. However, recently in Sardinia, this grasses compound is getting much attention in dairy sheep farms based on grazing. During winter and spring, in this region, pastures of C3 grasses reached very high values of WSC (often 25-30 % of DM). Thus, in recent years, WSC content is an important pasture quality parameter included in routine chemical analyses of pastures. If the most common nutritional issue related to grazing early stage pastures was the excess of protein, resulting in high blood and milk urea levels and dark colored diarrhea (Cannas et al., 1998), recently in Sardinia during winter and spring, a problem related to excess of grasses soluble sugars (WSC) predominated, leading to a decrease of the fat content in the milk, often resulting in an inversion of the milk fat to protein ratio and yellow/light diarrhea (Porcu et al., 2024), a useful field indicator of ruminal sub acidosis. The WSC group is very heterogeneous and their inclusion effects on ruminal fermentation and milk production are related to the source of WSC, their diet inclusion level and the total diet formulation (Klevenhusen and Zebeli, 2021). Different types of sugars have varying fermentation rates and are used with different efficiency by ruminal bacteria (Emanuele and Sniffen, 2014), thus affecting rumen pH and volatile fatty acids (VFA) profile in rumen (Oba, 2011). Fructans, in contrast to simple sugars, are not digestible by mammalian enzymes and are fermented by ruminal bacteria (Hall and Weimer, 2016). WSC are usually considered to be quickly degraded by ruminal microbes (fructanhydrolytic and saccharolytic) at fermentation rate of up to 300 %/hour, but the rate of fermentation likely varies among various types of WSC, depending on their DP and integrity of the cell wall, especially the NDF content of plant. Indeed, the WSC effects on rumen fermentation and pH are difficult to predict (Klevenhusen and Zebeli, 2021). High concentrations of sugars and/or starch in the

diet cause a rapid growth of Streptococcus bovis and Selenomonas ruminantium, leading to a shift of their fermentation to lactate production. This shift occurs slowly in the presence of adequate level of ammonia nitrogen in rumen, which is used as nitrogen source; to prevent lactate production, it is necessary to moderate the sugars levels in the ruminant diet and combine this with feed rich in protein (Emanuele and Sniffen, 2014). The grasses rich in WSC have low CP levels, as shown in Figure 1. Grazing these pastures can probably cause a shift of fermentation and reduction in ruminal pH, thus supplying a feed high in protein before grazing (third meal technique) might prevent this issue. A recent study in New Zealand conducted by Sun et al. (2023) showed that annual Italian ryegrass (*Lolium multiflorum* L.), the most common grasses used as pasture in Sardinian dairy sheep farms, has a higher portion of soluble nutrients, soluble sugars and starch, as well as a greater degradation rate of an insoluble but degradable fraction (B) of DM than hybrid and perennial ryegrasses (*Lolium perenne* L.). The same study showed that the DM in the soluble fraction (A) was higher in the Italian cultivars and tended to have lower NDF and shear strength, making it a highly and quickly degradable forage. Thus, these characteristics make it a high-quality feed but also a risk for ruminal acidosis due to WSC overload.

Forages high in WSC could increase palatability, dry matter intake (Lee et al., 2002), milk production (Ciavarella et al., 2000; Miller et al., 2001; Moorby, 2001; Taweel et al., 2005) and liveweight gain (Lee et al. 2001), probably due to a change in VFA profile produced by the WSC fermentation (Ciavarella et al., 2000). Ueda et al. (2016) showed that cows modified their diurnal grazing pattern according to the diurnal variation in WSC content, with longer grazing time in the evening (higher WSC content) than in morning (lower WSC content); indicating that palatability, and

consequently the grazing time and intake, was influenced by WSC content of the herbage. Other studies have shown that also in ewes, the temporal dynamics of herbage intake and grazing time is affected by diurnal changes in WSC, indicating that WSC content is an important parameter in the selection process by sheep (Orr et al., 1997; Ciavarella et al., 2000; Molle et al., 2022). Avondo et al. (2008) conducted a study on goats grazing annual ryegrass (*Lolium multiflorum* ssp. westervoldicum) for 4 hours/day and found that herbage intake was significantly higher in the afternoon, due to a higher WSC content in the afternoon (20.4 % DM) than in the morning (17.4 % DM). In the same study, the afternoon grazing group had significantly higher milk protein content and lower milk urea content, probably a result of a more balanced nitrogen to energy ratio from sugars. A recent study conducted in Sardinia (Italy) by Molle et al. (2022) during spring on lactating dairy ewes fed Italian ryegrass pasture (*Lolium multiflorum* Lam.) for 4 hours/day, showed higher post-grazing levels of propionic and butyric acids, glucose and insulin, and lower blood urea in ewes grazing in the afternoon (higher WSC levels) than grazing in the morning (lower WSC levels). Cannas et al. (2009) cited a study of Siever-Kelly et al. (1999) conducted on sheep, that showed higher intake of pasture treated with the herbicide glyphosate, which caused higher WSC concentrations in the plant. When offered different plants part, the sheep preferred the stems compared to leaves, due to their higher WSC content. In the same study, the use of treated herbage, with higher WSC content resulted in increased VFA production after feeding. Regarding conserved forage, Deroche et al. (2020) conducted a study on dairy cows which showed that hay with high total soluble carbohydrates content increased preference, digestibility and consequently, intake and productions of animals. The season affected chemical composition of pasture,

especially the WSC content, and consequently influenced ruminal fermentation pattern and animal performances. Maas et al (2001), in a study conducted on ewes, observed that autumn pasture with low soluble carbohydrates levels resulted in a ruminal fermentation pattern with a high acetate to propionate ratio, higher level of lipogenic VFA, a higher ruminal pH, and lower glucose and β-hydroxybutyrate (BHB) levels in blood than spring pasture (with higher WSC levels). Similarly, Beever et al. (1978) in a study on fistulated sheep that received herbage of perennial ryegrass harvested in different seasons and preserved by quick freezing, showed a higher yield of total VFA and propionate in sheep fed herbage cut in spring (with higher WSC content) than sheep fed herbage cut in autumn (with lower WSC content). Consistent with these results, an in vitro study conducted by Lee et al. (2003) with four different levels of WSC reported increased glucogenic to lipogenic VFA ratio and propionate and a linear decrease of the proportion of acetate and of ruminal pH as WSC levels increased. This is probably related to rapid degradation of WSC, which leads to a rapid increase of VFA and lactate. Additionally, the study showed that for the initial increases in WSC level there was an improvement in the efficiency of microbial protein synthesis (EMPS), but at the highest WSC addition, EMPS decreased dramatically, likely due to a decrease in cellulolytic activity. Several studies have reported the advantages of using selected "high-sugar" varieties to improve the synchronization of ruminal fermentations (protein and energy availability), the microbial conversion of dietary CP to microbial protein, and the efficiency of utilizing grass protein for milk production. These effects lead to a decrease in ammonia in the rumen, urea in plasma, and nitrogen losses in urine (Miller et al., 2001a; Miller et al., 2001b; Moorby, 2001; Taweel et al., 2005). A study by Lee et al. (2001) conducted on lambs grazing two varieties of

perennial ryegrass (*Lolium perenne* L.) with different WSC concentrations, showed a strong positive relationship between liveweight gain and herbage WSC content due to enhanced synchronization of microbial fermentation, protein synthesis and an improved glucogenic to lipogenic VFA ratio. Miller et al. (2001a) reported higher milk yield and milk protein yields in cows fed perennial ryegrass with high (16.5% of DM) than low (12.6% of DM) WSC concentrations. However, Merino et al. (2019) did not find differences on milk yield and composition in cows fed herbage with different sugars levels, consistent with the findings of Moorby et al. (2006). This study reported higher milk true protein yield in cows fed perennial ryegrass high in sugars (24.3 % DM) compared to the control group (16.1 % DM). However, grasses rich in WSC often have very low concentrations of CP (often < 10 % DM) (Satta et al., 2023; Figure 1) and fibre, resulting in an unbalanced feed in terms of energy and protein availability for microbes in the rumen, which can potentially lead to issues related to a lack of synchronization of fermentations. A study conducted on grazing Irish dairy cattle reported that low values of rumen pH is a common condition in cows grazing perennial ryegrass pasture, due to high digestibility, high contents of rapidly fermentable carbohydrate, and low levels of physically effective fibre, which may lead to subacute ruminal acidosis and subsequent animal health issues (O'Grady et al., 2008). Trevaskis et al. (2001) conducted a study on sheep fed ryegrass herbage (*Lolium multiflorum*) cut in the morning (with lower WSC) or in the afternoon (with higher WSC), in which the only differences were DM and WSC contents, showing significantly lower ruminal pH in sheep fed afternoon herbage, due to higher rumen-fermentable carbohydrates levels. Additionally, Molle et al. (2022) found a negative correlation between WSC intake and ruminal pH in grazing sheep on annual ryegrass; it is important to consider

that WSC levels in grazed grass of this study were higher compared to many experiments on grazing cattle. Rivero and Anrique (2015), in a review citing Agenas et al. (2002), reported the possibility of observing low ruminal pH and milk fat depression during critical times of the year, especially in early spring. In fact, this period is characterized by high-quality pastures in vegetative growth stage, characterised by low fibre content, high sugar content, high water content, and high polyunsaturated fatty acids (PUFA) content. Indeed, lush pastures often lead to a decrease in milk fat content (Rearte et al., 1984). A recent field study conducted on five pasture-based Sardinian sheep farms showed a negative correlation between milk fat concentration and WSC content of the pasture, but not to the NDF content (Satta et al., 2023; Cannas et al., 2023). Furthermore, grazing on young pastures involves high water intake that is associated with reduced production of saliva (Meyer et al., 1964 cited by Westwood and Lean, 2001) and consequently limited buffering action on rumen fluid. This mechanism may be related not only to high water content but also to low NDF and peNDF (Westwood and Lean, 2001), representing risk factors for SARA and consequent milk fat depression. Thoefner et al. (2004) studied the clinical response to an alimentary oligofructose overload in dairy heifers and observed a case of lameness, which showed strong similarities to the pathogenesis and pathophysiology of oligofructose induced acute laminitis in horses, both consequent to acidosis conditions. Also, Filho et al. (2019) inducted ruminal and metabolic acidosis and typical lesions of laminitis in calves through oligofructose overload. These studies confirm that fructan overload from grasses may cause ruminal acidosis, as oligofructose, a polymer with up to ten sugar subunits belonging to fructans (Filho et al., 2019), is the predominant carbohydrate in WSC of C3 grasses pastures.

Westwood et al. (2003), in a review, reported the occurrence of laminitis in pasture-fed dairy cows with low ruminal pH values, indicating that ruminal acidosis is a cause of laminitis in grazing animals. However, several studies reported that partially substituting dietary starch with sugar does not cause a decrease of ruminal pH (Emanuele and Sniffen, 2014; Sun et al., 2020). Sun et al. (2020) in an in vitro study replaced dietary corn starch with sugars (sucrose, fructose, and lactose) at different inclusion doses (3%, 6% and 9%) and observed an increase in butyrate production. In this study, the pH was not influenced by the treatments, except for the 9% sucrose substitution, that caused a decrease in pH and showed a lowest pH among the sugars tested. The 9% fructose increased the molar production of propionate and decreased the acetate to propionate ratio. These results confirm that sugar's effects on rumen fermentation are influenced by the type and concentration of sugar.

Objective of the Thesis

This thesis is structured into a general introduction, three chapters of experimental studies and general conclusions.

The first Chapter contains the general introduction, with the aim to expose the issue of lowering milk fat content in grazing dairy sheep in Sardinia and provide an overview about water soluble carbohydrates in grasses (Poaceae) and their implication in the nutrition of equine and ruminants.

The first research (Chapter 2) investigated if pasture WSC might affect milk fat synthesis and concentration.

The second research (Chapter 3) tested a nutritional technique (bicarbonate supplementation) to mitigate the negative effects of WSC overload on milk fat content.

The third research (Chapter 4) concerned a field survey that investigates the relationships between diet, grazed herbage, and milk composition, particularly the milk fat content of dairy ewes during the winter and spring months in Sardinia.

The general conclusions (Chapter 5) contain a summary of the main results obtained from the research of this thesis.

References

Abeynayake S. W., Etzerodt T. P., Jonavičienė K., Byrne S., Asp T., Boelt B. 2015. Fructan metabolism and changes in fructan composition during cold acclimation in perennial ryegrass. Frontiers in Plant Science, 6, 329.

Alende M. 2016. Evaluation of high sugar ryegrass varieties (Doctoral dissertation, Clemson University).

Allsop A. R. C., Nicol A. M., Edwards G. R. 2009. Effect of water soluble carbohydrate concentration of ryegrass on the partial preference of sheep for clover. Proceedings of the New Zealand Animal Production Society, 69, 20-23.

Atalay H. 2019. Milk fat/protein ratio in ketosis and acidosis. Balıkesir Sağlık Bilimleri Dergisi, 8(3), 143-146.

Avondo M., Bonanno A., Pagano R. I., Valenti B., Di Grigoli A., Alicata M. L., Galofaro V., Pennisi P. 2008. Milk quality as affected by grazing time of day in Mediterranean goats. Journal of Dairy Research, 75(1), 48-54.

Beever D. E., Terry R. A., Cammell S. B., & Wallace A. S. 1978. The digestion of spring and autumn harvested perennial ryegrass by sheep. The Journal of Agricultural Science, 90(3), 463-470.Ablondi M., Eriksson S., and Mikko S. 2022. Haplotype blocks and heterozygosity rich regions on ECA2 in Swedish Warmblood horses. In Proceedings of 12th World Congress on Genetics Applied to Livestock Production (WCGALP), Rotterdam, Netherlands. p. 3159–3162.

Cajarville C., Britos A., Errandonea N., Gutierrez L., Cozzolino D., Repetto J. L. 2015. Diurnal changes in water-soluble carbohydrate concentration in lucerne and tall

fescue in autumn and the effects on in vitro fermentation. New Zealand journal of agricultural research, 58(3), 281-291.

Cannas A., Pes A., Mancuso R., Vodret B., Nudda A. 1998. Effect of dietary energy and protein concentration on the concentration of milk urea nitrogen in dairy ewes. Journal of Dairy science, 81(2), 499-508.

Cannas A. 2004. Feeding of lactating ewes. In Dairy sheep nutrition (pp. 79-108). Wallingford UK: CABI Publishing.

Cannas A., Mereu A., Decandia M., Molle G. 2009. Role of the sensorial perceptions in feed selection and intake by ruminants. Italian Journal of Animal Science, 8 (suppl. 2), 243-251.

Cannas A., Ledda A., Sini M., Porcu M. A. 2023. Nutritional management of transition dairy sheep. Conference: Sheep-Goat Health and management congress. April 27-30, 2023 - Marmaris, Turkey.

Ciavarella T. A., Simpson R. J., Dove H., Leury B. J., Sims I. M. 2000. Diurnal changes in the concentration of water-soluble carbohydrates in Phalaris aquatica L. pasture in spring, and the effect of short-term shading. Australian Journal of Agricultural Research, 51(6), 749-756.

Deroche B., Bouchon M., Pomiès D., Martin B., Renaud J. P., Aoun M., & Baumont, R. 2020. High soluble carbohydrate concentration may increase hay intake, digestibility and milk production in dairy cows. In Meeting the future demands for grassland production (p. 240).

Deroche B., Morvan-Bertrand A., Le Morvan A., Wyss U., Aoun M., & Baumont R. 2022. Prediction of water-soluble carbohydrate contents in hay from their content in fresh forage and drying time. In Grassland at the heart of circular and

sustainable food systems. Proceedings of the 29th General Meeting of the European Grassland Federation, Caen, France, 26-30 June 2022, 2022, 128-130 ref. 5 ref.

Dhungana S. R., Braun D. M. 2021. Sugar transporters in grasses: Function and modulation in source and storage tissues. Journal of Plant Physiology, 266, 153541.

Duggan S., Downing T. W. 2022. A Horse Owner's Guide to Pasture-associated Laminitis. Oregon State University Extension Service.

Emanuele S. M., & Sniffen C. J. 2014. Feeding the rumen with sugar to increase ruminal fermentation efficiency. Penn State Dairy Cattle Nutrition Workshop.

Farghaly M. M., Hassan E. H., Abdo S. G. 2019. Influence of sodium bicarbonate supplementation on nutrients digestibility, milk production, rumen fermentation and some blood parameters in sheep. Egyptian Journal of Animal Production, 56(2), 71-77.

Francis S. A., Chapman D. F., Doyle P. T., Leury B. J., Egan A. R. 2002. Non-structural carbohydrate content of a perennial ryegrass cultivar bred for high sugar levels, compared to 'normal'perennial ryegrass and white clover. Animal production in Australia, 24, 73-76.

Geor R. J. 2009. Pasture-associated laminitis. Veterinary Clinics of North America: Equine Practice, 25(1), 39-50.

Geor R. J. 2010. Current concepts on the pathophysiology of pasture-associated laminitis. Veterinary Clinics of North America: Equine Practice, 26(2), 265-276.

Geor R. J. 2017. Internal Medicine and Clinical Nutrition. Equine Internal Medicine-E-Book, 191. Pages 191-217. ISBN 9780323443296. https://doi.org/10.1016/B978-0-323-44329-6.00005-X.

Ghajar S. M. 2020. The Suitability of Native Warm-Season Grasses for Equine (Doctoral dissertation, Virginia Tech).

Goñi A. M., Fernández J. A., Demarco P. A., Secchi M. A., Carcedo A. J., & Ciampitti I. A. 2024. Determination of water-soluble carbohydrates by near-infrared spectroscopy for canola, maize, and sorghum stem fractions. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 304, 123320.

Hall M. B. 2002. Rumen acidosis: carbohydrate feeding considerations. Proceedings of the 12th International Symposium on Lameness in Ruminants, Orlando, Florida, USA, 9th-13th January 2002, 2002, 51-61 ref. 25.

Hall M. B., Eastridge M. L. 2014. Invited review: Carbohydrate and fat: Considerations for energy and more. The Professional Animal Scientist, 30(2), 140-149.

Hall M. B., Weimer P. J. 2016. Divergent utilization patterns of grass fructan, inulin, and other nonfibre carbohydrates by ruminal microbes. Journal of dairy science, 99(1), 245-257.

Harris P., Bailey S. R., Elliott J., & Longland A. 2006. Countermeasures for pasture-associated laminitis in ponies and horses. The Journal of nutrition, 136(7), 2114S-2121S.

Hopkins C., Marais J. P., Goodenough D. C. W. 2002. A comparison, under controlled environmental conditions, of a *Lolium multiflorum* selection bred for high dry-

matter content and non-structural carbohydrate concentration with a commercial cultivar. Grass and forage Science, 57(4), 367-372.

Ivey Jennie L. 2017. Pasture-associated laminitis. Southern Equine Consortium. Vol. 2 No. 1 Issue Date: April 2017. University of Tennessee.

Jafari A. A. 2012. Environmental and genetic variation for water soluble carbohydrate content in cool season forage grasses. Carbohydrates—Comprehensive Studies on Glycobiology and Glycotechnology; IntechOpen: Rijeka, Croatia, 373-388.

Jakubowski H., Flatt P. 2023. Chapter 20.6: Biosynthesis of starch, sucrose and cellulose. In: Fundamentals of Biochemistry II—Bioenergetics and Metabolism. LibreTexts: Davis, CA, USA. Pp. 487-494.

Jensen K. B., Harrison P., Chatterton N. J., Bushman B. S., Creech J. E. 2014. Seasonal trends in nonstructural carbohydrates in cool-and warm-season grasses. Crop Science, 54(5), 2328-2340.

Johansen M., Jørgensen L. K., Hansen N. P., Bach Knudsen K. E., Weisbjerg M. R. 2022. Changes in sugar concentration over the day and the season in green forages. Grassland Science in Europe, Vol.27 – 29th General meeting. 26-30 June 2022 - Caen, France. Grassland at the heart of circular and sustainable food systems, 191-194.

Kagan I. A., Kirch B. H., Thatcher C. D., Teutsch C. D., Pleasant R. S. 2014. Chromatographic profiles of nonstructural carbohydrates contributing to the colorimetrically determined fructan, ethanol-soluble, and water-soluble carbohydrate contents of five grasses. Animal Feed Science and Technology, 188, 53-63.

Kagan I. A. 2022. Water-and Ethanol-Soluble Carbohydrates of Temperate Grass Pastures: a Review of Factors Affecting Concentration and Composition. Journal of Equine Veterinary Science, 110, 103866.

Kagan I. A., Anderson M. L., Kramer K. J., Seman D. H., Lawrence L. M., Smith S. R. 2020. Seasonal and diurnal variation in water-soluble carbohydrate concentrations of repeatedly defoliated red and white clovers in central kentucky. Journal of Equine Veterinary Science, 84, 102858.

Kagan I. A., Lawrence L. M., Seman D. H., Prince K. J., Fowler A. L., Smith S. R. 2018. Effects of sampling time, cultivar, and methodology on water-and ethanol-soluble carbohydrate profiles of three cool-season grasses in Central Kentucky. Journal of equine veterinary science, 61, 99-107.

Kara K. 2020. Milk urea nitrogen and milk fatty acid compositions in dairy cows with subacute ruminal acidosis. Veterinární medicína, 65(9).

Kitkas G. C., Valergakis G. E., Kritsepi-Konstantinou M., Gelasakis A. I., Arsenos G., Kalaitzakis E., Panousis N. 2019. Effects of ruminal pH and subacute ruminal acidosis on milk yield and composition of Holstein cows in different stages of lactation. Journal of the Hellenic Veterinary Medical Society, 70(2), 1551-1560.

Klevenhusen F., Zebeli Q. 2021. A review on the potentials of using feeds rich in water-soluble carbohydrates to enhance rumen health and sustainability of dairy cattle production. Journal of the Science of Food and Agriculture, 101(14), 5737-5746.

Kooman H. M. A. 2018. Subacute ruminal acidosis and the relationship with the detection of ruminal acidosis in milk based on milk-fat content, other milk production parameters and nutritional aspects in dairy cows (Master's thesis).

Lafiandra D., Masci S., Sissons M., Dornez E., Delcour Jan A. M., Courtin C., Caboni M. F. 2012. Chapter 6: Kernel Components of Technological Value, In American Associate of Cereal Chemists International, Durum Wheat (Second Edition), AACC International Press. Pages 85-124.

Lee M. R., Jones E. L., Moorby J. M., Humphreys M. O., Theodorou M. K., & Scollan N. D. 2001. Production responses from lambs grazed on Lolium perenne selected for an elevated water-soluble carbohydrate concentration. Animal Research, 50(6), 441-449.

Lee M. R. F., Harris L. J., Moorby J. M., Humphreys M. O., Theodorou M. K., MacRae J. C., Scollan N. D. 2002. Rumen metabolism and nitrogen flow to the small intestine in steers offered Lolium perenne containing different levels of water-soluble carbohydrate. Animal Science, 74(3), 587-596.

Lee M. R., Merry R. J., Davies D. R., Moorby J. M., Humphreys M. O., Theodorou M. K., MacRae J. C., Scollan N. D. 2003. Effect of increasing availability of water-soluble carbohydrates on in vitro rumen fermentation. Animal Feed Science and Technology, 104(1-4), 59-70.

Li F., Yang X. J., Cao Y. C., Li S. X., Yao J. H., Li Z. J., & Sun F. F. 2014. Effects of dietary effective fibre to rumen degradable starch ratios on the risk of sub-acute ruminal acidosis and rumen content fatty acids composition in dairy goat. Animal Feed Science and Technology, 189, 54-62.

Livingston D. P., Hincha D. K., Heyer A. G. 2009. Fructan and its relationship to abiotic stress tolerance in plants. Cellular and Molecular Life Sciences, 66, 2007-2023.

Longland A. C., Byrd B. M. 2006. Pasture nonstructural carbohydrates and equine laminitis. The Journal of nutrition, 136(7), 2099S-2102S.

Maas J. A., Wilson G. F., McCutcheon S. N., Lynch G. A., Burnham D. L., France J. 2001. The effect of season and monensin sodium on the digestive characteristics of autumn and spring pasture fed to sheep. Journal of Animal Science, 79(4), 1052-1058.

McIntosh B. J. 2006. Circadian and seasonal variation in pasture nonstructural carbohydrates and the physiological response of grazing horses (Doctoral dissertation, Virginia Tech).

Mele M., Buccioni A., Petacchi F., Serra A., Banni S., Antongiovanni M., Secchiari P. 2006. Effect of forage/concentrate ratio and soybean oil supplementation on milk yield, and composition from Sarda ewes. Animal Research, 55(4), 273-285.

Merino V. M., Balocchi O. A., & Rivero M. J. (2019). Milk production, milk quality, and behaviour of dairy cows grazing on swards with low and high water-soluble carbohydrates content in autumn: A pilot trial. Animals, 9(12), 1012.

Milinovich G. J., Trott D. J., Burrell P. C., Van Eps A. W., Thoefner M., Blackall L. L., Al Jassim R. A. M., Morton J. M., Pollitt C. C. 2006. Changes in equine hindgut bacterial populations during oligofructose-induced laminitis. Environmental microbiology, 8(5), 885-898.

Miller L. A., Moorby J. M., Davies D. R., Humphreys M. O., Scollan N. D., MacRae J. C., Theodorou M. K. 2001a. Increased concentration of water-soluble carbohydrate in perennial ryegrass (Lolium perenne L.): milk production from late-lactation dairy cows. Grass and Forage Science, 56(4), 383-394.

Miller L. A., Scollan, N. D., MacRae, J. C., Humphreys, M. O., Baker, D. H., Theodorou, M. K., & Moorby, J. M. 2001b. Efficiency of nitrogen use in dairy cows grazing ryegrass with different water soluble carbohydrate concentrations.

Molle G., Decandia M., Cabiddu A., Landau S. Y., Cannas A. 2008. An update on the nutrition of dairy sheep grazing Mediterranean pastures. Small Ruminant Research, 77(2-3), 93-112.

Molle G., Gregorini P., Cabiddu A., Decandia M., Giovanetti V., Sitzia M., Dattena M., Porcu C., Pasciu V., Gonzalez-Bulnes A., Berlinguer F., Cannas A. 2022. Does the timing of pasture allocation affect rumen and plasma metabolites and ghrelin, insulin and cortisol profile in dairy ewes?. Frontiers in Veterinary Science, 9, 969950.

Moorby J. 2001. Grass sugars make milk production sweeter. IGER Innovations, 5, 35-39.

Moorby J. M., Evans R. T., Scollan N. D., MacRae J. C., Theodorou M. K. 2006. Increased concentration of water-soluble carbohydrate in perennial ryegrass (*Lolium perenne* L.). Evaluation in dairy cows in early lactation. Grass and Forage Science, 61(1), 52-59.

Müller C. E., Nostell K., Bröjer J. 2016. Methods for reduction of water soluble carbohydrate content in grass forages for horses. Livestock Science, 186, 46-52.

Murray J.A. 2021. Your horse's gut: gastrointestinal structure and function In: The Journal for Equine Nutrition. January 2021.

Niyigena V., Ashworth A. J., Nieman C., Acharya M., Coffey K. P., Philipp D., Meadors L., Sauer T. J. 2021. Factors affecting sugar accumulation and fluxes

in warm-and cool-season forages grown in a silvopastoral system. Agronomy, 11(2), 354.

Oba M. (2011). Effects of feeding sugars on productivity of lactating dairy cows. Canadian Journal of Animal Science, 91(1), 37-46.

O'Grady L., Doherty M. L., Mulligan F. J. 2008. Subacute ruminal acidosis (SARA) in grazing Irish dairy cows. The Veterinary Journal, 176(1), 44-49.

Olszewska M. 2021. Effects of cultivar, nitrogen rate and harvest time on the content of carbohydrates and protein in the biomass of perennial ryegrass. Agronomy, 11(3), 468.

Orr R. J., Penning P. D., Harvey A., Champion R. A. 1997. Diurnal patterns of intake rate by sheep grazing monocultures of ryegrass or white clover. Applied Animal Behaviour Science, 52(1-2), 65-77.

Parsons A. J., Rasmussen S., Xue H., Newman J. A., Anderson C. B., Cosgrove G. P. 2004. Some high sugar grasses don't like it hot. In Proceedings of the New Zealand Grassland Association (pp. 265-271).

Pollock C. J., & Cairns A. J. 1991. Fructan metabolism in grasses and cereals. Annual Review of Plant Physiology and Plant Molecular Biology, 1991, Vol. 42, 77-101 ref. 126.

Porcu M.A, Ledda A., Puddu S., Canu M.E., Piras A., Zontini A., Cannas A., Atzori A.S. 2024. Optimizing herbage utilization in dairy sheep with a polyphenol-rich supplement. 75th Annual Meeting European Federation of Animal Science (EAAP), Florence, Italy. 1-5 September 2024.

Pulina G., Atzori A. S., Dimauro C., Ibba I., Gaias G. F., Correddu F., Nudda A. 2021. The milk fingerprint of Sardinian dairy sheep: Quality and yield of milk used for

Pecorino Romano PDO cheese production on population-based 5-year survey. Italian Journal of Animal Science, 20(1), 171-180.

Rech Â. F., de Arruda Cordoba U., Fávaro V. R., Flaresso J. A. 2022. Principais características de cultivares de azevém-anual utilizados no estado de Santa Catarina, Brasil. Agropecuária Catarinense, 35(1), 85-92.

Rivero M. J., Anrique R. 2015. Milk fat depression syndrome and the particular case of grazing cows: A review. Acta Agriculturae Scandinavica, Section A—Animal Science, 65(1), 42-54.

Rivero M. J., Balocchi O. A., Moscoso C. J., Siebald J. A., Neumann F. L., Meyer D., Lee M. R. 2019. Does the "high sugar" trait of perennial ryegrass cultivars express under temperate climate conditions?. Grass and Forage Science, 74(3), 496-508.

Rearte D. H., Kesler, E. M., & Stringer, W. C. 1984. Forage growth and performance of grazing dairy cows fed concentrates with or without sodium bicarbonate. Journal of Dairy Science, 67(12), 2914-2921.

Rearte D. H. 2005. New insights into the nutritional value of grass. In Utilisation of grazed grass in temperate animal systems (pp. 49-59). Wageningen Academic.

Satta F., Porcu M.A., Ledda A., Carta S., Deiana L., Cannas A. 2023. The concentration of water-soluble carbohydrates in the pasture grazed by lactating ewes might be a main cause of milk fat depression. ASPA Conference, Monopoli (Bari, Italy), 13-16 June 2023.

Schmidt V. A. M., Watson, C. A., Walker, R. L., Argo, C. M., Russell, W. R., Neacsu, M., P.A. Harris, Morrison, P. K. 2023. 50 Water-soluble carbohydrate content

in equine pasture: Impact of fertilization, season, and grass species. Journal of Equine Veterinary Science, 124, 104352.

Siciliano P. D., Gill J. C., Bowman M. A. 2017. Effect of sward height on pasture nonstructural carbohydrate concentrations and blood glucose/insulin profiles in grazing horses. Journal of Equine Veterinary Science, 57, 29-34.

Smith S., Prince K. 2017. How to Maximize Energy Content in Forage Grasses. Forage Extension Specialist and Masters Student University of Kentucky Lexington, KY. https://uknowledge.uky.edu/cgi/viewcontent.cgi?article=1072&context=forage_kca

Stein O., Granot, D. 2019. An overview of sucrose synthases in plants. Frontiers in plant science, 10, 95.

Striegel N. J. 2008. Sugar content in feed and forage affects horses' health. Colorado State University Extension.

Sun X. Q., Wang Y. P., Wei R. Y., Chen B., Zhao X. 2020. Effects of replacing starch with three sugars in a concentrate and forage diet on in vitro rumen fermentation, fatty acid composition and related bacteria. Animal Production Science, 60(9), 1173-1182.

Sun X., Chen A., Li J. 2023. Chemical, Physical, and Degradation Characteristics of Ryegrass Cultivars Grown in Autumn and Winter for Dairy Cows. Animals, 13(20), 3158.

Superchi P., Sabbioni A., Beretti V., Vecchi I., Simonini F. V. 2010. Hydrolyzable and fermentable carbohydrates in North Italian pastures for horses. Italian Journal of Animal Science, 9(3), e66.

Tabacco E., Borreani G., Valente M. E., Peiretti P. G. 2004. Dry matter and water-soluble carbohydrate contents of Italian ryegrass at cutting as affected by environmental factors. Ital. J. Agron, 8(1), 63-74.

Taweel H. Z., Tas B. M., Smit H. J., Elgersma A., Dijkstra J., Tamminga S. 2005. Effects of feeding perennial ryegrass with an elevated concentration of water-soluble carbohydrates on intake, rumen function and performance of dairy cows. Animal Feed Science and Technology, 121(3-4), 243-256.

Thoefner M. B., Pollitt C. C., Van Eps A. W., Milinovich G. J., Trott D. J., Wattle O., & Andersen P. H. 2004. Acute bovine laminitis: a new induction model using alimentary oligofructose overload. Journal of dairy science, 87(9), 2932-2940.

Trevaskis L. M., Fulkerson W. J., Gooden J. M. 2001. Provision of certain carbohydrate-based supplements to pasture-fed sheep, as well as time of harvesting of the pasture, influences pH, ammonia concentration and microbial protein synthesis in the rumen. Australian Journal of Experimental Agriculture, 41(1), 21-27.

Turner L. R., Donaghy D. J., Pembleton K. G., Rawnsley R. P. 2014. Longer defoliation interval ensures expression of the 'high sugar'trait in perennial ryegrass cultivars in cool temperate Tasmania, Australia. The Journal of Agricultural Science, 153(6), 995-1005.

Ueda K., Mitani T., Kondo S. 2016. Effect of water-soluble carbohydrate content in orchardgrass pasture on grazing time and rumen fermentation in dairy cows. Animal Science Journal, 87(9), 1122-1129.

Undersander D. 2013. Grass varieties for horses. Journal of Equine Veterinary Science, 33(5), 315-320.

Van Eps A. W., Pollitt C. C. 2006. Equine laminitis induced with oligofructose. Equine veterinary journal, 38(3), 203-208.

Van Soest P. J. 1982. Nutritional Ecology of the Ruminant. OB Books, Corvallis, pp. 95-117.

Watts K. A. 2004. Forage and pasture management for laminitic horses. Clinical techniques in equine practice, 3(1), 88-95.

Watts K. A. 2005. A review of unlikely sources of excess carbohydrate in equine diets. Journal of Equine Veterinary Science, 25(8), 338-344.

Watts K. A., Chatterton N. J. 2004. A review of factors affecting carbohydrate levels in forage. J. Equine Vet. Sci, 24(2), 84-87.

Watts, K. 2008. The influence of solar radiation and temperature on the diurnal fluctuation of NSC in grass. Copyright 2008. Rocky Mountain Research & Consulting, Inc. https://static1.squarespace.com/static/5e7fddf934de306c2b8cc313/t/5e8c82935a255f19a8a2cb9f/1586266771795/DiurnalFluctNSC.pdf

Watts K. 2009. Grass in Shade is Lower in Sugar. Sarfergrass.org. Rocky mountain research and consulting, Inc.

Watts K. 2010. Pasture management to minimize the risk of equine laminitis. Veterinary Clinics: Equine Practice, 26(2), 361-369.

Weinmann H. 1961. Total available carbohydrates in grasses and legumes. Herbage Abstracts, v.31, p.255-261, 1961.

Wei ß K., Alt M., Sommer G., Kroschewski B., Richardt W., Wein R., & Kalzendorf C. 2018. Determination of water-soluble carbohydrates in forages-comparison of methods.

Westwood C. T., Bramley E., Lean I. J. 2003. Review of the relationship between nutrition and lameness in pasture-fed dairy cattle. New Zealand Veterinary Journal, 51(5), 208-218.

Westwood C. T., & Lean, I. J. 2001. Nutrition and lameness in pasture-fed dairy cattle. In Proceedings-new zealand society of animal production (Vol. 61, pp. 128-134). New Zealand Society of Animal Production; 1999.

CHAPTER 2

Annual ryegrass rich in water soluble carbohydrates can induce milk fat depression of lactating dairy ewes

Abstract

Modern annual ryegrass varieties tend to have high concentrations of water-soluble carbohydrates (WSC), especially in years in which winter and early spring are characterized by cold and sunny days. These climatic conditions, increasingly frequent in the Mediterranean regions where most dairy sheep are raised, have been also associated with widespread cases of milk fat depression in lactating ewes. Thus, an experiment was conducted to explore the causal relationships between the utilization of ryegrass rich in WSC and milk fat synthesis. A tetraploid annual ryegrass field (*Lolium multiflorum* Lam. ssp. westervoldicum) seeded in October 2022 was used to feed 10 Sarda ewes in the second month of lactation with ryegrass herbage cut daily during February and March 2023. The animals were fed indoors with automatic Biocontrol AS (Rakkestad, Norway) feeders, which continuously recorded individual intake of the ewes throughout the day. The ewes received freshly cut grass ad libitum from 1:00 PM to 7:00 AM of the following day. With the aim to manipulate the WSC content of the grass administered to the groups, after an initial adaptation period during which all ewes received the same grass and an ad libitum dry total mixed ration, 5 ewes continued to receive ryegrass herbage cultivated under normal conditions (SUN treatment), while the other 5 received shaded ryegrass herbage (SHADE treatment). The WSC concentrations were higher in the SUN grass compared to the SHADE grass and varied daily based on the hours of sunshine and cloudiness (mean 30.6% vs. 24.0% DM basis, for SUN and SHADE, respectively; P = 0.007). Both the grass intake (SUN 997 g DM/d, SHADE 953 g DM/d), particularly high in the first hours after it was supplied, and the daily milk yield (SUN 2426 g/d, SHADE 2360 g/d) were not affected by the treatments. The WSC intake was higher in the SUN treatment compared to

SHADE (SUN 300 g DM/d, SHADE 226 g DM/d; P < 0.001). The daily milk fat content was affected by the treatment, with SUN having lower values than SHADE (SUN 5.28%, SHADE 5.55%; P = 0.003), with marked difference in the afternoon milking (SUN 5.79%, SHADE 6.19%; P = 0.009) three hours after the grass supply, and no differences in the subsequent morning milking. The C18:1 trans-10 tended to be higher in SUN than in SHADE group (0.34 vs. 0.29 g/100 g of total fatty acids methyl esters (FAME), respectively; P = 0.06).

In conclusion, these results indicate that the high WSC of ryegrass might be one of the causative factors responsible for the milk fat depression often observed in dairy ewes grazing this species during winter and early spring in Sardinia.

Keywords

Water soluble carbohydrates, dairy sheep, annual ryegrass, herbage, milk fat depression

Introduction

The dairy sheep breeding system in Sardinia is characterized by the use of natural or artificial pastures with a seasonal production pattern (from autumn to spring) which coincides with the lactation period of the ewes (Molle et al., 2008). In fact, milk yield and the chemical composition of milk are strongly related to the chemical composition and biomass of pasture, which are directly influenced by the season (Pulina et al., 2021). In recent years, Sardinian sheep farms have increasingly adopted the use of modern varieties of tetraploid annual ryegrass for grazing. These grasses, in years with low temperatures and sunny days and especially when night frosts occur, tend to accumulate high levels of water-soluble carbohydrates (WSC) (Watts, 2005; Watts, 2008; Smith and Prince, 2017) during late winter and early spring in Mediterranean area (Satta et al., 2023). The WSC are a heterogeneous group of carbohydrates including monosaccharides (glucose, fructose), disaccharides (sucrose), oligosaccharides and fructans, which are the predominant WSC in grasses (Klevenhusen and Zebeli, 2021; Kagan, 2022). The WSC, and in particularly fructans, have been widely studied in equine nutrition due to their involvement in pasture-associated laminitis. In fact, excessive consumption of pastures high in WSC can lead to laminitis in horses (Schmidt et al., 2023), as fructans are incompletely degraded in the foregut and rapidly fermented in the hindgut (Undersander, 2013), leading to a drop in intestinal pH and intestinal disturbances, that can trigger laminitis (Geor, 2009). The fermentation rate of fructans is influenced by their molecular weight, degree of polymerisation (DP), and linear or branched structure, which varies between grass species; ryegrass, for example, contains fructans with a low DP (30 to 40) that favour rapid fermentation (Geor, 2010; Klevenhusen and Zebeli, 2021). Research on

the effects of WSC in ruminants has predominantly focused on their impact on palatability, intake, and production. However, studies on the impacts of WSC overload on animal health and milk composition are very few. These studies have reported that high WSC content in grasses can increase palatability and dry matter intake (Hight et al. 1968; Moorby, 2001; Ciavarella et al., 2000b; Lee et al., 2002), improve milk production (Miller et al., 2001) and increase liveweight gain (Hight et al. 1968), probably due to WSC being a rapidly available source of energy for microorganisms, and for the changes in volatile fatty acid (VFA) profile resulting from their degradation (Ciavarella et al., 2000a; Lee et al., 2001). In fact, increased WSC intake is linked to a higher glucogenic to lipogenic VFA ratio, with a decrease in the proportion of acetate and an increase in propionate (Lee et al., 2002; Lee et al., 2003), as well as elevated glucose and insulin levels (Molle et al., 2022). Moreover, WSC intake has been inversely correlated with ruminal pH (Lee et al., 2003; Molle et al., 2022), indicating a potential risk of SARA. A consequence of SARA is the reduction of milk fat precursors, which can lead to milk fat depression. In grazing cows, milk fat depression can occur during periods when pastures are in the vegetative stage and are nutrient-dense: high in water, sugar and polyunsaturated fatty acids (PUFAs) but low in fibre, such as in early spring (Rivero and Anrique, 2015). Indeed, results from a field study on pasture-fed sheep showed that milk fat content is inversely associated with the WSC content of the grass, but not with its neutral detergent fibre (NDF) content (Satta et al., 2023). Recently, a decrease in milk fat content, often accompanied by an inversion of the fat to protein ratio, has been observed in Sardinian sheep farms during late winter and early spring, leading to lower cheese yield. This could be due to the increase in milk yield of the ewes, due to genetic selection and improved nutrition, with associated

"dilution effect", but also by the diffusion of SARA. In grazing dairy sheep, low milk fat content could result from the use of modern tetraploid annual ryegrass varieties, along with climatic conditions that, promoting WSC accumulation in grasses, may be contribute to SARA and subsequent milk fat depression. The aim of this study was specifically to investigate if pasture WSC might affect milk fat synthesis and concentration.

Materials and methods

Experimental site and treatment

The experiment was conducted at the experimental farm of the Department of Agricultural Sciences of University of Sassari, located in the North-West of Sardinia (Ottava, Sassari, Italy; 40°48'41.4"N 8°17'50.7"E) from February to March 2023. On the 18 of October 2022, a 0.8 ha field was sown with tetraploid annual ryegrass (*Lolium multiflorum* Lam. ssp. westervoldicum) at a seeding rate of 30 kg/ha and fertilized with 130 kg/ha of nitrogen applied at sowing. Nitrogen was applied in the form of urea (46% N) through a fertilizer containing also diciandiammide (inhibitor of nitrification). From the 18 of February 2024, a random area of the field was covered, starting at 1 pm, for 48 hours by two mobile greenhouses, each measuring 4 m wide by 4.5 m long (covering an area of 36 m^2). From 19 February 2023, another area of the field was covered daily for 48 hours under two additional mobile greenhouses. This went on until the 15 March, to obtain grass with the same 48 h shade treatment for each experimental day, according to Ciavarella et al. (2000a) that created different shade treatments from 38.5 hours to 46.5 hours to manipulate WSC concentrations. The greenhouses were totally covered with a porous shading net, made of high-density

polyethylene stabilized for UV (Picture 1), intercepting 87% of solar radiation, measured using a SpectroSense 2+ 8-channel logging display meter (Skye Instruments Ltd, Llandrindod Wells, Powys, UK). The rest of the field was cultivated under normal conditions, receiving 100% of sunlight, to create two treatments: SUN and SHADE, aimed at manipulating the WSC content of the grass. The shaded and unshaded grass were harvested daily at 12:00 AM, to allow sufficient accumulation of WSC, with an Attila AT 900 MF (Brumar srl, Asti, Italy) mowing machine at cutting height of 8-12 cm from the ground and subsequently taken to the barn. Weather data (maximum and minimum temperature, rainfall and solar radiation) were recorded at the experimental farm meteorological station, located about 150 m from the field (Figure 1, Table 1).

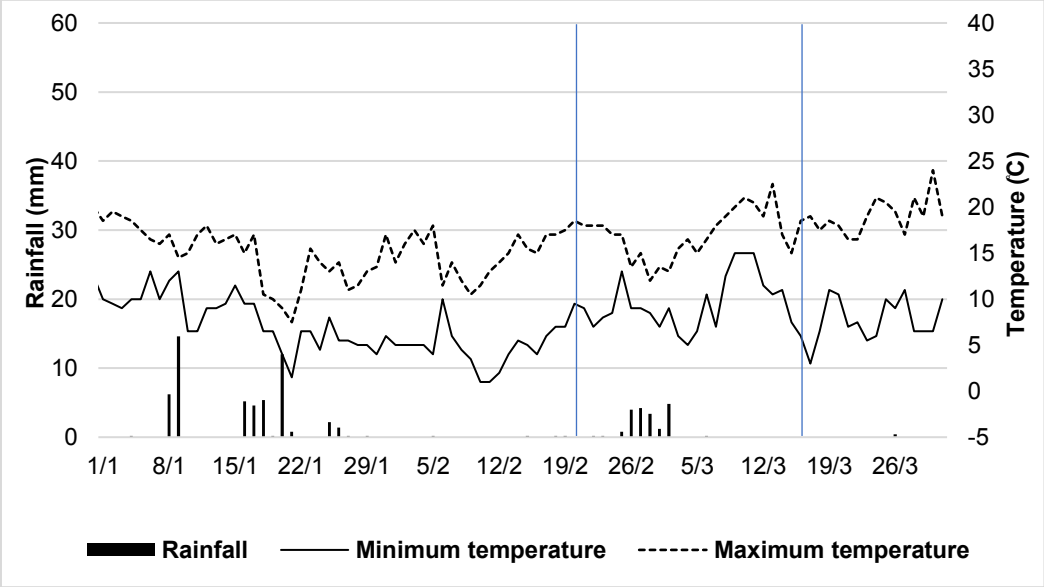

Figure 1. The minimum and maximum temperatures (◦C) and rainfall (mm) at the experimental farm during the period from January 2023 to March 2023 (20 February 2023 start of the trial and grass mowing; 16 March 2023 end of the trial).

Table 1. Monthly mean values for minimum and maximum temperatures, solar radiation and rainfall sum at experimental farm during the period from October 2022 (18 October sowing date) to March 2023 (20 February 2023 start of the trial and grass mowing).

	October	**November**	**December**	**January**	**February**	**March**
Mean minimum temperatures (°C)	15.5	12.3	10.4	7.8	6.1	8.8
Mean maximum temperatures (°C)	26	20.4	18.7	14.8	15.3	18.3
Mean solar radiation (MJ/m^2 per day)	14	8.7	7.9	7.9	11.3	16.2
Rainfall, sum (mm/month)	15.6	200.3	113.9	53.2	13.6	6.6

Picture 1. The greenhouses totally covered with a porous shading net made of high-density polyethylene stabilized for UV, used during the experimental period (from 20 February to 16 March) to obtain the 48 h SHADE treatment.

Animals and diets

Ten Sarda dairy ewes in the second month of lactation were selected from a group of ewes housed indoors and fed ad libitum a dry total mixed ration (TMR), composed of 53% of forage and 47% of concentrates. Based on pre-experimental measurements of milk yield (2856 ± 447 g/ewe per day), body weight (55.5 ± 6.28 kg), body condition score (2.93 ± 0.30 units), and milk fat content (5.34 ± 0.39%), the ewes were divided into two homogeneous groups of five animals per treatment. The ewes were assigned to two treatments in a completely randomized design: feeding shaded ryegrass herbage (SHADE) and unshaded ryegrass herbage (SUN). The experimental period was preceded by 10 days of adaptation, during which all ewes received increasing amounts of the same ryegrass herbage and dry TMR ad libitum. During the experimental phase, from the 20 February to the 16 March 2023 (25 experimental days), one group

continued to receive the freshly cut unshaded ryegrass (SUN), while the other group received freshly cut shaded ryegrass (SHADE). All ewes were housed and fed indoors and were machine-milked twice daily at 7:30 AM and 4:30 PM. Fresh grass was offered to the sheep ad libitum from 1:00 PM to 7:00 AM of the following day using ten automatic Biocontrol AS mangers (Rakkestad, Norway), which continuously recorded individual intake. During the experimental period, after eight days of transition in which part of the mangers were dedicated to the total mixed ration (Table 2), each ewe had one dedicated manger. In addition, the ewes received a supplementation of 400 g/d per head of soybean meal and 550 g/d per ewe of whole corn grains (as-fed basis). Starting from the 14th experimental day, 300 g/d per ewe of beet pulp was added to increase the diet fibre content, because liquid feces, probably caused by ruminal acidosis, were observed in some ewes. The soybean meal was fed individually, split into two equal meals supplied at milking, while the corn grains and beet pulp were also split into two equal meals and fed individually in a manger after each of the daily milkings and left available for 20 minutes. Orts of concentrates were quantified individually at each meal. The ewes had continuous access to fresh water. The composition of the experimental diet and the diet sequence per animal are reported in Table 2.

Table 2. Experimental diet sequence of both experimental groups during experimental period.

Experimental day	**7:30 AM**	**8:00 AM**	**1:00 PM**	**4:30 PM**	**5:00 PM**	**From 6:00 PM to 7:00 AM**
Day 0 to 8			Ad libitum dry total mixed ration			
Days 0 to 25			**Ryegrass herbage supply**			
Days 0 to 25	Milking	Corn grain		Milking	Corn grain	
Days 0 to 25	Soybean meal			Soybean meal		
Days 14 to 25		Beet pulp			Beet pulp	

Measurements, sampling and analyses

Representative samples of the grass offered to each group were collected daily within an hour after mowing time (12:00 AM). The samples were divided into two subsamples, one immediately frozen at -20 °C and the other dried immediately at 65 °C, then grinded to pass a 1 mm screen. The herbage samples were analyzed in correspondence of the milk sampling days, twice a week. The dry matter (DM) content was determined by oven-drying at temperature of 105 °C for 24 h. The neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL), crude protein (CP), soluble protein, ash, ether extract (EE) and acid detergent insoluble crude protein (ADICP) were determined by near-infrared reflectance spectroscopy (NIRS) in Cargill Laboratories (Fiorenzuola D'Arda, Italy). Non-fibre carbohydrates (NFC)

were calculated as: 100 − (NDF + CP - ADICP + ash + EE). The water-soluble carbohydrates (WSC) content of herbage samples was determined by the anthrone sulfate colorimetric method (Deriaz, 1961) using glucose as a standard. The absorbance value was colorimetrically determined at 625 nm. The individual intake and intake rate of grass were monitored and recorded continuously with automatic Biocontrol AS (Rakkestad Norway) feeders. The intake of supplements was recorded individually by weighing manually the offer and the refusals after each meal. Individual milk yield (g/ewe) was measured daily at each milking (morning milking at 7:30 AM and afternoon milking at 4:30 PM) by using appropriate vessels to collect the milk of each ewe and then weighting it. Individual milk samples were collected twice a week (8 sampling days in total, corresponding to experimental days 2, 4, 8, 11, 15, 18, 22 and 25), and each sample was divided into two subsamples. One was refrigerated at 4 °C and analyzed in the same day for the determination of fat, protein, lactose, urea using a Milkoscan 6000 instrument (Foss Electric, Hillerød, Denmark), and for somatic cell count (SCC) using a Fossomatic 360 instrument (Foss Electric); the second aliquot was frozen at -20°C for gas chromatography (GC) analysis. The determination of fatty acids (FA) first involved the extraction of milk fat, which was performed according to Nudda et al. (2005). The preparation of fatty acids methyl esters (FAME), including their separation and quantification, were performed according to Correddu et al. (2019), using a 7890A GC System (Agilent Technologies, Santa Clara, CA, USA) provided by a 7693 autosampler (Agilent Technologies), capillary column (CP-Sil 88; 100 m × 0.250 μm i.d., 0.25 μm film thickness, Agilent Technologies) and a flame ionization detector (FID). As reported by Correddu et al. (2019), the initial oven temperature was set at 45 °C, maintained for 4 min, and then

it increased until 175 °C at a rate of 13 °C/min. This temperature was held for 27 min, after which the temperature increased at 4 °C/min until reaching 215 °C, and it was held for 35 min. The gas used as carrier was helium at flow rate of 1 mL/min and a pressure of 28 psi. The sample injected was equal to 1 µl with split ratio 1:80. The temperatures of injector and detector were set to 250 °C. The identification of individual FA was performed using ChemStation Upgrade software (OpenLAB CDS GC; Agilent Technologies), comparing their retention times with those of standards methyl ester and previously published isomeric profile as detailed by Nudda et al. (2005). The individual FAME was expressed as g/100 g of total FAME. The FA groups were calculated as following described and expressed as g FA/100 g of milk: saturated fatty acids (SFA), as sum of the individual saturated FA; unsaturated fatty acid (UFA), as sum of the individual unsaturated FA; monounsaturated fatty acid (MUFA), as sum of the individual monounsaturated FA; polyunsaturated fatty acids (PUFA), as sum of the individual polyunsaturated FA; PUFA n-3 as sum of the individual n-3 FA; PUFA n-6 as sum of the individual n-6 FA; total conjugated linoleic acid (CLA) as sum of the individual CLA; trans fatty acids (TFA), as sum of the individual trans FA; branched-chain fatty acids (BCFA), as sum of the individual branched-chain FA); odd-chain fatty acids (OCFA), as sum of the individual odd-chain FA); OBCFA (sum of the individual odd- and branched-chain FA); short-chain fatty acids (SCFA; sum of the individual FA from C4:0 to C10:0); medium-chain fatty acids (MCFA; sum of the individual FA from C11:0 to C17:0). De novo and preformed FA were calculated as described by Woolpert et al. (2016) (de novo from C4:0 to C14:0, and preformed ≥C18:0) and expressed as g FA/100 g of milk. The FA profile of lyophilized herbage ryegrass samples was determined according to Kramer et al.

(1997), with modification described by Correddu et al. (2015). The GC analysis of herbage samples was performed with the same equipment and methods described for milk samples. ChemStation Upgrade software (OpenLAB CDS GC; Agilent Technologies) was used to calculate the retention time and area of every individual FAME, and then identified by comparing the retention times with those of known standards and with the published studies as described by Nudda et al. (2008). The BW was measured weekly with a precision scale Biocontrol AS (Rakkestad Norway), and body condition score (BCS) was evaluated using a scale from 0 to 5 by the same three trained persons.

Statistical analysis

Statistical analyses were performed using the statistical software Minitab 21.4.1 (© 2023 Minitab, LLC). Data on chemical composition of herbage were analyzed with a General Linear Model (GLM), with an experimental design based on ANOVA with the fixed effect of treatment (2 levels) and the error term. Data on intake and milk fatty acid profile were analyzed with a General Linear Model (GLM), with an experimental design based on ANOVA which included the fixed effects of treatment and day, their interaction and the random effect of animal. Data are expressed as means and the group mean differences were assessed using the Tukey Pairwise Comparisons Test, a P-value < 0.05 considered significant, and a trend was considered for $0.05 <$ P-value ≤ 0.10. The statistical mixed model used was as follows:

$$\boldsymbol{Y_{ijk} = \mu + treat_i + day_j + treat_i \, x \, day_j + anim_k + Error_{ijk}}$$

where: μ is the mean, $treat_i$ is the treatment effect (i = SUN; SHADE), day_j is the experimental day of data collection (j = 2; 4; 8; 11; 15; 18; 22; 25), $treat_i$ x day_j is the interaction, $anim_k$ is the random effect of animal and $Error_{ijk}$ is the experimental error.

Additionally, data on milk yield, milk composition, BW and BCS were covariate-adjusted using pre-experimental measurements.

The statistical mixed model used was as follows:

$$Y_{ijk} = \mu + bx_{ijk} + treat_i + day_j + treat_i \, x \, day_j + anim_k + Error_{ijk}$$

where: μ is the mean, b is the regression slope, x_{ijk} is the covariate (i.e. pre-experimental data), $treat_i$ is the treatment effect (i = SUN; SHADE), day_j is the experimental day of data collection (j = 2; 4; 8; 11; 15; 18; 22; 25), $treat_i$ x day_j is the interaction, $anim_k$ is the random effect of animal and $Error_{ijk}$ is the experimental error. The relationship between WSC intake and fat content of milk was analyzed using linear regression analysis.

Results

Water soluble carbohydrates and other chemical components of grass

The chemical composition of ryegrass herbage is reported in Table 3. The dry matter content significantly differed between the treatments and was higher in SUN than in SHADE (17.5 and 14.4%, respectively; P = 0.003). The WSC concentration was strongly affected by the treatment and was significantly higher in SUN than in the SHADE treatment (30.6 and 24.0% DM, respectively; P =0.007). The WSC content varied daily, influenced by the hours of sunshine and cloudiness. Figure 2 shows that

shaded grass showed a lower WSC content compared to unshaded grass, but this difference was absent or very small between treatments on cloudy days, when solar radiation was low (e.g., experimental day 8, Figure 2, Figure 3). Additionally, the NFC content was higher in SUN than in SHADE group (38.7 and 31.2% DM, respectively; P = 0.009). Conversely, the CP content was higher in shaded grass compared to unshaded grass (10.6 vs. 8.9% DM, respectively; P = 0.02), but the values were very low in both treatments, considering the plants were in the vegetative stage. Instead, the soluble CP (sum of A and B1 protein fractions) and ADICP did not differ between treatments. NDF and ADF were not affected by the treatments. Specifically, NDF did not vary during the trial (Figure 2), while ADL was higher in SUN than in SHADE (4.4 vs. 3.3%, respectively; P = 0.001). The fatty acids profile of ryegrass herbage was not affected by the treatment (Table 3).

Table 3. Chemical composition of 28 fresh ryegrass herbage samples from SUN and SHADE treatments from 14 experimental days, and fatty acid profile (total fatty acid methyl esters, FAME; g/100 g of total FAME) of 10 fresh ryegrass herbage samples from SUN and SHADE treatments. All variables were analyzed by using NIRs method, except for WSC which was analyzed using a chemical method (anthrone method), and fatty acids, which were analyzed by gas-chromatography analysis.

Variable	Treatment		SEM	P-value
	SUN	SHADE		
DM (% of fresh matter)	17.6	14.4	0.68	***0.003***
CP (% DM)	8.9	10.6	0.48	***0.02***
Soluble CP (% DM)	4.2	4.7	0.21	***0.09***
ADICP (% DM)	0.5	0.5	0.03	***0.63***
EE (% DM)	2.5	2.7	0.13	***0.2***
Ash (% DM)	10.4	10.7	0.38	***0.66***
NDF (% DM)	40.4	42.5	1.32	***0.28***
ADF (% DM)	22.5	23.3	0.68	***0.43***
ADL (% DM)	4.4	3.3	0.22	***0.001***
NFC (% DM)	38.7	31.2	1.9	***0.009***
WSC (% DM)	30.6	24	1.59	***0.007***
Fatty acid (g/100 g of total FAME)				
C12:0	0.72	0.66	0.07	***0.63***
C14:0	0.70	0.65	0.07	***0.62***
C15:0	0.12	0.11	0.01	***0.51***
C16:0	17.5	17.9	0.62	***0.68***
C16:1 cis-7	1.44	1.35	0.04	***0.20***
C16:1 cis-9	0.25	0.27	0.04	***0.69***
C17:0	0.25	0.24	0.02	***0.81***
C17:1 cis-10	0.11	0.12	0.01	***0.54***
C18:0	1.50	1.55	0.08	***0.7***
C18:1 cis-9	1.93	2.06	0.19	***0.64***

Table 3. Continuation.

Variable	Treatment		SEM	P-value
	SUN	SHADE		
C18:2 n-6	12.0	12.2	0.32	***0.72***
C20:0	0.34	0.42	0.06	***0.39***
C18:3 n-3	62.2	61.6	1.25	***0.67***
C20:3 n-3 (10, 14, 17)	0.07	0.06	0.01	***0.46***
C24:0	0.80	0.86	0.07	***0.55***

DM: dry matter; CP: crude protein; ADICP: acid detergent insoluble crude protein; EE: ether extract; NDF: neutral detergent fibre; ADF: acid detergent fibre; ADL: acid detergent lignin; NFC: non-fibre carbohydrates; WSC: water-soluble carbohydrates; FAME: fatty acids methyl esters.

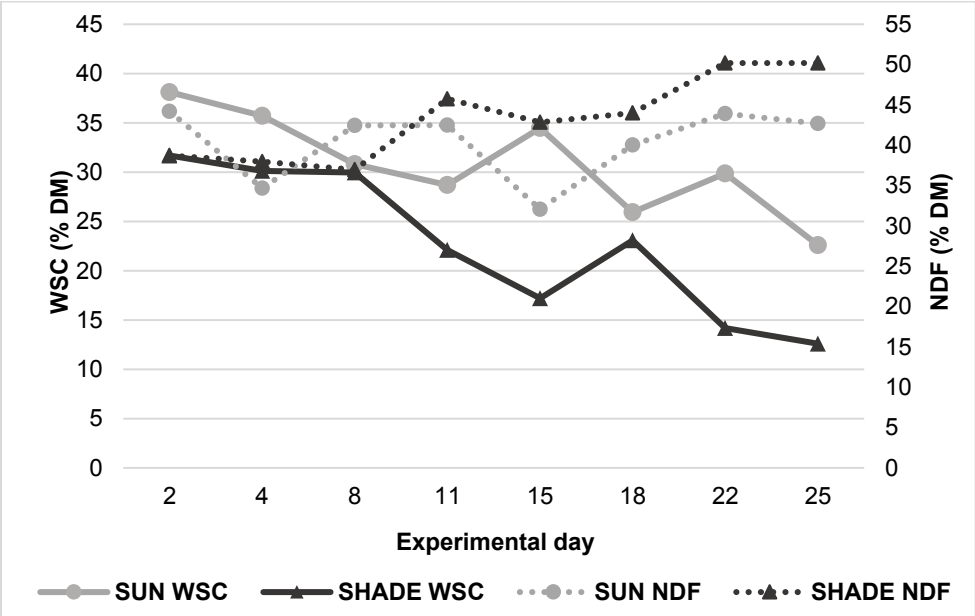

Figure 2. Variation in WSC content (% DM) and NDF content (% DM) of ryegrass from SUN and SHADE treatments in the 8 sampling days of the experiment, which lasted 25 days (20th of February 2023 to 16th of March 2023), during which milk samplings were also carried out.

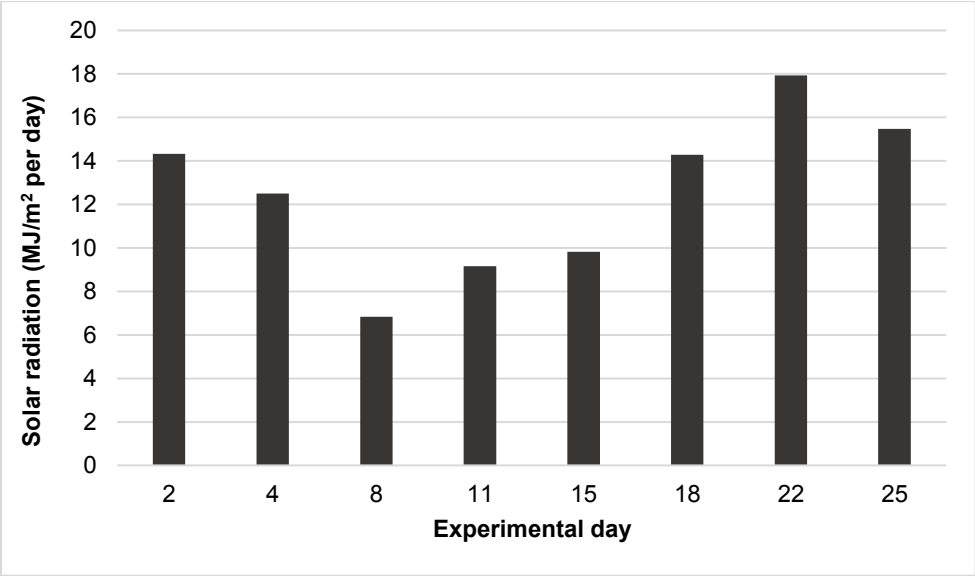

Figure 3. Solar radiation (MJ/m^2 per day) in the 8 sampling days of the experiment, which lasted 25 days (20th of February 2023 to 16th of March 2023), during which milk samplings were also carried out.

Intake

Table 4 reports the mean data of intake and dietary composition of ingested ration.

The total DMI was not affected by the treatment, with values of 2015 g/d for SUN and 2055 g/d for SHADE. The daily intake of concentrates did not differ between the groups. Particularly, the daily intake of soybean meal and corn grain did not differ between the two groups. In contrast, the daily intake of dry TMR, supplied only during the first 8 experimental days (sampling days 1, 2, and 3), was significantly higher in the SHADE group than in the SUN groups (489 and 300, respectively; P = 0.045). Additionally, the daily intake of beet pulps, supplied from 14th experimental day (sampling days 5, 6, 7, 8), was significantly higher in SHADE than in SUN (212 and 156, respectively; P = 0.002). The daily grass DMI did not differ between the two

groups, with values of 997 and 953 g/d for SUN and SHADE treatments, respectively. However, grass intake in the afternoon, before the afternoon milking (from 1:00 PM to 4:00 PM), was higher in the SHADE group compared to the SUN group (483 and 422 g/DM per day, respectively; $P = 0.004$). In both groups, the grass intake during the day was not homogeneous. In the first hour after grass supply, grass intake was 16% of daily grass intake for SUN and 19% for SHADE (Figure 4). Grass intake before the afternoon milking (from 1:00 PM to 4:00 PM) accounted for 44% of daily grass intake in the SUN and 51% in the SHADE (Figure 4). The daily intake of WSC was affected by the treatment and was significantly higher in the SUN than in SHADE (300 and 226 g/d, respectively; $P < 0.001$). The NDF intake, on the contrary, was higher in the SHADE treatment than in SUN treatment (706 and 634 g/d, respectively; $P = 0.007$). Additionally, the CP intake was also higher in the SHADE treatment compared to the SUN treatment (384 and 352 g/d for SHADE and SUN, respectively; $P < 0.001$). As regards the composition of the diet ingested by the two groups, the dietary WSC content was significantly higher for SUN group compared to the SHADE group (15 and 10.6%, DM basis, respectively; $P < 0.001$). Conversely, the dietary NDF content in the ration ingested by the SHADE group was higher compared to the SUN group (33.5 and 31.3% DM, respectively; $P < 0.001$), as well as the dietary CP content (18.4 and 17.6% of DM for SHADE and SUN respectively; $P = 0.003$).

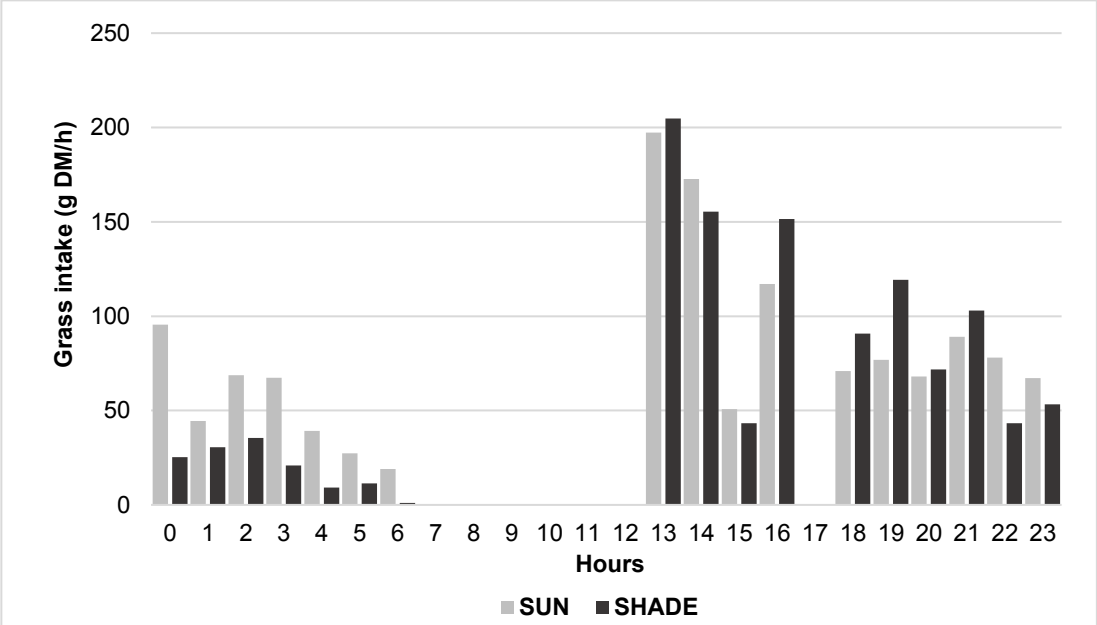

Figure 4. Average hourly grass intake in 5 of the 8 sampling days (25 total experimental days) for the two groups: SUN and SHADE.

Table 4. Total intake, grass intake and dietary intake and composition of the ration ingested during the experimental period by the SUN and SHADE groups.

Variable	Treatment	Experimental day								*Mean*	*SEM*	P-value
		2	**4**	**8**	**11**	***15***	***18***	***22***	***25***			**T**
Total DMI (g DM/d) [1]	**SUN**	2138	1672	2117	1832	1915	2077	2306	2064	**2015**	43	***0.51***
	SHADE	2322	1995	2453	2055	1610	2006	2036	1966	**2055**		
Daily concentrate intake	**SUN**	823	841	836	841	956	871	1035	1042	**906**	10.5	***0.37***
(g DM/d)	**SHADE**	828	841	837	811	869	994	1079	1093	**919**		
Daily dry TMR intake	**SUN**	480	95	324	-	-	-	-	-	**300**	63.4	***0.04***
(g DM/d)[2]	**SHADE**	696	204	568	-	-	-	-	-	**489**		
Daily beet pulp intake	**SUN**	-	-	-	-	159	59	198	211	**156**	11.7	***0.002***
(g DM/d) [3]	**SHADE**	-	-	-	-	149	149	243	259	**212**		
Daily soybean meal intake	**SUN**	339	358	355	358	357	347	358	347	**352**	3.14	***0.29***
(g DM/d)	**SHADE**	344	358	354	352	334	334	354	350	**347**		
Daily corn grain intake	**SUN**	483	483	481	483	441	465	480	483	**475**	5.51	***0.21***
(g DM/d)	**SHADE**	483	483	483	459	386	461	482	483	**465**		
Daily grass intake (g DM/d)	**SUN**	836	736	957	991	959	1206	1271	1023	**997**	29.7	***0.29***
	SHADE	798	950	1048	1245	741	1012	957	873	**953**		
Grass intake, afternoon[4]	**SUN**	471	322	499	391	284	377	571	460	**422**	14.4	***0.004***
(g DM/d)	**SHADE**	538	504	625	470	343	400	483	499	**483**		

Table 4. Continuation.

Variable	Treatment	Experimental day								*Mean*	*SEM*	P-value T
		2	**4**	**8**	**11**	***15***	***18***	***22***	***25***			
WSC intake (g DM/d)	**SUN**	304	263	290	274	307	338	396	231	**300**	8.31	***< 0.001***
	SHADE	263	286	271	338	169	225	146	110	**226**		
NDF intake (g DM/d)	**SUN**	791	448	711	503	527	612	801	677	**634**	18.5	***0.007***
	SHADE	905	604	823	676	536	686	719	700	**706**		
CP intake (g DM/d)	**SUN**	378	317	379	324	331	349	370	365	**352**	5.96	***< 0.001***
	SHADE	451	360	428	382	332	349	384	391	**384**		
Dietary WSC, % DM	**SUN**	15.5	15.1	15.0	14.3	18.0	14.3	16.3	11.2	**15.0**	0.41	***< 0.001***
	SHADE	13.3	13.2	13.5	12.6	8.6	11.9	6.4	5.6	**10.7**		
Dietary NDF, % DM	**SUN**	34.8	26.8	33.3	30.0	27.1	30.8	34.5	32.8	**31.3**	0.38	***< 0.001***
	SHADE	34.3	30.3	32.6	33.6	32.3	33.8	35.8	35.6	**33.6**		
Dietary CP, % DM	**SUN**	16.7	19.0	17.7	19.4	17.0	17.5	16.0	17.7	**17.6**	0.18	***0.002***
	SHADE	17.1	18.0	16.9	19.0	20.0	17.2	19.1	19.9	**18.4**		

[1] The ewes received a supplementation of 358 g/d per head of soybean meal and 483 g/d per head of whole corn grains, and from the 14th experimental day was added 265 g/d per head of beet pulps (on DM basis).

[2] TMR ad libitum only during the first 8 experimental days (1, 2 and 3 sampling days).

[3] From 14th experimental day (5, 6, 7 and 8 sampling days).

[4] Afternoon: from 1:00 PM to 4:00 PM (before the 4:30 PM afternoon milking).

Body weight and milk production and composition

The covariate-adjusted means data on BW, BCS, milk production and composition are reported in Table 5. The body weight (BW) was significantly affected by the treatment, with means of 55.8 kg for SUN and 54.5 kg for SHADE (P = 0.004). However, no difference was found in the BCS, with values of 2.88 for SUN and 2.91 for SHADE group respectively. The daily milk yield was not affected by the treatment, with means of 2426 and 2360 g/d for SUN and SHADE groups, respectively. Additionally, there was no difference between morning and evening milk production. The daily milk fat content was significantly higher in SHADE compared to SUN group (5.55% vs. 5.28%, respectively; P = 0.003). Furthermore, the milk fat content of afternoon milking was strongly affected by the treatment and was significantly higher in SHADE than in SUN (6.19% vs. 5.79%, respectively; P = 0.009), despite there was no difference between the groups in milk production of afternoon milking. No difference was found between SUN and SHADE for milk fat concentration of morning milking (5.09% and 5.07% for SUN and SHADE, respectively). The milk fat yield was not affected by the treatment in each milking. A negative correlation between WSC intake (g DM/d) and fat concentration in the milk of afternoon milking was observed (Figure 5). The daily milk protein concentration and yield and those of each milking were not affected by the treatment and were low in both groups. The milk fat to protein ratio in the afternoon milking was significantly higher in the SHADE group compared to the SUN group (1.37 and 1.28, respectively; P = 0.009) but did not differ in the morning milking (1.13 and 1.14 for SUN and SHADE, respectively). In Table 6 is reported the milk fatty acid profile. The complete milk fatty acid profile is reported in the Supplementary Table

S1. The C4:0 tended to be higher in SHADE than in SUN group (2.56 vs. 2.46 g/100 g of total FAME, respectively; P = 0.072). The C16:0 was significantly higher in SHADE group than in SUN (25.4 vs. 23.9 g/100 g of total FAME, respectively; P = 0.02). The C18:1 trans-10 tended to be higher in SUN than in SHADE group (0.34 vs. 0.29 g/100 g of total FAME, respectively; P = 0.062). Also, C18:1 cis-11 was in tendency higher in SUN than in SHADE group (0.40 vs. 0.37 g/100 g of total FAME, respectively; P = 0.082). In Table 7 are reported the milk fatty acids groups expressed as g FA/100 g of milk. SCFA did not differ between groups, while MCFA tended to be higher in SHADE than in SUN (1.86 and 1.65, respectively; P = 0.08). De novo and preformed fatty acids, as well as BCFA, OCFA, and OBCFA were not affected by the treatment.

Table 5. Covariate-adjusted means of dairy ewe's performances and milk composition in SUN and SHADE groups.

Variable	Treatment		SEM	*P-value*
	SUN	SHADE		
BW (kg)	55.8	54.5	0.28	***0.004***
BCS	2.88	2.91	0.04	***0.71***
Daily milk yield (g/d)	2426	2360	32.9	***0.16***
Milk yield, afternoon (g/milking)	869	853	18.9	***0.54***
Milk yield, morning (g/milking)	1557	1507	21.9	***0.11***
Daily milk fat content (%)	5.28	5.55	0.06	***0.003***
Milk fat content, afternoon (%)	5.79	6.19	0.39	***0.009***
Milk fat content, morning (%)	5.09	5.07	0.06	***0.79***
Daily fat yield (g/d)	128.7	129.0	2.21	***0.92***
Fat yield, afternoon (g/milking)	50.6	52.4	1.37	***0.37***
Fat yield, morning (g/milking)	79	75.8	1.32	***0.09***
Daily milk protein content (%)	4.53	4.49	0.04	***0.48***
Milk protein content, afternoon (%)	4.52	4.55	0.03	***0.59***
Milk protein content, morning (%)	4.52	4.47	0.04	***0.41***
Daily protein yield (g/d)	109.1	106.6	1.82	***0.35***
Milk protein yield, afternoon (g/milking)	39.2	38.8	0.94	***0.79***
Milk protein yield, morning (g/milking)	69.4	68.3	1.22	***0.55***
Daily milk fat to protein ratio	1.19	1.22	0.02	***0.12***
Milk fat to protein ratio, afternoon	1.28	1.37	0.02	***0.009***
Milk fat to protein ratio, morning	1.13	1.14	0.02	***0.55***

BW: Body weight; BCS: Body condition score; Afternoon: 4:30 pm milking milk; Morning: 7:30 am milking milk.

Table 6. Fatty acids profile (total fatty acid methyl esters, FAME; g/100 g of total FAME) of milk samples (of each morning and afternoon milking) from two sampling days in the SUN and SHADE groups.

Variable	Treatment		SEM	P value
	SUN	SHADE		
C4:0	2.46	2.56	0.04	***0.07***
C6:0	2.13	2.20	0.03	***0.14***
C8:0	2.18	2.21	0.06	***0.70***
C10:0	7.35	7.15	0.27	***0.59***
C12:0	4.37	4.21	0.15	***0.45***
C14:0	10.9	11	0.22	***0.96***
C15:0	1.11	1.11	0.03	***0.99***
C16:0	23.9	25.4	0.45	***0.02***
C16:1 cis-9	0.66	0.61	0.02	***0.07***
C17:0	0.90	0.88	0.02	***0.56***
C18:0 (SA)	11.6	10.8	0.23	***0.02***
C18:1 trans 6-8	0.22	0.21	0.007	***0.18***
C18:1t9	0.20	0.20	0.20	***0.67***
C18:1 trans 10	0.34	0.29	0.02	***0.06***
C18:1 trans 11 (VA)	1.28	1.19	0.06	***0.33***
C18:1 trans 12	0.30	0.31	0.01	***0.48***
C18:1 cis-9	18.9	18.8	0.72	***0.96***
C18:1 cis-11	0.40	0.37	0.01	***0.08***
C18:1 cis-12	0.18	0.18	0.006	***0.34***
C18:2 n-6 (LA)	2.01	1.93	0.05	***0.29***
C18:3 n-3 (LNA)	0.63	0.63	0.01	***0.90***
CLA cis-9, trans-11 (RA)	0.55	0.55	0.03	***0.98***
CLA C18:2 trans 10, cis-12	0.01	0.01	0.001	***0.57***
C22:5n-3 (EPA)	0.05	0.05	0.002	***0.19***
C22:5n-3 (DPA)	0.09	0.09	0.004	***0.49***
C22:6n-3 (DHA)	0.02	0.02	0.002	***0.34***

SA = stearic acid; VA = vaccenic acid; LA = linoleic acid; LNA = linolenic acid; RA = rumenic acid; EPA = eicosapentaenoic acid; DPA = docosapentaenoic acid; DHA = docosahexaenoic acid.

Table 7. Fatty acids groups (g fatty acid/100 g of milk) of individual milk samples (taken each morning and afternoon milking) from two sampling days (experimental days: 11 and 22) in the SUN and SHADE groups.

Variable	Treatment		SEM	P value
	SUN	SHADE		
SCFA	0.72	0.77	0.03	0.28
MCFA	1.65	1.86	0.08	0.08
De novo	1.29	1.39	0.06	0.24
Preformed (LCFA)	1.48	1.61	0.09	0.29
De novo/preformed	0.88	0.89	0.03	0.84
BCFA	0.08	0.09	0.004	0.34
OCFA	0.10	0.11	0.004	0.22
OBCFA	0.18	0.19	0.009	0.26
TFA	0.19	0.20	0.01	0.56
SFA	2.66	2.93	0.12	0.14
UFA	1.11	1.22	0.06	0.23
MUFA	0.93	1.03	0.06	0.24
PUFA	0.18	0.20	0.01	0.19
PUFA n-3	0.03	0.03	0.001	0.17
PUFA n-6	0.09	0.10	0.005	0.30
n-6/n-3	3.43	3.39	0.08	0.74
Total CLA	0.03	0.03	0.001	0.33

SCFA (short-chain fatty acids; from C4:0 to C10:0); MCFA (medium-chain fatty acids; from C11:0 to C17:0); De novo: from C4:0 to C14:0; preformed ≥C18:0; BCFA (sum of individual branched-chain FA); OCFA (sum of individual odd-chain FA); OBCFA (sum of individual odd- and branched-chain FA); TFA: sum of individual trans FA; SFA: sum of the individual saturated FA; UFA: sum of the individual unsaturated FA; MUFA: sum of the individual monounsaturated FA; PUFA: sum of the individual polyunsaturated FA; PUFA n-3: sum of individual n-3 FA; PUFA n-6: sum of individual n-6 FA; total CLA: sum of individual CLA.

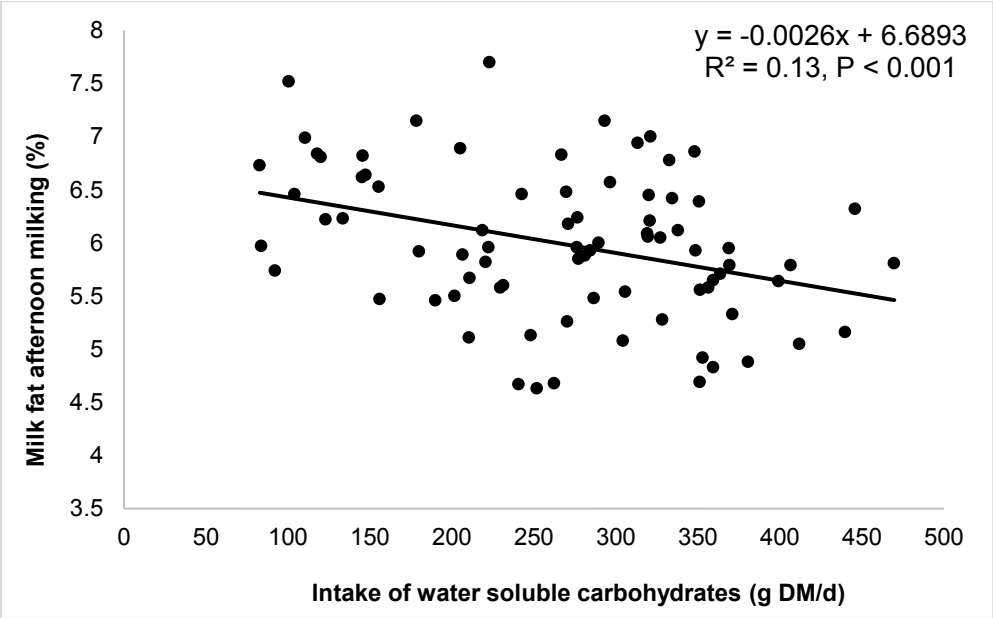

Figure 5. Relationship between the milk fat concentration of afternoon milking (%) and intake of water soluble carbohydrates (WSC; g DM/d) in dairy ewes from the SUN and SHADE groups.

Discussion

Effect of shade on water soluble carbohydrates and other chemical components of grass

The DM content of herbage was higher in SUN treatment (Table 3), consistent with the result obtained by Ciavarella et al. (2000a), which explains this difference considering that under shadow might occur stomatal closure and the lack of evaporation and transpiration, after sunrise, of droplets resulting from guttation and condensation of water under shading, resulting in higher moisture content. Additionally, the study by Lee et al. (2002) showed higher DM values in grass cut at

14:00 compared to that cut at 10:00 AM, confirming the effect of the longer time available for evaporation and transpiration, as well as the physiological daily fluctuations in DM content. The CP content was significantly higher in SHADE compared to SUN, in accordance with study by Abraham et al. (2014). Similarly, Mayland and Grunes (1974) and Ciavarella et al. (2000a) found that the nitrogen concentration in shaded pasture was higher compared to unshaded pasture. In this study, the NDF content did not vary between the two treatments, consistent with the findings of Ciavarella et al. (2000b), although results are considerably variable across different studies. For instance, Allsop et al. (2009) reported higher NDF and ADF content in the SHADE treatment and lower WSC content, indicating a shift from non-structural to structural carbohydrates. However, in our study, lignin (ADL) was higher in the SUN treatment, consistent with the studies by Hight et al., (1968) and Abraham et al. (2014), who found that shade reduced ADL content. Indeed, low light levels reduce lignification, causing shaded plants to be less lignified (Moore, 2001). Shading resulted in a decrease in the amount of WSC, which was the component most affected by this treatment, confirming the results of Ciavarella et al. (2000a), Ciavarella et al. (2000b), and Allsop et al. (2009), as well as in low NSC levels, as reported by Watts (2004) and Longland and Byrd (2006). In the SUN treatment, WSC levels reached values higher than those mentioned in most studies of the literature. These levels were within the range of 20-40% DM reported for high-sugar grasses by Lee et al. (2003), as well as with values reaching 30% DM reported by Van Soest (1982) for perennial ryegrass, and by Berry and Hoveland (1969); Walsh and Birrell (1987); Chatterton et al. (1989) cited by Ciavarella et al. (2000a) for Phalaris pasture. Specifically, in this study the average WSC content in SUN was 30.6% DM, with a maximum value that

reached 38.1% DM, similarly to what observed in the study by Miller et al. (2001) conducted in UK, which compared a variety of "high-sugar" (HS) perennial ryegrass with a control variety. In this study the WSC concentrations of HS grass peaked at over 350 g kg^{-1} DM, while in the control herbage, the WSC concentrations reached a peak at about 240 g kg^{-1} DM. In our study, despite the significant difference between treatments, with higher levels for SUN, the SHADE treatment also had a high average WSC content: 24% DM (Table 3). The WSC levels observed in our study in a Mediterranean environment are comparable to those observed in a study conducted in five northern European countries, where WSC concentrations ranged from below 100 g/kg DM to over 385 g/kg DM, with the highest concentrations recorded at cooler temperatures (personal communication of Longland et al. to McIntosh, 2006). In our study, we chose to use a tetraploid annual Italian ryegrass (*Lolium multiflorum* Lam. ssp. westervoldicum) for its potential to accumulate high levels of WSC, as we observed in the pasture of these species on Sardinian sheep farms during winter and spring. Sun et al. (2023), in a comparison of nine ryegrass cultivars, showed that Italian ryegrass (*Lolium multiflorum*) had the highest levels of soluble nutrients, soluble sugars, and starch. Ploidy also influences the WSC content of the plant, with tetraploids having higher WSC levels compared to diploids (Olszewska et al., 2021; Kagan, 2022; Rech et al., 2022). An additional explanation for the WSC concentrations observed in this study is that the crop was not cut or grazed, from the time of sowing in October 2022 until the start of experimental period. Defoliation, either through mowing or grazing, leads to a decrease in WSC content, likely due to the use of WSC for regrowth (Siciliano et al., 2017; Kagan, 2022), while the mature stems of intact plants can contain more fructans compared to grasses that are regularly

grazed or cut (Harris et al., 2006). In fact, the frequency of defoliation is one of the ways to modify crop composition, especially WSC and CP contents (Merino et al., 2019). Additionally, the transition from vegetative to reproductive development tends to result in higher WSC content (Harris et al., 2006). In our study, the crop had extensive time to accumulate WSC, remaining in the field intact (no mowing) until the start of the trial (February 20, 2023), and was at a vegetative stage. These factors likely also contributed to the higher lignin content observed in SUN. A negative correlation between WSC and CP content was observed (r^2= 60%, $P < 0.001$), as demonstrated in numerous other studies on grasses (Taweel et al., 2005; Satta et al., 2023). In this study the CP content was very low in both treatments, although significantly higher in the SHADE group, confirming the inverse relationship with WSC. The NFC content was also higher in SUN, with an average value of 39.2% DM compared to 31.7% DM in SHADE, likely due to the higher WSC concentration in SUN. The variations in WSC content between treatments and across different experimental days were substantial (Figure 2), in contrast to the relatively stable NDF content.

Influence of different WSC contents on dairy ewes' intake, performances, and milk composition

The total DMI and grass intake (g DM/d) were not influenced by the treatment, in contrast with numerous studies that reported increased palatability and DMI of forages with a high content of WSC (Hight et al. 1968; Moorby, 2001; Lee et al., 2002). In this study, the intake between treatments probably was not affected because the level of WSC was high in both treatments (WSC mean 30.6 vs. 24% DM for SUN and

SHADE, respectively). However, WSC intake was higher for SUN than SHADE ($P < 0.001$), due to the significant difference in WSC content between the two treatments. In contrast, total NDF concentration ($P < 0.001$) and intake of the ration ($P = 0.007$) was significantly higher in SHADE than in SUN, despite the NDF content did not differ between the two herbages. This probably is due to the higher intake of dry TMR ($P = 0.045$), supplied only during the first 8 experimental days (1, 2 and 3 sampling days), and beet pulps ($P = 0.002$), supplied starting on the 14th experimental day (5, 6, 7 and 8 sampling days), in SHADE compared to SUN. The higher WSC intake in SUN group may have an indirect effect on fibre intake of the supplements, due to a lower ruminal pH, which decreases fibre fermentation and, as a consequence, decreases the passage rate from the rumen. The higher CP content of shaded grass resulted in a significantly higher CP intake for SHADE groups. The significant difference in BW, which was higher for the SUN group, could be caused by a different acetate to propionate ratio at the ruminal level and higher blood glucose and insulin, resulting from the higher WSC ingestion in the SUN group, as observed in the studies by Lee et al. (2002) and Molle et al. (2022). In fact, Molle et al. (2022) showed that sheep grazing in the afternoon (higher WSC concentration) compared to those grazing in the morning (lower WSC concentration) had higher post-grazing propionic and butyric acid in the rumen and higher basal levels of blood glucose and insulin. This metabolic-hormonal pattern could be compatible with deposition of nutrients and consequent increased BW. Furthermore, this explanation could help to account for the reduction in milk fat observed in the SUN group. In fact, one of the theories involved in milk fat depression is the glucogenic-insulin theory, which explains that the increase in propionate in the rumen and the rates of hepatic gluconeogenesis may result in an

increase in blood insulin levels. Given its ability to inhibit lipolysis, this leads to increased utilization of acetate, β-hydroxybutyrate, and dietary long-chain fatty acids (LCFA) at the adipose tissue level, thereby reducing the amount of lipogenic precursors available for milk fat synthesis and limiting the preformed fatty acids available for the mammary gland (Koch and Lascano, 2018). The increase in the glucogenic to lipogenic VFA ratio, resulting from higher intake of WSC, as observed by Lee et al., 2002 in a study on steers, could also be a cause of reduction in milk fat precursors. Indeed, daily milk fat content (%), especially milk fat content of afternoon milking, was higher in ewes that ingested shaded grass. However, this difference was not observed in milk from morning milking, as well as for the morning milk fat to protein ratio. This might be explained from the fact that 42% of SUN and 51% of SHADE total daily grass intake occurred within the 3 hours before the afternoon milking (1630 h), i.e. from the time of its supply (1:00 PM) to 4:00 PM. This means that 40-50% of the total WSC intake was consumed very quickly in a short period of time, likely causing a drop in ruminal pH caused by a variation in the VFA produced in the rumen and consequent different hormone-levels, which can lead to a decrease in milk fat synthesis in the SUN group, as previously explained. Indeed, ewes that ingested unshaded grass with extremely high levels of WSC probably experienced problems related to WSC overload, as indicated by some diarrhoea cases observed in this group. Additionally, the lower NDF intake in the SUN than in SHADE group may be a factor that exacerbates this condition. However, the fat to protein ratio remained above the inversion threshold of 1.00, despite a significant difference in favour of the SHADE group, which had a higher value in afternoon milking ($P = 0.009$). In fact, a low milk fat to protein ratio is a milk indicator of ruminal acidosis (Atalay, 2019; Kara,

2020). This is probably related to the very low milk protein content observed in both groups, resulting in a high fat to protein ratio, even at low fat values. During conditions of ruminal acidosis, not only does the milk fat content decrease, but the milk protein content can also decline (Caja and Bocquier, 2000; Dong et al., 2013), due to reduced bacterial protein flow from the rumen to the intestine. Indeed, microbial amino acids have a very important role in milk protein synthesis. The milk fatty acid (FA) profile showed that C18:1 trans-10 tended to be higher in the SUN group than in SHADE group ($P = 0.062$). This fatty acid is an intermediate product of the secondary biohydrogenation of C18:2n-6, which is abundant in high-concentrates diets or under conditions of low rumen pH and is associated with diets that induce milk fat depression (MFD) (Bauman et al., 2011; Fievez et al., 2012; Miccoli et al., 2022). In fact, at lower ruminal pH, the accumulation of FA during rumen biohydrogenation shifts toward the C18:1 trans-10 pathway, which increases with higher amounts of concentrate in the diet (Colman et al., 2010). Bauman et al. (2011) reported that the decrease in milk fat content during MFD is highly related to rumen outflow and the milk fat content of C18:1 trans-10. Indeed, in this study ewes fed unshaded grass had a higher tendency for C18:1 trans-10, indicating a shift in rumen biohydrogenation caused by WSC overload, which consequently reduced milk fat. Molle et al. (2022), showed that as WSC intake from grasses increased, ruminal pH decreased, whereas most studies showed that sugars do not have the same potential to decrease ruminal pH as starch (Oba, 2011). The results in literature are varied. It must be considered that the quantity and type of sugar influence rumen VFA profile and consequently ruminal pH, and that most studies have focused on simple sugars rather than WSC or specifically on fructans. The effect of WSC quantity also depends on the NDF content of feeds. In

fact, Klevenhusen et al. (2021) reported that WSC-rich ryegrass hay did not cause SARA due to its high level of peNDF, which has a positive effect on rumination. Therefore, the low NDF and peNDF content of herbage represents an additional risk factor for SARA when the feed is rich in WSC. In this study, a negative correlation was found between WSC intake (g DM/d) and milk fat content of afternoon milking (Fig. 5) but not with NDF intake. The grass intake in the afternoon (from 1:00 PM to 4:00 PM, before the 4:30 PM milking) was significantly higher in SHADE than in SUN, probably because one of the effects of SARA is to reduce intake. Despite the higher intake (422 and 483 g DM/d for SUN and SHADE, respectively), the difference was not sufficient to compensate for the substantial difference in the WSC content between the two treatments. The rest of the total grass intake occurred over a long period of time (14 hours), from 5:00 PM to 7:00 AM of the following day, without causing ruminal WSC excess and any consequent subacidosis symptom; this could explain the lack of differences in milk fat content during the morning milking between the two treatments. In studies conducted on dairy cows by Miller et al. (2001), Taweel et al. (2005), Taweel et al. (2006), Moorby et al. (2006), and Merino et al. (2019), milk fat was not affected by the level of WSC. However, in these studies the levels of WSC and/or difference in WSC between treatments were too low to influence the milk fat content. A recent review (Klevenhusen and Zebeli, 2021) showed that there are no studies reporting a negative effect of WSC on the milk fat content of lactating dairy cows, probably because the levels of WSC in the diet of cattle are usually low. However, Rivero and Anrique (2015) reported that in certain periods of the year, such as spring, pastures exhibit all conditions that can lead to a decrease in milk fat content, such as low fibre content, high levels of sugars and water, representing a critical season

for milk fat content. Nevertheless, there are no studies regarding the effect of WSC overload on milk fat synthesis in ewes. In our study, the WSC levels of the grass supply were very high, for the reasons already explained, and this could have caused effects on ruminal pH and/or on rumen VFA, with a shift in rumen biohydrogenation, glucose and insulin level and consequently on milk fat synthesis. This hypothesis could be supported by the fact that the effects were marked three hours after the supply of grass, in afternoon milking when the highest grass intake occurred.

Conclusions

The lower milk fat content observed in ewes fed unshaded grass with higher WSC content (SUN) compared to those fed shaded grass with lower WSC content (SHADE), especially during afternoon milking (three hours after grass feeding), along with the tendency for higher levels of C18:1 trans-10 in the SUN group, an indicator of diet induced MFD, suggests that WSC overload from ryegrass may be a cause of the MFD observed in dairy ewes grazing during the winter and early spring. These seasons, when characterized by low temperatures and sunny days, favor the accumulation of WSC to very high levels in grasses, posing a risk for pasture-fed sheep. Additionally, this study highlights that WSC content is an important herbage quality parameter to consider when formulating rations for pasture-fed dairy sheep. Further research should focus on characterizing this complex class of carbohydrates, particularly the quantity and types of simple sugars and fructans in these commonly used as pasture crops, as well as their differential utilization at the ruminal level and their subsequent effects on animal health and milk composition, as WSC overload represents a new nutritional issue for grazing ewes in the Mediterranean area.

References

Allsop A. R. C., Nicol A. M., Edwards G. R. 2009. Effect of water soluble carbohydrate concentration of ryegrass on the partial preference of sheep for clover. Proceedings of the New Zealand Animal Production Society, 69, 20-23.

Atalay H. 2019. Milk fat/protein ratio in ketosis and acidosis. Balıkesir Sağlık Bilimleri Dergisi, 8(3), 143-146.

Bauman D. E., Harvatine K. J., & Lock A. L. 2011. Nutrigenomics, rumen-derived bioactive fatty acids, and the regulation of milk fat synthesis. Annual review of nutrition, 31(1), 299-319.

Caja G., & Bocquier F. 2000. Effects of nutrition on the composition of sheep's milk. Cahiers Options Méditerranéennes, 52(59-74).

Ciavarella T. A., Simpson R. J., Dove H., Leury B. J., Sims I. M. (2000a). Diurnal changes in the concentration of water-soluble carbohydrates in Phalaris aquatica L. pasture in spring, and the effect of short-term shading. Australian Journal of Agricultural Research, 51(6), 749-756.

Ciavarella T. A., Dove H., Leury B. J., & Simpson R. J. (2000b). Diet selection by sheep grazing Phalaris aquatica L. pastures of differing water-soluble carbohydrate content. Australian Journal of Agricultural Research, 51(6), 757-764.

Colman E., Fokkink W. B., Craninx M., Newbold J. R., De Baets B., & Fievez V. 2010. Effect of induction of subacute ruminal acidosis on milk fat profile and rumen parameters. Journal of dairy science, 93(10), 4759-4773.

Correddu F., Nudda A., Battacone G., Boe R., Francesconi A. H. D., & Pulina G. 2015. Effects of grape seed supplementation, alone or associated with linseed, on

ruminal metabolism in Sarda dairy sheep. Animal feed science and technology, 199, 61-72.

Correddu F., Cellesi M., Serdino J., Manca M. G., Contu M., Dimauro C., Ibba I., & Macciotta N. P. P. 2019. Genetic parameters of milk fatty acid profile in sheep: Comparison between gas chromatographic measurements and Fourier-transform IR spectroscopy predictions. Animal, 13(3), 469-476.

Deriaz R. E. 1961. Routine analysis of carbohydrates and lignin in herbage. Journal of the Science of Food and Agriculture, 12(2), 152-160.

Dong H., Wang S., Jia Y., Ni Y., Zhang Y., Zhuang S., Shen X., & Zhao R. 2013. Long-term effects of subacute ruminal acidosis (SARA) on milk quality and hepatic gene expression in lactating goats fed a high-concentrate diet. PloS one, 8(12), e82850.

Fievez V., Colman E., Castro-Montoya J. M., Stefanov I., & Vlaeminck B. 2012. Milk odd-and branched-chain fatty acids as biomarkers of rumen function—An update. Animal Feed Science and Technology, 172(1-2), 51-65.

Geor R. J. 2009. Pasture-associated laminitis. Veterinary Clinics of North America: Equine Practice, 25(1), 39-50.

Geor R. J. 2010. Current concepts on the pathophysiology of pasture-associated laminitis. Veterinary Clinics of North America: Equine Practice, 26(2), 265-276.

Hight G. K., Sinclair D. P., & Lancaster R. J. 1968. Some effects of shading and of nitrogen fertiliser on the chemical composition of freeze-dried and oven-dried herbage, and on the nutritive value of oven-dried herbage fed to sheep. New Zealand Journal of Agricultural Research, 11(2), 286-302.

Kagan I. A. 2022. Water-and Ethanol-Soluble Carbohydrates of Temperate Grass Pastures: a Review of Factors Affecting Concentration and Composition. Journal of Equine Veterinary Science, 110, 103866.

Kara K. 2020. Milk urea nitrogen and milk fatty acid compositions in dairy cows with subacute ruminal acidosis. Veterinární medicína, 65(9).

Klevenhusen F., Zebeli Q. 2021. A review on the potentials of using feeds rich in water-soluble carbohydrates to enhance rumen health and sustainability of dairy cattle production. Journal of the Science of Food and Agriculture, 101(14), 5737-5746.

Koch L. E., & Lascano G. J. 2018. Milk fat depression: etiology, theories, and soluble carbohydrate interactions. Journal of Animal Research and Nutrition, 3(2), 2.

Kramer J. K., Fellner V., Dugan M. E., Sauer F. D., Mossoba M. M., & Yurawecz M. P. 1997. Evaluating acid and base catalysts in the methylation of milk and rumen fatty acids with special emphasis on conjugated dienes and total trans fatty acids. Lipids, 32, 1219-1228.

Lee M. R., Merry R. J., Davies D. R., Moorby J. M., Humphreys M. O., Theodorou M. K., ... Scollan N. D. 2003. Effect of increasing availability of water-soluble carbohydrates on in vitro rumen fermentation. Animal Feed Science and Technology, 104(1-4), 59-70.

Lee M. R. F., Harris L. J., Moorby J. M., Humphreys M. O., Theodorou M. K., MacRae J. C., & Scollan N. D. 2002. Rumen metabolism and nitrogen flow to the small intestine in steers offered Lolium perenne containing different levels of water-soluble carbohydrate. Animal Science, 74(3), 587-596.

Lee M. R., Jones E. L., Moorby J. M., Humphreys M. O., Theodorou M. K., & Scollan N. D. 2001. Production responses from lambs grazed on Lolium perenne selected for an elevated water-soluble carbohydrate concentration. Animal Research, 50(6), 441-449.

Longland A. C., & Byrd B. M. 2006. Pasture nonstructural carbohydrates and equine laminitis. The Journal of nutrition, 136(7), 2099S-2102S.

Mayland H. F., & Grunes D. L. 1974. Shade-induced grass-tetany-prone chemical changes in Agropyron desertorum and Elymus cinereus. Rangeland Ecology & Management/Journal of Range Management Archives, 27(3), 198-201.

McIntosh, B.J., 2006. Circadian and seasonal variation in pasture nonstructural carbohydrates and the physiological response of grazing horses. PhD thesis, Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

Merino V. M., Balocchi O. A., & Rivero M. J. 2019. Milk production, milk quality, and behaviour of dairy cows grazing on swards with low and high water-soluble carbohydrates content in autumn: A pilot trial. Animals, 9(12), 1012.

Miccoli F. E., Pérez C. D., Vargas-Bello-Pérez E., Danelón J. L., Cantet J. M., Martínez R., Bargo F., Colombatto D., & Palladino, R. A. 2022. Effects of high fibre energy supplements on production performance, milk composition and milk fatty acid profile from dairy ewes fed fresh cut Lolium multiflorum. Small Ruminant Research, 209, 106640.

Miller L. A., Moorby J. M., Davies D. R., Humphreys M. O., Scollan N. D., MacRae J. C., Theodorou M. K. 2001. Increased concentration of water-soluble carbohydrate in perennial ryegrass (Lolium perenne L.): milk production from late-lactation dairy cows. Grass and Forage Science, 56(4), 383-394.

Molle G., Decandia M., Cabiddu A., Landau S. Y., Cannas A. 2008. An update on the nutrition of dairy sheep grazing Mediterranean pastures. Small Ruminant Research, 77(2-3), 93-112.

Molle G., Gregorini P., Cabiddu A., Decandia M., Giovanetti V., Sitzia M., Dattena M., Porcu C., Pasciu V., Gonzalez-Bulnes A., Berlinguer F., Cannas A. 2022. Does the timing of pasture allocation affect rumen and plasma metabolites and ghrelin, insulin and cortisol profile in dairy ewes?. Frontiers in Veterinary Science, 9, 969950.

Moorby J. 2001. Grass sugars make milk production sweeter. IGER Innovations, 5, 35-39.

Moorby J. M., Evans R. T., Scollan N. D., MacRae J. C., & Theodorou M. K. 2006. Increased concentration of water-soluble carbohydrate in perennial ryegrass (Lolium perenne L.). Evaluation in dairy cows in early lactation. Grass and Forage Science, 61(1), 52-59.

Moore K. J., & Jung H. J. G. 2001. Lignin and fiber digestion. Journal of Range Management 54, 420–430.

Nudda A., McGuire M. A., Battacone G., & Pulina G. 2005. Seasonal variation in conjugated linoleic acid and vaccenic acid in milk fat of sheep and its transfer to cheese and ricotta. Journal of Dairy Science, 88(4), 1311-1319.

Nudda A., Palmquist D. L., Battacone G., Fancellu S., Rassu S. P. G., & Pulina G. 2008. Relationships between the contents of vaccenic acid, CLA and n− 3 fatty acids of goat milk and the muscle of their suckling kids. Livestock Science, 118(3), 195-203.

Oba, M. 2011. Effects of feeding sugars on productivity of lactating dairy cows. Canadian Journal of Animal Science, 91(1), 37-46.

Olszewska M. 2021. Effects of cultivar, nitrogen rate and harvest time on the content of carbohydrates and protein in the biomass of perennial ryegrass. Agronomy, 11(3), 468.

Pulina G., Atzori A. S., Dimauro C., Ibba I., Gaias G. F., Correddu F., Nudda A. 2021. The milk fingerprint of Sardinian dairy sheep: Quality and yield of milk used for Pecorino Romano PDO cheese production on population-based 5-year survey. Italian Journal of Animal Science, 20(1), 171-180.

Rech Â. F., de Arruda Cordoba U., Fávaro V. R., Flaresso J. A. 2022. Principais características de cultivares de azevém-anual utilizados no estado de Santa Catarina, Brasil. Agropecuária Catarinense, 35(1), 85-92.

Rivero M. J., Anrique R. 2015. Milk fat depression syndrome and the particular case of grazing cows: A review. Acta Agriculturae Scandinavica, Section A—Animal Science, 65(1), 42-54.

Satta F., Porcu M.A., Ledda A., Carta S., Deiana L., Cannas A. 2023. The concentration of water-soluble carbohydrates in the pasture grazed by lactating ewes might be a main cause of milk fat depression. (Abstract). ASPA Conference, Monopoli (Bari, Italy), 13-16 June 2023.

Schmidt V. A. M., Watson, C. A., Walker, R. L., Argo, C. M., Russell, W. R., Neacsu, M., ... & Morrison, P. K. 2023. 50 Water-soluble carbohydrate content in equine pasture: Impact of fertilization, season, and grass species. Journal of Equine Veterinary Science, 124, 104352.

Siciliano P. D., Gill J. C., Bowman M. A. 2017. Effect of sward height on pasture nonstructural carbohydrate concentrations and blood glucose/insulin profiles in grazing horses. Journal of Equine Veterinary Science, 57, 29-34.

Smith S., Prince K. 2017. How to Maximize Energy Content in Forage Grasses. Forage Extension Specialist and Masters Student University of Kentucky Lexington, KY. https://uknowledge.uky.edu/cgi/viewcontent.cgi?article=1072&context=forage_kca

Sun X., Chen A., & Li J. 2023. Chemical, Physical, and Degradation Characteristics of Ryegrass Cultivars Grown in Autumn and Winter for Dairy Cows. Animals, 13(20), 3158.

Taweel H. Z., Tas B. M., Smit H. J., Elgersma A., Dijkstra J., & Tamminga S. 2005. Effects of feeding perennial ryegrass with an elevated concentration of water-soluble carbohydrates on intake, rumen function and performance of dairy cows. Animal Feed Science and Technology, 121(3-4), 243-256.

Taweel H. Z., Tas B. M., Smit H. J., Elgersma A., Dijkstra J., & Tamminga S. 2006. Grazing behaviour, intake, rumen function and milk production of dairy cows offered Lolium perenne containing different levels of water-soluble carbohydrates. Livestock Science, 102(1-2), 33-41.

Undersander D. 2013. Grass varieties for horses. Journal of Equine Veterinary Science, 33(5), 315-320.

Van Soest P. 1982. Nutritional ecology of the ruminant. O & B Books Inc., Corvallis, OR, USA.

Watts K. A. 2004. Forage and pasture management for laminitic horses. Clinical techniques in equine practice, 3(1), 88-95.

Watts K. A. 2005. A review of unlikely sources of excess carbohydrate in equine diets. Journal of Equine Veterinary Science, 25(8), 338-344.

Watts K. 2008. The influence of solar radiation and temperature on the diurnal fluctuation of NSC in grass. Copyright 2008. Rocky Mountain Research & Consulting, Inc. https://static1.squarespace.com/static/5e7fddf934de306c2b8cc313/t/5e8c82935a255f19a8a2cb9f/1586266771795/DiurnalFluctNSC.pdf

Watts K. A. 2009. Grass in Shade is Lower in Sugar. Sarfergrass.org. Rocky mountain research and consulting, Inc. https://static1.squarespace.com/static/5e7fddf934de306c2b8cc313/t/5e87480367d06f1dfe7d5ae0/1585924099224/Shade.pdf.

Woolpert M. E., Dann H. M., Cotanch K. W., Melilli C., Chase L. E., Grant R. J., & Barbano D. M. 2016. Management, nutrition, and lactation performance are related to bulk tank milk de novo fatty acid concentration on northeastern US dairy farms. Journal of dairy science, 99(10), 8486-8497.

Appendix Chapter 2

Supplementary Table 1. Complete milk fatty acids profile (total fatty acid methyl esters, FAME; g/100 g of total FAME).

Supplementary Table 1. Complete milk fatty acids profile (total fatty acid methyl esters, FAME; g/100 g of total FAME).

Item	Treatment		SEM	P-value
	SUN	SHADE		
C4:0	2.46	2.56	0.04	**0.072**
C6:0	2.13	2.20	0.03	**0.137**
C7:0	0.03	0.02	0.001	**0.027**
C8:0	2.18	2.21	0.06	**0.695**
C9:0	0.21	0.21	0.007	**0.65**
C10:0	7.36	7.13	0.27	**0.594**
C10:1	0.01	0.01	0.0006	**0.535**
C11:0	0.29	0.29	0.01	**0.915**
C12:0	4.37	4.21	0.15	**0.454**
iso-C13:0	0.02	0.02	0.002	**0.897**
anteiso-C13:0	0.04	0.03	0.002	**0.709**
iso-C14:0	0.16	0.15	0.008	**0.435**
C14:0	10.9	11	0.22	**0.961**
iso-C15:0	0.32	0.32	0.01	**0.733**
anteiso-C15:0	0.58	0.55	0.02	**0.23**
C14:1c9	0.12	0.13	0.005	**0.621**
C15:0	1.11	1.11	0.03	**0.994**
C15:1 c9	0.07	0.07	0.003	**0.863**
iso-C16:0	0.44	0.40	0.01	**0.08**
C16:0	23.9	25.4	0.46	**0.02**
C16:1t4	0.01	0.01	0.001	**0.363**
C16:1t5	0.02	0.02	0.001	**0.026**
C16:1t6-7	0.03	0.02	0.002	**0.077**
iso-C17:0	0.05	0.04	0.002	**0.46**
C16:1t9	0.63	0.61	0.01	**0.185**
C16:1t10	0.01	0.01	0.001	**0.624**
C16:1t11-t12	0.07	0.08	0.003	**0.27**
C16:1c7	0.31	0.29	0.007	**0.029**
anteiso-C17:0	0.59	0.58	0.02	**0.652**
C16:1c9	0.66	0.61	0.02	**0.071**
C16:1c10	0.03	0.03	0.002	**0.704**
C16:1c11	0.01	0.01	0.001	**0.056**
3,7,11,15-Tetramethyl-16:0	0.04	0.04	0.001	**0.551**
C17:0	0.90	0.89	0.02	**0.564**

Supplementary Table S1. Continuation.

Item	Treatment		SEM	P-value
	SUN	SHADE		
iso-C18:0	0.09	0.10	0.01	**0.207**
C17:1 c6-7	0.02	0.01	0.001	**0.098**
C17:1c8	0.07	0.06	0.001	**0.418**
C17:1c9	0.26	0.24	0.01	**0.133**
C18:0	11.6	10.8	0.23	**0.023**
C18:1t4	0.01	0.01	0.001	**0.67**
C18:1t5	0.013	0.012	0.001	**0.238**
C18:1t6-8	0.22	0.21	0.007	**0.183**
C18:1t9	0.20	0.20	0.01	**0.669**
C18:1t10	0.34	0.29	0.02	**0.062**
C18:1t11	1.28	1.19	0.06	**0.33**
C18:1t12	0.30	0.31	0.01	**0.48**
C18:1t13-t14	0.86	0.82	0.05	**0.617**
C18:1c9	18.9	18.9	0.72	**0.963**
C18:1c11	0.40	0.37	0.01	**0.082**
C18:1c12	0.18	0.18	0.006	**0.336**
C18:1c13	0.06	0.06	0.002	**0.593**
C18:1t16-c14	0.44	0.43	0.02	**0.776**
C19:0/C18:1c15	0.27	0.28	0.01	**0.513**
C18:2t10t14	0.04	0.02	0.002	**0.003**
C18:2t11t15	0.03	0.03	0.002	**0.998**
C18:2t9t12	0.01	0.01	0.001	**0.06**
C18:2c9t13	0.32	0.32	0.02	**0.849**
C17cyclo	0.11	0.12	0.01	**0.434**
C18:2t8c13	0.15	0.16	0.01	**0.511**
C18:2c9t12	0.09	0.10	0.004	**0.234**
C18:1c16	0.02	0.02	0.001	**0.616**
C18:2t9c12	0.02	0.02	0.001	**0.192**
C18:2t11c15	0.17	0.19	0.01	**0.415**
C18:2n6	2.01	1.93	0.05	**0.288**
C18:2t12c15	0.04	0.04	0.002	**0.003**
C18:2c12c15	0.016	0.021	0.001	**0.003**
C20:0	0.22	0.21	0.003	**0.001**
Δ7,9 17:2	0.02	0.02	0.001	**0.857**

Supplementary Table S1. Continuation.

Item	Treatment		SEM	P-value
	SUN	SHADE		
C18:3n6	0.06	0.05	0.003	**0.023**
C20:1c9	0.009	0.008	0.001	**0.443**
C20:1c11	0.04	0.04	0.001	**0.194**
C18:3n3	0.63	0.63	0.01	**0.897**
CLAc9t11	0.55	0.55	0.03	**0.981**
C20:1c15	0.013	0.012	0.001	**0.049**
CLAt9c11/C21:0	0.07	0.067	0.001	**0.013**
CLAt10c12	0.015	0.014	0.001	**0.574**
CLAt12t14	0.02	0.02	0.001	**0.444**
CLAt11t13	0.025	0.025	0.002	**0.841**
C20:2n9	0.02	0.02	0.001	**0.867**
CLAt9t11*	0.04	0.03	0.003	**0.085**
C18:4n3	0.009	0.01	0.001	**0.238**
C20:2n6	0.02	0.02	0.001	**0.794**
C20:3n9	0.05	0.05	0.002	**0.481**
C22:0	0.11	0.11	0.002	**0.434**
C20:3n6	0.03	0.03	0.001	**0.278**
10,14,17 C20:3*	0.01	0.009	0.0004	**0.092**
C22:1n9	0.01	0.01	0.0005	**0.213**
C20:3n3	0.008	0.007	0.0004	**0.653**
C20:4n6	0.15	0.16	0.01	**0.519**
C23:0	0.06	0.06	0.001	**0.921**
C20:4n3	0.008	0.008	0.001	**0.575**
C22:2n6	0.15	0.14	0.005	**0.122**
EPA	0.06	0.05	0.002	**0.188**
C24:0	0.05	0.05	0.001	**0.069**
C22:3n6	0.007	0.007	0.001	**0.796**
C24:1c15	0.019	0.02	0.001	**0.66**
C22:4n6	0.015	0.016	0.001	**0.286**
C25:0	0.01	0.01	0.001	**0.846**
C26:0	0.04	0.04	0.001	**0.986**
DPA	0.09	0.09	0.004	**0.492**
DHA	0.02	0.02	0.002	**0.339**

Abbreviations: EPA = eicosapentaenoic acid; DPA = docosapentaenoic acid; DHA = docosahexaenoic acid.

CHAPTER 3

Sodium bicarbonate limited milk fat depression of lactating dairy ewes fed ryegrass herbage rich in water soluble carbohydrates

Abstract

The use of grass pastures rich in water soluble carbohydrates (WSC) in ewes can induce ruminal sub-acidosis, resulting in a decrease in milk fat content. With the aim to prevent sub-acidosis conditions from WSC overload, the effect of supplying sodium bicarbonate ($NaHCO_3$), as rumen buffer, to sheep fed fresh cut ryegrass (*Lolium multiflorum* Lam. ssp. westervoldicum) herbage rich in WSC (mean of 28% DM) was studied. Ten lactating ewes of the Sarda breed in their third month of lactation were selected, based on milk production and body weight. They were then divided into two homogeneous groups: control (CNT) and sodium bicarbonate (BIC). The animals were fed indoors with automatic Biocontrol AS (Rakkestad, Norway) feeders, which recorded continuously during the day the individual intake of grass of the ewes. The ewes received ad libitum freshly cut grass from 12:30 PM to 7:00 AM of the subsequent day. In addition, they received, in two separate meals at milking (7:30 AM and 16:30 PM), a total of 400 g/d of soybean meal (SBM) and 550 g/d (as fed values) of whole corn grains. The BIC group also received a supplement of 25 g/day per ewe of sodium bicarbonate, just before the ryegrass supply. Data on intake and milk yield and composition were covariate-adjusted using pre-experimental measurements. Daily DM intake of grass (BIC 1488 g of DM/d, CNT 1340 g of DM/d; $P = 0.03$), particularly high in the first hours after its supply, was higher in BIC than in CNT. Milk yield (CNT 1859 g/d, BIC 1838 g/d) was not affected by the treatment. Milk fat concentration of afternoon (BIC 6.52%, CNT 6.01%; $P < 0.001$) and morning (BIC 5.39%, CNT 5.06%; $P = 0.03$) milking was higher in the BIC than CNT. Moreover, milk fat yield of afternoon milking was higher in the BIC than CNT (BIC 42.2 g/d, CNT 38.7 g/d; $P = 0.03$) but there were no differences in morning milking (BIC 64.7 g/d, CNT 60.6 g/d).

The fat to protein ratio of the afternoon (BIC 1.35, CNT 1.25; $P < 0.001$) and morning (BIC 1.13, CNT 1.06; $P = 0.01$) milk was higher in BIC than in CNT. In conclusion, sodium bicarbonate, supplied just before the grass supply, can be used to mitigate the negative effects of WSC overload, due to its significant ability to reduce milk fat depression with strong, even though short-term, action. Additionally, this study highlights that WSC content is an important herbage quality parameter to be considered when formulating rations for pasture-fed dairy sheep.

Keywords

Sodium bicarbonate, water-soluble carbohydrates overload, annual ryegrass, milk fat depression, grazing dairy ewes.

Introduction

Grazing on grasses rich in water-soluble carbohydrates (WSC) can lead to ruminal subacute ruminal acidosis (SARA) and subsequent milk fat depression (MFD), a problem increasingly common in Sardinian dairy sheep farms in recent years. In particular, specific grass species, especially tetraploid annual ryegrass commonly used as forage crops for grazing in Sardinia, can accumulate extremely high WSC levels (>30% DM) during winters and springs characterized by low temperatures and sunny days, which favor photosynthetic processes and limit night respiration (Watts, 2005; Watts, 2008). As a result, WSC overload from grasses in grazing dairy ewes represents a new nutritional issue. Issues related to WSC overload, particularly from fructans, are well studied in horses because they are implicated in the onset of pasture-associated laminitis. Studies inducing laminitis through oligofructose overload models, which mimic laminitis resulting from the intake of fructan-rich pasture, have shown a link between field cases of laminitis and fructan content of grass (Thoefner et al., 2004; Van Eps and Pollitt, 2006; Pollitt and Milinovich, 2017; Noronha et al., 2019). Oligofructose is a fructose polymer with a degree of polymerization (DP) of up to ten and is present in temperate grasses (Niness et al., 1999; Noronha et al., 2019). In these studies, the clinical and metabolic effects are similar to those observed after starch overload, with the onset of clinical laminitis occurring after the peak of plasma D-lactate, an isomer produced exclusively by bacteria considered a marker of carbohydrate fermentation in the hindgut (Van Eps and Pollitt, 2006). Studies using these models to induce laminitis in cows suggest that the pathogenesis of oligofructose-induced laminitis is similar in both equine and cows, in both being preceded by acidosis conditions (Thoefner et al., 2004; Noronha et al., 2019). Molle

et al. (2022) showed a negative correlation between WSC intake and ruminal pH, as well as a lower acetate to propionate ratio and higher glucose and insulin in the blood, of dairy ewes grazing on afternoon pasture (higher in WSC for the photosynthetic activity during the day) than on morning pasture (lower WSC). In a field study on grazing dairy ewes in Sardinia, the WSC content in pasture was found to be inversely related to milk fat content (Satta et al., 2023). Similarly, Chapter 2 of this thesis reported a negative correlation between WSC intake and milk fat content of ewes fed fresh ryegrass. In dairy ruminants, it seems that WSC overload is likely a cause of SARA and milk fat depression, like starch overload. Feeding techniques to prevent the negative effects of carbohydrate overload include ensuring adequate levels of NDF and peNDF in the diet, evaluating the physical characteristics of the feed and forages used, and the use of feeds rich in NDF and soluble fibre (e.g., beet pulps) to promote chewing activity, rumination, salivation and rumen motility. The addition of buffers to the diet is a common practice in ruminants, particularly when using diets high in concentrates and low in forages, which pose a risk for development of SARA and milk fat depression. In fact, such diets or pastures with low NDF and peNDF, and high NFC and moisture content, may lead to a reduction in chewing time, rumination, and salivation (Westwood and Lean, 2001; Westwood et al., 2003; Kleen et al., 2003; Rivero and Anrique, 2015; Annatte et al., 2021). Buffers increase water intake and salivation, which consequently raise the rumen liquid dilution rate. This leads to an increased outflow of fermentable carbohydrates with the liquid phase from the rumen, reducing their absorption rate. As a result, ruminal propionate decreases, ruminal pH increases and milk fat production is enhanced (Hart and Doyle, 1985; Russell and Chow, 1993; Calsamiglia et al., 2012; Jaramillo-Lopez et al., 2017; Vicente et al.,

2023). One of the most common buffer salts used in ruminant rations is sodium bicarbonate ($NaHCO_3$) (Annatte et al., 2021), which is used to prevent a reduction of ruminal pH and, consequently, ruminal acidosis and decrease of milk fat content (Kalscheur et al., 1997; Calsamiglia et al., 2012; Jaramillo-López et al., 2017). Sodium bicarbonate has a rapid buffering action on ruminal fluid that prevents post-prandial increases of H^+ (Russell and Chow, 1993) and inhibits the over-growth of acid-tolerant lactobacilli, thus preventing pH decrease (Kleen et al., 2003). It can also increase passage and fluid dilution rate of nutrients (Russell and Chow 1993; Vicente et al., 2023) and improve the outflow of ruminal fluid, reducing the accumulation of VFA and lactate (Vicente et al., 2023). Studies investigating the effects of sodium bicarbonate on ruminants have shown increases in DMI, nutrient digestibility, ruminal pH and TVFA, as well as higher acetate to propionate ratio, milk yield, and milk fat content (Hart and Doyle, 1985; Calsamiglia et al., 2012; Farghaly et al., 2019; Annatte et al., 2021; Vicente et al., 2023; Ameen et al., 2023). However, the response to sodium bicarbonate supplementation varies according to the diet type, with greater effects observed at lower ruminal pH levels (Tripathi et al., 2004; Hu and Murphy, 2005; Calsamiglia et al., 2012). We hypothesize that sodium bicarbonate, supplied as a ruminal buffer, could prevent sub-acidosis conditions and milk fat depression caused by WSC overload in lactating grazing ewes. Thus, the aim of this study was to test the effect of sodium bicarbonate supplementation as a ruminal buffer on milk fat synthesis in a trial involving lactating Sarda ewes fed fresh herbage of tetraploid annual ryegrass with a high WSC concentration.

Materials and methods

Experimental site, animals and diet

The experiment was conducted at the experimental farm of the Department of Agricultural Sciences of University of Sassari, located in the north-west of Sardinia (Ottava Sassari, Italy; 40°48'41.4"N 8°17'50.7"E) from 6 to 17 April 2023 (12 experimental days). A field of tetraploid annual ryegrass (*Lolium multiflorum* Lam. ssp. westervoldicum) was sown on 18 October 2022 and used to feed the ewes during the experimental phase. The seeding rate used was 30 kg/ha, and the nitrogen fertilization rate used was 130 kg/ha, applied at sowing. Nitrogen was applied in the form of urea (46% N) through a fertilizer containing also diciandiammide (inhibitor of nitrification). The herbage was harvested daily at 11:30 AM, to have a greater level of WSC, with a mowing machine Attila AT 900 MF (Brumar srl, Asti, Italy) at cutting height of 8-12 cm from the ground, and subsequently taken to the barn. Weather data (maximum and minimum temperature, rainfall and solar radiation) were recorded at the meteorological station of the experimental farm, located approximately 150 m from the field, from the sowing date to the end of the trial. Mean monthly weather conditions from the sowing date to the end of the trial (October 2022 to 17 April 2023), are presented in Table 1. Ten Sarda ewes in the third month of lactation were selected from a group of ewes housed indoors and fed ryegrass herbage and concentrates based on pre-experimental measurements of milk yield (2123±254 g/ewe per day), body weight (58.6 ± 6.7 kg) and milk fat content (5.50 ± 0.46%). They were divided into two homogeneous groups of 5 ewes per treatment: control (CNT) and sodium bicarbonate (BIC). They were assigned to the treatments in a completely randomized design. Sheep were fed ad libitum ryegrass herbage immediately after cutting, from

12:30 AM to 7:00 AM of the subsequent day, using automatic Biocontrol AS (Rakkestad, Norway) feeders, that continuously recorded the individual intake of sheep. In addition, the ewes received a total of 400 g/day per ewe of soybean meal, 550 g/day per ewe of whole corn grains and 300 g/day per ewe of beet pulps (as-fed values), split in two separate equal meals at milking. The BIC group was supplemented with 25 g/day per ewe of sodium bicarbonate (about 1% of total predicted DMI) just before the ryegrass supply. Sodium bicarbonate was dissolved in water and administered orally via syringe, so that all animals received the same amount of the supplement, while CNT group did not receive any supplementation before herbage supply. All animals were housed and fed indoors and machine-milked twice daily at 7:30 AM and 4:30 PM. The composition of the experimental diet and the diet sequence per animal are reported in Table 2.

Table 1. Monthly mean weather values for minimum and maximum temperatures, solar radiation and rainfall sum at experimental farm of the Department of Agriculture of University of Sassari (Ottava, Sassari, Italy) during the period from sowing date (18 October 2022) to 17 April 2023 (Start of trial: 06 April 2023; End of trail: 17 April 2023).

Variable	**Month**						
	October	**November**	**December**	**January**	**February**	**March**	**April**
Mean minimum temperatures (°C)	15.5	12.3	10.4	7.8	6.1	8.8	7.6
Mean maximum temperatures (°C)	26	20.4	18.7	14.8	15.3	18.3	17.3
Mean solar radiation (MJ/ m^2 per day)	14	8.7	7.9	7.9	11.3	16.2	20.4
Rainfall, sum (mm/month)	15.6	200.3	113.9	53.2	13.6	6.6	4.6

Table 2. Experimental diet sequence of CNT and BIC groups during experimental period.

Hour	CNT	BIC
7:30 AM Morning milking	Soybean meal 200 g	Soybean meal 200 g
8:00 AM	Corn grains 275 g Beet pulps* 150 g	Corn grains 275 g Beet pulps* 150 g
12:00 AM	---	**Sodium bicarbonate**
From 12:30 PM to 7:00 AM	**Ryegrass herbage supply**	
4:30 PM Afternoon milking	Soybean meal 200 g	Soybean meal 200 g
5:00 PM	Corn grains 275 g Beet pulps* 150 g	Corn grains 275 g Beet pulps* 150 g

*Until 7 April (2nd experimental day).

Measurements, sampling and analyses

The samples of herbage supplied to ewes were collected daily, immediately after mowing, and were divided into two subsamples, one immediately frozen at -20 °C and the other dried immediately at 65 °C, and then grinded to pass in 1 mm screen. The herbage samples were analyzed in correspondence of milking sampling days. The dry matter (DM) content was determined by oven drying at temperature of 105 °C for 24 h. The neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL), crude protein (CP), soluble protein, ash, ether extract (EE) and acid detergent insoluble crude protein (ADICP) were determined by near-infrared reflectance spectroscopy (NIRS) in Cargill Laboratories (Fiorenzuola D'Arda, Italy). Non-fibre

carbohydrates (NFC) were calculated as: 100 − (NDF + CP – ADICP + ash + EE). The water-soluble carbohydrate (WSC) content of herbage samples was determined by the anthrone sulfate colorimetric method (Deriaz, 1961) using glucose as a standard. The absorbance value was colorimetrically determined at 625 nm. The individual intake and intake rate of herbage were monitored and recorded continuously with automatic Biocontrol feeders. The supplements intake was recorded by weighing manually the offer and the refusals after each meal. Individual milk yields (g/ewe) were measured daily at each milking (morning milking at 7:30 AM and afternoon milking at 4:30 PM), using appropriate vessels to collect the milk of each ewe and then weighing it. Individual milk samples were collected twice a week, and each sample was divided into two subsamples. One was refrigerated at 4 °C and analyzed in the same day for the determination of fat, protein, lactose, and urea using a Milkoscan 6000 instrument (Foss Electric, Hillerød, Denmark), and for somatic cell count (SCC) using a Fossomatic 360 instrument (Foss Electric); the second aliquot was frozen at -20 °C for subsequent gas chromatographic (GC) analysis. The determination of fatty acid (FA) first involved the extraction of milk fat, which was carried out in accordance with Nudda et al. (2005). The preparation of fatty acids methyl esters (FAME), including their separation and quantification, were performed according to Correddu et al. (2019), using a 7890A GC System (Agilent Technologies, Santa Clara, CA, USA) provided by a 7693 autosampler (Agilent Technologies), capillary column (CP-Sil 88; 100 m × 0.250 μm i.d., 0.25 μm film thickness, Agilent Technologies) and a flame ionization detector (FID). As reported by Correddu et al. (2019), the oven initial temperature was set at temperature of 45 °C, maintained for 4 min, and then it increased until 175 °C at a rate of 13 °C/min. This temperature was manteined for 27

min, after which the temperature increased at 4 °C/min until reaching 215 °C, and it was held for 35 min. The gas used as carrier was helium at flow rate of 1 mL/min and a pressure of 28 psi. The sample injected was equal to 1 µl with split ratio 1:80. The temperatures of injector and detector were set at 250 °C. The identification of individual FA was performed using ChemStation Upgrade software (OpenLAB CDS GC; Agilent Technologies) by comparison of their retention times with those of methyl ester standards and previously published isomeric profile as detailed by Nudda et al. (2005). The individual FAME was expressed as g/100 g of total FAME. FA groups were determined as follows and expressed as g/100 g of total FAME (Table 6) and as g of FA/100 g of milk (Table 7): SFA as sum of the individual saturated FA; UFA as sum of the individual unsaturated FA; MUFA as sum of the individual monounsaturated FA; PUFA as sum of the individual polyunsaturated FA; anteiso FA as sum of individual anteiso FA; PUFA n-3 as sum of the individual n-3 FA; PUFA n-6 as sum of the individual n-6 FA; total CLA as sum of the individual CLA; SCFA (sum of the individual FA from C4:0 to C10:0), MCFA (sum of the individual FA from C11:0 to C17:0), BCFA as sum of the individual branched-chain FA; OCFA as sum of the odd chain FA; OBCFA as sum of the individual odd- and branched-chain FA. De novo and preformed FA were determined as described by Woolpert et al., (2016): de novo as sum from C4:0 to C14:0, and preformed ≥C18:0, and expressed as g FA/100 g of milk.

The FA profile of lyophilized herbage ryegrass samples was determined in accordance with Kramer et al. (1997) with modification described to Correddu et al. (2015). The GC analysis of herbage samples was performed with the same equipment described for milk samples. To calculate the retention time and area of each individual FAME,

the ChemStation Upgrade software (OpenLAB CDS GC; Agilent Technologies) software was used, and then identified by comparing retention times with those of known standards and with published studies, as described by Nudda et al. (2008). Body weight (BW) was measured weekly with a precision scale Biocontrol AS (Rakkestad Norway), and body condition score (BCS) was evaluated using a scale from 0 to 5 by the same three trained persons.

Statistical analyses

Statistical analyses were carried out using the statistical software Minitab 21.4.1 (© 2023 Minitab, LLC). Data on intake, milk yield and composition, BW and BCS were subjected to General Linear Model (GLM), which included the fixed effects of treatment and day, their interaction and the random effect of animal and were covariate-adjusted using the mean of two pre-experimental measurements (2 April and 5 April), except for the milk fatty acids profile. Data are expressed as means and the group mean differences were assessed using the Tukey Pairwise Comparisons Test. with a P-value < 0.05 considered significant.

The mixed model used was as follows:

$$\mathbf{Y_{ijk} = \mu + bx_{ijk} + treat_i + day_j + treat_i \times day_j + anim_k + Error_{ijk}}$$

where: μ is the mean, b is the regression slope, x_{ijk} is the covariate (i.e. pre-experimental data), $treat_i$ is the treatment effect (i = CNT; BIC), day_j is the experimental day of data collection (j = 3; 6; 9; 12), $treat_i$ x day_j is the interaction, $anim_k$ is the random effect of animal and $Error_{ijk}$ is the experimental error.

Results

Chemical composition of ryegrass herbage

The chemical composition of the ryegrass herbage supplied to the two experimental groups is reported in Table 3.

The dry matter content was relatively high, with a mean of 22.7% of DM and a maximum value of 25.5% of DM. The crude protein (CP) content was very low, with a mean of 7.5% DM and a minimum registered value of 6.7% DM. In contrast, the WSC content was much higher, with an average value of 28% of DM and a maximum recorded value of 29.8% of DM. The NFC content was also elevated, with a mean of 33.2% of DM and a maximum recorded value of 35.6 % of DM. The mean NDF content was 46.8% DM, with a maximum value of 49% of DM. Additionally, the mean ADF content was 27% of DM, and the mean ADL content was 5.7% DM.

Table 3. Average chemical composition, minimum and maximum values and fatty acid profile of the ryegrass herbage (4 grass samples) supplied to the two experimental groups during the experimental period (6-17 April 2023) in correspondence with milk sampling days (3, 6, 9, 12).

Component	Mean	Min	Max
DM (% of fresh matter)	22.7	21.6	25.5
CP (% DM)	7.5	6.7	8.7
Soluble CP (% DM)	3.9	3.4	4.5
ADICP (% DM)	0.66	0.61	0.76
EE (% DM)	1.9	1.7	2.1
Ash (% DM)	9.8	8.9	11.3
NDF (% DM)	46.8	45	49
ADF (% DM)	27	24.7	29.4

Table 3. Continuation.

Component	Mean	Min	Max
ADL (% DM)	5.7	5	6.3
NFC (% DM)	33.2	29.7	35.6
WSC (% DM)	28	26.2	29.8
Fatty acid (g/100 g of total FAME)			
C12:0	0.66	0.51	0.81
C14:0	0.70	0.45	1.03
C15:0	0.17	0.13	0.26
C16:0	20.1	18.3	25.7
C16:1 cis-7	1.53	1.45	1.67
C16:1 cis-9	0.51	0.34	0.73
C17:0	0.28	0.21	0.39
C17:1 cis-10	0.12	0.11	0.15
C18:0	2.03	1.39	2.58
C18:1 cis-9	4.08	2.37	6.55
C18:2 n-6	14.1	13.1	15.3
C20:0	0.50	0.37	0.73
C18:3 n-3	53.5	43.4	60.2
C20:3 n-3 (10, 14, 17)	0.05	0.04	0.05
C24:0	0.85	0.69	1.04

DM, dry matter; CP, crude protein; ADICP, acid detergent insoluble crude protein; EE, ether extract; NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin; NFC, non-fibre carbohydrates; WSC, water-soluble carbohydrates; FAME: fatty acids methyl esters.

Intake

Table 4 reports the covariate-adjusted means of intake data.

The total DMI (g of DM/d) was positively affected by the treatment, with the BIC group showing a higher value compared to the CNT group (2319 vs. 2178 g/DM per

d, respectively; P = 0.03). The daily grass intake was significantly higher in BIC than in CNT group (1488 vs. 1340 g/DM per d, respectively; P = 0.03). Daily concentrate intake was not affected by the treatment, indicating that the difference in total DMI between the groups was due to higher grass intake in the BIC group. Figure 1 reports the grass feeding behavior, as average hourly grass intake (g of DM/hour). The grass intake throughout the day was not uniform. Grass intake before afternoon milking (from 12:30 PM to 4:00 PM) accounted for 35% and 39% of total daily grass intake for CNT and BIC, respectively. However, the grass intake before afternoon milking did not differ between the CNT and BIC groups (Table 4). The WSC intake was significantly higher in the BIC group compared to the CNT group, with values of 418 and 377 g of DM/d, respectively, due to the higher grass intake in the BIC group. Similarly, NDF intake was higher in the BIC group (857 vs. 791 g of DM/d, for BIC and CNT respectively; P = 0.04). Conversely, CP intake did not differ between the two groups.

Table 4. Covariate-adjusted means of intakes from two experimental groups (CNT, BIC) during experimental period (12 days: from 6 to 16 April 2023).

Variable	Treatment		SEM	*P-value*
	CNT	BIC		
Total DMI (g DM/d)	2178	2319	44.2	***0.03***
Daily grass intake (g DM/d)	1340	1488	45.9	***0.03***
Daily concentrate intake (g DM/d)	843	826	17	***0.49***
Grass intake, afternoon (g DM/d)	584	607	36.8	***0.65***
WSC intake (g DM/d)	377	418	12.8	***0.03***
NDF intake (g DM/d)	791	857	21.8	***0.04***
CP intake (g DM/d)	331	335	6.3	***0.64***

Afternoon: from 12:30 PM to 4:00 PM (before the 4:30 PM afternoon milking).

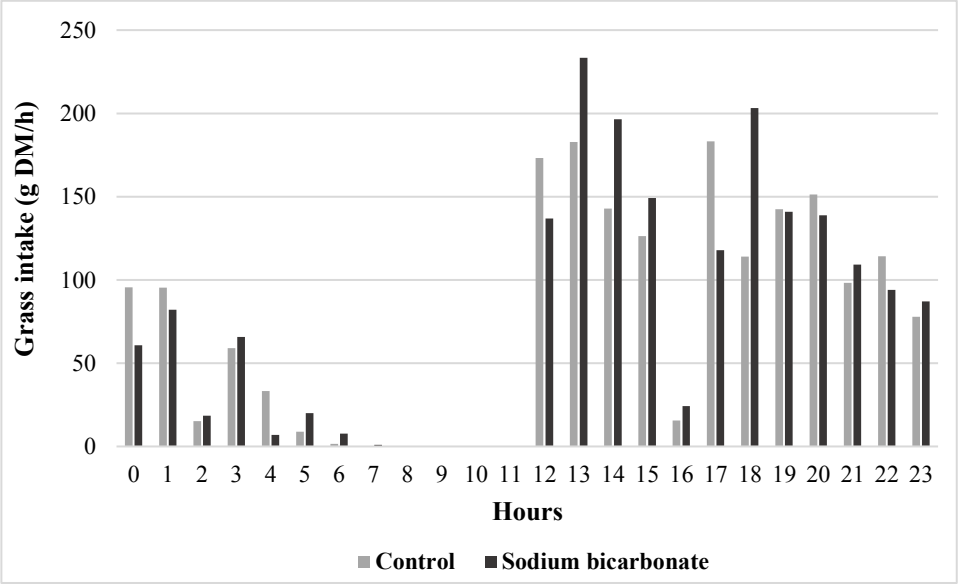

Figure 1. Hourly grass intake as average of 4 experimental days (3, 6, 9, 12), corresponding with milk samplings, for the two treatment groups CNT and BIC.

Milk yield and chemical composition

The covariate-adjusted mean data on milk yield and composition are reported in Table 5. Daily milk yield was not affected by the treatment, with means values of 1859 and 1838 g/d for the CNT and BIC groups, respectively. Additionally, there were no differences in morning or afternoon milk production between the two groups. The daily milk fat content was positively affected by the treatment and was significantly higher in the BIC group compared to the CNT group, with values of 5.79 and 5.41%, respectively ($P = 0.002$). Furthermore, the milk fat content of afternoon milking was higher in BIC group than in CNT group (6.52 vs. 6.01%, respectively; $P < 0.001$). The milk fat content for morning milking was also higher in the BIC group (5.39 and 5.06%, for BIC and CNT, respectively; $P = 0.027$). Daily fat yield was significantly higher in the BIC than in the CNT group (106.5 vs. 99.7 g/milking, respectively; P =

0.051). Additionally, the fat yield for afternoon milking was significantly higher in BIC group compared to the CNT group (42.2 vs. 38.7 g/milking, respectively; P = 0.033). However, the fat yield for morning milking was not affected by the treatment. The daily milk protein content and the protein content of milk from each milking were not influenced by the use of sodium bicarbonate and were low in both groups. The daily milk fat to protein ratio was significantly influenced by the treatment and was higher in the BIC group than in the CNT group (1.21 and 1.13, respectively; P < 0.001), as well as the milk fat to protein for afternoon milking (1.35 vs. 1.25, respectively; P< 0.001) and for morning milking (1.13 vs. 1.06, respectively; P = 0.011), due to the higher milk fat content values in BIC group. Table 6 reported milk fatty acids profile. The complete milk fatty acid profile is reported in Supplementary Table 1. The C17:0 FA was significantly higher in CNT than in BIC group (0.90 vs. 0.82 g/100 g FAME, respectively; P = 0.038). The C17:1 cis-9 was in tendency higher in CNT compared to BIC group (0.29 vs. 0.25 g/100 g FAME, respectively; P = 0.075). The sum of C17:0 and C17:1 cis-9 was significantly higher in CNT than in BIC group (1.19 vs. 1.07 g/100 g FAME, respectively; P = 0.045). The anteiso C17:0 was significantly higher in CNT than in BIC group (0.51 vs. 0.45 g/100 g FAME, respectively; P = 0.003). The linoleic acid (C18:2n-6) was significantly higher in CNT compared to BIC group (2.18 vs. 1.90 g/100 g FAME, respectively; P = 0.023), as well as linolenic acid (C:18:3n-3; CNT: 0.77 and BIC: 0.59 g/100 g FAME; P < 0.001). The total PUFA was significantly higher in CNT than in BIC group (4.64 vs. 4.14 g/100 g FAME, respectively; P = 0.002). Additionally, PUFA n-3 (CNT: 0.87, BIC: 0.69 g/100 g FAME; P < 0.001) and PUFA n-6 (CNT: 2.72, BIC: 2.40 g/100 g FAME; P = 0.015) were significantly higher in CNT than in BIC group. The n-6/n-3 ratio was significantly higher in BIC than in

CNT group (BIC: 3.48, CNT: 3.12 g/100 g FAME; P = 0.038). The OCFA was significantly higher in CNT compared to BIC group (2.77 vs. 2.60 g/100 g FAME, respectively; P = 0.028). Also, the BCFA (CNT: 2.08 and BIC: 1.95 g/100 g FAME; P = 0.005) and OBCFA (CNT: 3.68 and BIC: 3.46 g/100 g FAME; P < 0.001) were significantly higher in CNT than in BIC group. The anteiso FAs were higher in CNT compared to BIC group (1.07 vs. 0.98 g/100 g FAME, respectively; P = 0.007).

In Table 7 are reported the fatty acid groups expressed as g FA/100 g of milk. The BCFA, OCFA and OBCFA were not affected by the treatment. The SFA tends to be higher in BIC than in CNT (2.86 and 2.47 g FA/100 g milk, respectively; P = 0.078). SCFA and MCFA did not differ between CNT and BIC groups. However, the de novo fatty acid group was in tendency higher in BIC group compared to CNT group (1.39 and 1.22 g FA/100 g milk, respectively; P = 0.086). Conversely, the preformed fatty acid group was not affected by the treatment.

Table 5. Covariate-adjusted means of BW, BCS, milk productions and composition in CNT and BIC groups.

Variable	Treatment		SEM	*P-value*
	CNT	BIC		
BW (kg)	57.8	58.2	0.34	***0.41***
BCS	3.1	2.9	0.10	***0.20***
Daily milk yield (g/d)	1859	1838	28.6	***0.62***
Milk yield, afternoon (g/milking)	649	647	16.1	***0.91***
Milk yield, morning (g/milking)	1206	1194	24.5	***0.74***
Daily milk fat content (%)	5.41	5.79	0.08	***0.002***
Milk fat content, afternoon (%)	6.01	6.52	0.09	***< 0.001***
Milk fat content, morning (%)	5.06	5.39	0.10	***0.03***
Daily fat yield (g/d)	99.7	106.5	2.34	***0.05***
Fat yield, afternoon (g/milking)	38.7	42.2	1.12	***0.03***
Fat yield, morning (g/milking)	60.6	64.7	2.03	***0.18***
Daily milk protein content (%)	4.81	4.81	0.04	***0.92***
Milk protein content, afternoon (%)	4.78	4.87	0.03	***0.07***
Milk protein content, morning (%)	4.83	4.77	0.04	***0.34***
Daily milk fat to protein ratio	1.13	1.21	0.01	***< 0.001***
Milk fat to protein ratio, afternoon	1.25	1.35	0.02	***< 0.001***
Milk fat to protein ratio, morning	1.06	1.13	0.02	***0.01***

BW: Body weight; BCS: Body condition score; Afternoon: 4:30 PM milking milk; Morning: 7:30 AM milking milk.

Table 6. Fatty acids profile (total fatty acid methyl esters, FAME; g/100 g of total FAME) of milk samples from 12th experimental days of four sampling days in the CNT and BIC groups.

Variable	Treatment		SEM	P value
	CNT	BIC		
C4:0	2.37	2.39	0.04	***0.74***
C6:0	2.23	2.22	0.06	***0.86***
C8:0	2.30	2.30	0.10	***0.95***
C10:0	7.46	7.88	0.333	***0.39***
C12:0	4.29	4.59	0.187	***0.28***
iso C13:0	0.03	0.02	0.002	***0.88***
C14:0	11.4	11.2	0.21	***0.43***
iso C14:0	0.16	0.14	0.01	***0.22***
C15:0	1.17	1.09	0.04	***0.16***
iso C15:0	0.35	0.33	0.01	***0.28***
C16:0	25.3	26	0.63	***0.44***
C16:1 cis-9	0.78	0.82	0.04	***0.49***
iso C16:0	0.37	0.37	0.01	***0.71***
C17:0	0.90	0.82	0.02	***0.04***
C17:1 cis-9	0.29	0.25	0.01	***0.07***
C17:0 + C17:1 cis-9	1.19	1.07	0.04	***0.04***
C17:1 cis-9/ C17:0	0.32	0.30	0.01	***0.23***
anteiso C17:0	0.51	0.45	0.01	***0.003***
C18:0 (SA)	9.55	10.1	0.25	***0.10***
C18:1 trans 6-8	0.19	0.21	0.007	***0.04***
C18:1t9	0.17	0.18	0.006	***0.26***
C18:1 trans 10	0.28	0.30	0.02	***0.48***
C18:1 trans 11 (VA)	0.80	0.80	0.04	***0.97***
C18:1 trans 12	0.23	0.25	0.01	***0.24***
C18:1 cis-9	19.6	18.7	0.69	***0.27***
C18:1 cis-11	0.38	0.36	0.01	***0.23***
C18:1 cis-12	0.16	0.17	0.008	***0.54***
CLA C18:2 trans 10, cis-12	0.007	0.008	0.0007	***0.67***
C18:2 n-6 (LA)	2.18	1.90	0.07	***0.02***
C18:3 n-3 (LNA)	0.77	0.59	0.02	***< 0.001***
CLA cis-9, trans-11 (RA)	0.45	0.43	0.02	***0.33***
C22:5n-3 (EPA)	0.07	0.06	0.003	***0.07***
C22:5n-3 (DPA)	0.11	0.09	0.003	***0.005***
C22:6n-3 (DHA)	0.02	0.02	0.002	***0.52***
SFA	70.4	71.9	0.64	***0.12***
UFA	30.1	28.7	0.61	***0.11***
MUFA	25.5	24.5	0.59	***0.28***
PUFA	4.64	4.14	0.09	***0.002***

Table 6. Continuation.

Variable	Treatment		SEM	P value
	CNT	BIC		
PUFA n-3	0.87	0.69	0.02	***< 0.001***
PUFA n-6	2.72	2.40	0.08	***0.01***
n-6/n-3 ratio	3.12	3.48	0.11	***0.04***
Total CLA	0.62	0.61	0.02	***0.68***
anteiso FAs	1.07	0.98	0.02	***0.007***
OCFA	2.77	2.60	0.05	***0.03***
BCFA	2.08	1.95	0.03	***0.005***
OBCFA	3.68	3.46	0.03	***< 0.001***

SA = stearic acid; VA = vaccenic acid; LA = linoleic acid; LNA = linolenic acid; RA = rumenic acid; EPA = eicosapentaenoic acid; DPA = docosapentaenoic acid; DHA = docosahexaenoic acid.

Table 7. Fatty acids groups (g fatty acid/100 g milk) of milk samples (of each morning and afternoon milking) from one sampling day (12th experimental day) in the CNT and BIC groups.

Variable	Treatment		SEM	P value
	CNT	BIC		
SCFA	0.68	0.78	0.04	**0.10**
MCFA	1.59	1.82	0.10	**0.12**
De novo fatty acids	1.22	1.39	0.07	**0.086**
Preformed fatty acids (LCFA)	1.32	1.46	0.08	**0.28**
De novo/preformed	0.95	0.97	0.03	**0.69**
BCFA	0.07	0.07	0.004	**0.42**
OCFA	0.10	0.10	0.005	**0.41**
OBCFA	0.17	0.18	0.009	**0.41**
TFA	0.14	0.16	0.01	**0.13**
SFA	2.47	2.86	0.14	**0.08**
UFA	1.05	1.12	0.07	**0.47**
MUFA	0.88	0.95	0.06	**0.42**
PUFA	0.17	0.17	0.01	**0.89**
PUFA n-3	0.03	0.03	0.001	**0.13**
PUFA n-6	0.09	0.09	0.01	**0.92**
n-6/n-3 ratio	3.12	3.49	0.11	**0.04**
Total CLA	0.02	0.02	0.001	**0.22**

SCFA (short-chain fatty acids; from C4:0 to C10:0); MCFA (medium-chain fatty acids; from C11:0 to C17:0); De novo: from C4:0 to C14:0; preformed ≥C18:0; BCFA (sum of individual branched-chain FA); OCFA (sum of individual odd-chain FA); OBCFA (sum of individual odd- and branched-chain FA); TFA: sum of individual trans FA; SFA: sum of the individual saturated FA; UFA: sum of the individual unsaturated FA; MUFA: sum of the individual monounsaturated FA; PUFA: sum of the individual polyunsaturated FA; PUFA n-3: sum of individual n-3 FA; PUFA n-6: sum of individual n-6 FA; total CLA: sum of individual CLA.

Discussion

Nutritional composition of ryegrass herbage

The relatively high DM content, with a mean of 22.7% of DM and a maximum recorded value of 25.5% of DM, was due to the phenological stage (from vegetative to reproductive stage) of the plants at harvest. As the harvest date advances to a later growth stage and plant matures, DM yield, fibre content, and WSC increases, while CP content decreases compared to grass harvested at an earlier vegetative stage (King et al., 2012; Jafari, 2012). Additionally, plant maturity coincides with a reduction in the leaf to stem ratio, and WSC contents are higher in stems (Jafari, 2012). The CP content was very low, with a mean of 7.5% DM and a minimum recorded value of 6.7% DM. The only nitrogen fertilization applied to the field was 130 kg/ha at sowing, and no top-dressing fertilization was performed. However, the WSC content was much higher than that of CP, with an average value of 28% of DM and a maximum value of 29.8% of DM, confirming the negative phenotypic and genotypic correlation between these two nutrients in grasses (Taweel et al., 2005; Jafari, 2012). King et al., (2012) reported that Italian ryegrass (*Lolium multiflorum*), especially when growth without nitrogen fertilizer, is characterized by a high WSC content. In fact, increasing nitrogen fertilizer applications raises CP and nitrate content but reduces herbage DM, WSC and fructans (King et al., 2012; Loaiza et al., 2017; Olszewska, 2021). Additionally, another factor that probably influenced the chemical composition of herbage in this study is that the field was sown in October 2022, and the grass remained uncut and ungrazed until it was mowed at the start of the experimental trial in April 2023. Indeed, defoliation, either through mowing or grazing, leads to a decrease in WSC content, likely due to the use of WSC for regrowth (Siciliano et al., 2017; Kagan, 2022). Mature

stems can contain more fructans compared to grasses that are regularly grazed or cut (Harris et al., 2006). In fact, in plants that are defoliated less frequently, the higher WSC content during late spring coincides with greater stem development, where fructans accumulate (Pollock and Cairns, 1991; Loaiza et al., 2017). The frequency of defoliation is one of the ways to modify crop composition, particularly WSC and CP contents (Loaiza et al., 2017; Merino et al., 2019). In addition, the high fibre components (mean values: NDF 46.8, ADF 27, ADL 5.7% of DM) are consistent with the advanced growth stage at the time of harvest in April. The plant species used in this trial, annual Italian ryegrass (*Lolium multiflorum* Lam. ssp. westervoldicum) of tetraploid type, is one of the most commonly used grass species in Sardinian dairy sheep farm as annual forage crops for grazing, it has a great capacity to accumulate WSC during the winter and spring under low temperatures and sunny days. Indeed, Sun et al. (2023) in a study conducted in New Zealand, characterized by a temperate climate, showed that Italian ryegrass (*Lolium multiflorum*) had the highest levels of soluble nutrients, soluble sugars, and starch in a comparison of nine ryegrass cultivars. Additionally, ploidy influences the WSC content of the plant, and tetraploid types generally achieve higher WSC levels compared to diploid types (Olszewska et al., 2021; Kagan, 2022; Rech et al., 2022). Species and cultivar of the plant, rate of nitrogen fertilization, stage of maturity at harvest, and defoliation frequencies are four of the main factors affecting the yield and composition of plants (King et al., 2012; Merino et al., 2019). The high content of DM, fibre components, NFC and WSC, and very low CP content observed in this study were the result of the grass species and variety used, phenological growth stage at harvest, harvest date, nitrogen fertilization, and the weather conditions. In fact, the mean of minimum and maximum temperatures

in April, the month when the grass was mowed for this trial, were lower than in March, but the mean of solar radiation was higher than in March (Table 1). Additionally, the sum of precipitation recorded from February to April decreased, with very low values. These weather conditions are consistent with the WSC content observed in this trial, given that the WSC content of grasses is negatively related to increases in temperature (Olszewska, 2021), as well as to moisture stress, which limits grass growth and consequently leads to an increase in WSC (Jafari, 2012).

Effect of sodium bicarbonate ($NaHCO_3$) supplementation on intake, milk yield, and composition

The total DMI (g of DM/d) was positively affected by the sodium bicarbonate supplementation, with higher values for the BIC group compared to CNT group (2319 vs. 2178 g/DM per d, respectively; P = 0.03). This result is consistent with the study of Farghaly et al. (2019), conducted on sheep, where supplementation with sodium bicarbonate at doses of 1.5 or 3% increased the DMI of roughage and total DMI. Additionally, the study conducted by Ameen et al. (2023) on lamb fed high-concentrate diets, showed that the addition of dietary sodium bicarbonate (2%) resulted in an increase in total DMI. Furthermore, the study by Sarwar et al. (2007), conducted on buffaloes, reported increased DM and water intakes with increasing sodium bicarbonate levels (0.5%, 1%, and 1.5%). The water intake in our study was not measured. Daily grass intake was significantly higher in the BIC group (1488 vs. 1340 g/DM/d, for BIC and CNT respectively; P = 0.03), while daily concentrate intake was not affected by the treatment, indicating that the difference in total DMI between the groups was due to higher grass intake in the BIC group. Similarly, in the study by

Farghaly et al. (2019), concentrate intake was not influenced, whereas roughage intake was positively affected. In the study by Wittayakun et al. (2015), the addition of 1.2% sodium bicarbonate to the diet of dairy cows fed forage and concentrate did not affect total intake but positively affected roughage intake. In our study, WSC and NDF intake were significantly higher in the BIC group compared to the CNT group, reflecting the greater grass intake in the BIC group, while CP intake did not differ between the groups (Table 4). The higher intake of forage may be related to greater dilution rate of rumen fluid caused by sodium bicarbonate supplementation, which reduces the retention time of DM (Rogers and Davis, 1982; Vicente et al., 2023). Daily milk yield, as well as milk yield from afternoon and morning milking, was not affected by the treatment, in accordance with the results of Wittayakun et al. (2015) obtained with 1.2% sodium bicarbonate in dairy cows, despite the higher DMI in the supplemented group in both their study and ours. In contrast, Farghaly et al. (2019), reported that supplementation with 1.5 and 3% sodium bicarbonate increased milk yield, with 3% resulting in a significantly higher daily milk yield compared to the 1.5% dose. Additionally, the study by Sarwar et al. (2007) reported an increase in milk production in buffaloes fed 1.5% sodium bicarbonate, attributed to higher DMI. This effect is likely due to the higher dosages used in their studies compared to ours, which was approximately 1.0% of total DMI.

The milk fat content was positively affected by sodium bicarbonate supplementation. In fact, despite the significantly higher WSC intake in the BIC group (Table 4), which in other studies was negatively correlated with milk fat content (Satta et al., 2023; Chapter 2 of this Thesis), the daily milk fat content in the BIC group was higher compared to the CNT group (Table 5). However, NDF intake was also significantly

higher in the BIC group than in the CNT group. Therefore, the increased NDF intake likely contributed positively to the rise in milk fat content, as it enhances chewing activity, rumination, and salivation, which help to maintain a better ruminal pH and milk fat production, likely resulting in a combined effect with the use of buffer salt. Milk fat percentage in each milking was significantly higher in the BIC group, even though the milk production did not differ in any milking between the groups, indicating that this is not a dilution related effect. In particular, a strong positive effect was observed in the afternoon milking (6.52 vs. 6.01% for BIC and CNT, respectively; $P < 0.001$), which occurred four hours after sodium bicarbonate supplementation. Fargharly et al. (2019) reported a significant increase in milk fat percentage in ewes fed 3% of $NaHCO_3$, but not with dosage of 1.5%. The positive response to the 1% dosage used in our study can be ascribed to the fact that the effect of dietary buffers on increasing ruminal pH is greater in low-forage diets compared to high-forage diets (Kalscheur et al., 1997), as well as being more pronounced at lower pH and less at higher pH levels (Tripathi et al. 2004; Hu and Murphy, 2005; Calsamiglia et al., 2012). In our study, the animals were fed exclusively ryegrass herbage rich in WSC (28% DM) available ad libitum and concentrates, while in the study by Fargharly et al. (2019) the animals had a diet based on wheat straw ad libitum and concentrates. In our study the forage component was entirely represented by fresh grass, the levels of NDF and likely peNDF were lower, and the moisture content was higher. Our results are consistent with those of Sarwar et al. (2007), who found that milk fat percentage increased in diets supplemented with 1% and 1.5% sodium bicarbonate in buffaloes, despite being fed wheat straw and concentrates. Hu and Murphy (2005), in a metanalysis of early- and mid-lactation dairy cows, describe a positive impact of

sodium bicarbonate on DMI and productivity in cows fed solely or predominantly maize silage-based diets, but found no impact in cows fed different types of roughage, indicating that the response to sodium bicarbonate varies based on the type of diet, whether low or high in forage content, and may be related to differing fibre contents of the forages. In this study, the milk fat to protein ratio for each milking was significantly higher in BIC than in CNT group, although it remained above the inversion threshold of 1.00 in both groups. The low fat to protein ratio is a milk indicator of ruminal acidosis (Atalay, 2019; Kara, 2020). In our study the ratio remained positive, likely due to the low milk protein content observed in both groups, which resulted in a high fat to protein ratio, even at low fat concentration values. In fact, during conditions of rumen acidosis, not only does the milk fat content decreases, but the milk protein content can also decrease (Caja and Bocquier, 2000; Dong et al., 2013) as a consequence of reduced bacterial flow from the rumen to the gut, and this cause a reduction of the essential amino acids required by the mammary gland for its protein synthesis. Daily milk fat production (g/d) was also positively influenced by the treatment and was higher in the BIC group. Additionally, the fat yield in the afternoon milking was significantly higher in the BIC group (42.2 vs. 38.7 g/milking for BIC and CNT, respectively; $P = 0.03$). However, the fat yield in the morning milking was not affected by the treatment. The positive effect of sodium bicarbonate supplementation on both milk fat yield and milk fat percentage was most marked in the afternoon milking, four hours after sodium bicarbonate supplementation. This could be explained by its rapid buffering action at ruminal level, which prevents post-prandial increases of H^+ (Russell and Chow 1993). Additionally, it is important to consider that in the hours before the afternoon milking (from 12:30 to 4:00 PM), grass

intake accounted for 35% and 39% of the total daily grass intake (g DM/d) for CNT and BIC, respectively. This indicates that 35-39% of the daily WSC intake was consumed in four hours, while the rest was ingested over the following 14 hours (from 5:00 PM to 7:00 AM the subsequent day). Furthermore, during the nighttime hours (from 12:00 AM to 7:00 AM), before morning milking, grass intake was very low, accounting for 17% and 14% of the total daily grass intake for CNT and BIC, respectively (Figure 1). Therefore, it is likely that ruminal pH was lower in the hours preceding the afternoon milking compared to morning milking. Since the response to sodium bicarbonate is influenced by the diet and is greater at low ruminal pH (Tripathi et al., 2004; Hu and Murphy, 2005; Calsamiglia et al., 2012), this could further explain the marked effect observed in the afternoon milking. As regards the milk fatty acids profile, the concentrations of C17:0 ($P = 0.038$), the sum of C17:0 and C17:1 cis-9 ($P = 0.045$), and the anteiso C17:0 ($P = 0.003$) were significantly higher in CNT than in BIC, and C17:1 cis-9 ($P = 0.075$) showed a tendency to be higher in CNT group. The rumen amylolytic bacteria are enriched in odd-chain fatty acids, such as C15:0, C17:0 and/or anteiso-branched-chain FAs (Vlaeminck et al., 2006b; Fievez et al., 2012; Civico et al., 2017; Toral et al., 2020). Indeed, C15:0 and C17:0 is positively correlated with the proportion of propionate, with an increase in C17:0 as the amount of concentrate in diets increases (Vlaeminck et al., 2006b; Colman et al., 2020). Additionally, anteiso C17:0 is an indicator of the consumption of high-starch diets, due to the more active elongation of anteiso C15:0 to anteiso C17:0 that may occur as a consequence of consuming high-starch diets (Colman et al., 2010; Civico et al., 2017). Furthermore, odd-chain FAs are formed by the elongation of propionate and valerate and tend to be higher in diets with high concentrates (Vlaeminck et al., 2006a;

Enjalbert et al., 2008; Fievez et al., 2012). The higher milk proportions of C15:0, C17:0 and/or C17:1 cis-9 (and their sum), and anteiso FAs have been reported as biomarkers and indicators of ruminal acidosis, with elevated levels of C17:0 also indicating protein deficiency (Fievez et al., 2012; Toral et al., 2020). In this study, the anteiso FA (g/100 g FAME; $P = 0.007$) were significantly higher in CNT compared to BIC group (Table 6). The C18:3 n-3 was significantly higher in the CNT group than in BIC group ($P < 0.001$; Table 6), despite the greater intake of grass in BIC group. In fact, high forage diets generally tend to produce milk with a greater content of C18:3 n-3 (Toral et al., 2020). Additionally, the levels of PUFA ($P = 0.002$), PUFA n-3 ($P < 0.001$) and PUFA n-6 ($P = 0.015$) were significantly higher in CNT group (g/100 g FAME; Table 6), probably as result from a higher rate of rumen biohydrogenation process in the BIC group compared to CNT group, leading to lower levels of polyunsaturated FAs in the milk of supplemented ewes. The higher PUFA n-6 is due to higher C18:2 n-6 in CNT than in BIC group ($P = 0.023$), that in milk is typically elevated in case of high-concentrates diets and/or low rumen pH (Enjalbert et al., 2008; Sterk et al., 2011; Fievez et al., 2012). Additionally, the de novo fatty acids (g/100 g milk) tended to be higher in BIC compared to CNT ($P = 0.086$; Table 7). De novo fatty acids were found positively associated with milk fat and protein content (Barbano et al., 2014; Woolpert et al., 2016) and are an indicator of ruminal fermentation conditions (Woolpert et al., 2016), being synthesized in the mammary gland from acetate and butyrate from rumen origin. This group of FA is strongly related to nutrition and may be depressed by high dietary carbohydrate and PUFA (Harvatine and Bauman, 2011, cited by Woolpert et al., 2017). The milk FA profile suggests that sodium bicarbonate supplementation improves the ruminal environment and microbial activity, consequently leading to

improved ruminal pH, as reflected in the FA profile. The milk protein content was not affected by the treatment, in accordance with Hadjipanayiotou (1982), Sarwar et al. (2007), Fargharly et al. (2019), Matamoros et al. (2020). However, the milk fat to protein ratio was positively influenced by the $NaHCO_3$ and was higher in the BIC group at each milking, especially in the afternoon milking (1.35 vs. 1.25 for BIC and CNT, respectively; $P < 0.001$). This difference was related to the higher milk fat content, as the milk protein content was not affected by the treatment and the value was low in both groups. The positive effect of sodium bicarbonate on milk fat production can be explained as a consequence of increased water intake and saliva production derived from the use of buffer salt, which leads to a subsequent rise in the rumen liquid dilution rate (Russell and Chow, 1993; Jaramillo-Lopez et al., 2017). This increase in liquid dilution rate increase the outflow of fermentable carbohydrates with the liquid phase from the rumen, which decreases the rate of propionate production and absorption in the rumen, as well as the accumulation of VFA and lactate, resulting in an increase in ruminal pH and, consequently, higher milk fat production (Hart and Doyle, 1985; Russell and Chow, 1993; Calsamiglia et al., 2012; Jaramillo-Lopez et al., 2017; Vicente et al., 2023). The increased ruminal pH resulting from sodium bicarbonate supplementation has been well documented (Rogers and Davis, 1982; Tripathi et al., 2004; Marden et al., 2008; Farghaly et al. 2019, Tayeb et al., 2020; Vicente et al., 2023; Ameen et al., 2023), although it was not observed in some studies (Hadjipanayiotou, 1982; Wittayakun et al., 2015). Additionally, the consequent increase in ruminal pH due to the use of sodium bicarbonate may have increased the acetate to propionate ratio in favour of acetate, which has a positive relationship with milk fat (Hadjipanayiotou, 1982; Hu and Murphy, 2005; Sarwar et

al., 2007; Fargharly et al., 2019). Considering that the response to $NaHCO_3$ use depends on the type of diet and is greater at low ruminal pH (Tripathi et al., 2004; Hu and Murphy, 2005; Calsamiglia et al., 2012), our results indirectly confirm that grazing on WSC-rich grasses can lower ruminal pH, and the use of buffers acting at the ruminal level is beneficial for milk fat content. Additionally, the high moisture content of fresh herbage and its limited stimulus to rumination, may have contributed to a lower pH, as these factors tend to decrease saliva production per kg DM eaten, consequently reducing the buffering action provided by bicarbonate ions present in saliva (Westwood and Lean, 2001; Westwood et al., 2003). In our study, we chose to use sodium bicarbonate due to its rapid buffering action at ruminal level, as WSCs are commonly considered a group of rapidly degraded carbohydrates by ruminal microbes, with fermentation rate of up to 300%/hour, although the rate of fermentation probably differs between different types of WSC depending on their DP, cell-wall integrity, and NDF content (Klevenhusen and Zebeli, 2021). Combining rapid-acting ruminal buffers like sodium bicarbonate with alkalizers such as magnesium oxide (MgO), which have slower action (effective after 24 hours) and a postabsorptive rather than a rumen effect (Calsamiglia et al., 2012), could potentially have an additive effect on milk fat content in pasture-fed ewes, resulting in longer-lasting effects. Additionally, providing hay with high NDF and peNDF content before grazing WSC-rich grasses can be beneficial by increasing chewing time, rumination, salivation, thereby exerting a buffering action in the rumen. However, it is important to consider that this trial was conducted indoors, and the feeding behaviour of grazing ewes may differ in terms of species and plant selection, grass intake rate, and be influenced by time-restricted access to pasture. Thus, WSC intake during grazing under field conditions, in terms of

both quantity and hourly intake rate, and the negative effects of WSC overload could be exacerbated in grazing ewes compared to those observed indoors.

Conclusions

The inclusion of sodium bicarbonate, supplied as a ruminal buffer just before the herbage supply, in diet of ewes fed ryegrass herbage rich in WSC (28% DM) improves total dry matter intake, herbage intake, milk fat percentage, and milk fat yield, especially in afternoon milking, probably due to its stronger short-term action. Sodium bicarbonate supplementation also improves the ruminal environment and microbial activity, consequently leading to improved ruminal pH, as reflected in the the FA profile. Thus, sodium bicarbonate may be used to prevent ruminal sub acidosis and milk fat depression from WSC overload in lactating grazing ewes, when pastures are rich in WSC and low in CP, with the restriction that it must be administered prior to grazing access due to its rapid effect in the rumen. In fact, it could be beneficial to test the combination of rapid-action ruminal sodium bicarbonate with slow-action alkalinizers, such as magnesium oxide, which could exert a long-lasting additive effect, as well as to test different levels of their inclusion in a diet. Additionally, this study highlights that WSC content is an important herbage quality parameter to be considered when formulating rations for pasture-fed dairy sheep, especially due to its influence on milk fat content.

References

Ameen K. A., Safwat M., Salama R., & Awad A. A. 2023. Effect of sodium bicarbonate and ionophore supplementation on nutrients digestibility and growth performance of lambs fed high concentrate diets. Egyptian Journal of Nutrition and Feeds, 26(3), 261-271.

Annatte I., Nuhu S., Ahmed A. H., Saidu S., NA I., NB S., & KB U. 2021. Lactic acidosis-induced laminitis in dairy cattle. In Annual Congress (p. 37).

Atalay H. 2019. Milk fat/protein ratio in ketosis and acidosis. Balıkesir Sağlık Bilimleri Dergisi, 8(3), 143-146.

Barbano D. M., Melilli C. and Overton T. R. 2014. Advanced use of FTIR spectra of milk for feeding and health management. Pages 105–113 in Proc. Cornell Nutrition Conf., Syracuse, NY. Cornell University, Ithaca, NY.

Caja G., & Bocquier F. 2000. Effects of nutrition on the composition of sheep's milk. Cahiers Options Méditerranéennes, 52(59-74).

Calsamiglia S., Blanch M., Ferret A., & Moya D. 2012. Is subacute ruminal acidosis a pH related problem? Causes and tools for its control. Animal feed science and technology, 172(1-2), 42-50.

Cívico A., Núñez Sánchez N., Gómez-Cortés P., de la Fuente M. A., Peña Blanco F., Juárez M., Schiavone A., & Martínez Marín A. L. 2017. Odd-and branched-chain fatty acids in goat milk as indicators of the diet composition. Italian Journal of Animal Science, 16(1), 68-74.

Colman E., Fokkink W. B., Craninx M., Newbold J. R., De Baets B., & Fievez V. 2010. Effect of induction of subacute ruminal acidosis on milk fat profile and rumen parameters. Journal of dairy science, 93(10), 4759-4773.

Correddu F., Nudda A., Battacone G., Boe R., Francesconi A. H. D., & Pulina G. 2015. Effects of grape seed supplementation, alone or associated with linseed, on ruminal metabolism in Sarda dairy sheep. Animal feed science and technology, 199, 61-72.

Correddu F., Cellesi M., Serdino J., Manca M. G., Contu M., Dimauro C., Ibba I., & Macciotta, N. P. P. 2019. Genetic parameters of milk fatty acid profile in sheep: Comparison between gas chromatographic measurements and Fourier-transform IR spectroscopy predictions. Animal, 13(3), 469-476.

Deriaz R. E. 1961. Routine analysis of carbohydrates and lignin in herbage. Journal of the Science of Food and Agriculture, 12(2), 152-160.

Dong H., Wang S., Jia Y., Ni Y., Zhang Y., Zhuang S., Shen X., & Zhao R. 2013. Long-term effects of subacute ruminal acidosis (SARA) on milk quality and hepatic gene expression in lactating goats fed a high-concentrate diet. PloS one, 8(12), e82850.

Enjalbert F., Videau Y., Nicot M. C., & Troegeler-Meynadier A. 2008. Effects of induced subacute ruminal acidosis on milk fat content and milk fatty acid profile. Journal of animal physiology and animal nutrition, 92(3), 284-291.

Farghaly M. M., Hassan E. H., & Abdo S. G. 2019. Influence of sodium bicarbonate supplementation on nutrients digestibility, milk production, rumen fermentation and some blood parameters in sheep. Egyptian Journal of Animal Production, 56(2), 71-77.

Fievez V., Colman E., Castro-Montoya J. M., Stefanov I., & Vlaeminck B. 2012. Milk odd-and branched-chain fatty acids as biomarkers of rumen function—An update. Animal Feed Science and Technology, 172(1-2), 51-65.

Hadjipanayiotou M. 1982. Effect of sodium bicarbonate and of roughage on milk yield and milk composition of goats and on rumen fermentation of sheep. Journal of Dairy Science, 65(1), 59-64.

Hart S. P., & Doyle J. J. 1985. Adaptation of early-weaned lambs to high-concentrate diets with three grain sources, with or without sodium bicarbonate. Journal of Animal Science, 61(4), 975-984.

Hu W., & Murphy M. R. 2005. Statistical evaluation of early-and mid-lactation dairy cow responses to dietary sodium bicarbonate addition. Animal feed science and technology, 119(1-2), 43-54.

Jafari A. A. 2012. Environmental and genetic variation for water soluble carbohydrate content in cool season forage grasses. Carbohydrates—Comprehensive Studies on Glycobiology and Glycotechnology; IntechOpen: Rijeka, Croatia, 373-388.

Jaramillo-López E., Itza-Ortiz M. F., Peraza-Mercado G., & Carrera-Chávez J. M. 2017. Ruminal acidosis: strategies for its control. Austral journal of veterinary sciences, 49(3), 139-148.

Kagan I. A. 2022. Water-and Ethanol-Soluble Carbohydrates of Temperate Grass Pastures: a Review of Factors Affecting Concentration and Composition. Journal of Equine Veterinary Science, 110, 103866.

Kalscheur K. F., Teter B. B., Piperova L. S., & Erdman R. A. 1997. Effect of dietary forage concentration and buffer addition on duodenal flow of trans-C18: 1 fatty acids and milk fat production in dairy cows. Journal of dairy science, 80(9), 2104-2114.

Kara K. 2020. Milk urea nitrogen and milk fatty acid compositions in dairy cows with subacute ruminal acidosis. Veterinární medicína, 65(9).

King C., McEniry J., Richardson M., & O'kiely P. 2012. Yield and chemical composition of five common grassland species in response to nitrogen fertiliser application and phenological growth stage. Acta Agriculturae Scandinavica, Section B–Soil & Plant Science, 62(7), 644-658.

Kleen J. L., Hooijer G. A., Rehage J., & Noordhuizen J. P. T. M. 2003. Subacute ruminal acidosis (SARA): a review. Journal of Veterinary Medicine Series A, 50(8), 406-414.

Klevenhusen F., Zebeli Q. 2021. A review on the potentials of using feeds rich in water-soluble carbohydrates to enhance rumen health and sustainability of dairy cattle production. Journal of the Science of Food and Agriculture, 101(14), 5737-5746.

Kramer J. K., Fellner V., Dugan M. E., Sauer F. D., Mossoba M. M., & Yurawecz M. P. 1997. Evaluating acid and base catalysts in the methylation of milk and rumen fatty acids with special emphasis on conjugated dienes and total trans fatty acids. Lipids, 32, 1219-1228.

Loaiza P. A., Balocchi O., & Bertrand A. 2017. Carbohydrate and crude protein fractions in perennial ryegrass as affected by defoliation frequency and nitrogen application rate. Grass and Forage Science, 72(3), 556-567.

Marden J. P., Julien C., Monteils V., Auclair E., Moncoulon R., & Bayourthe C. 2008. How does live yeast differ from sodium bicarbonate to stabilize ruminal pH in high-yielding dairy cows?. Journal of Dairy Science, 91(9), 3528-3535.

Matamoros C., Cai J., Patterson A. D., & Harvatine K. J. 2021. Comparison of the effects of short-term feeding of sodium acetate and sodium bicarbonate on milk fat production. Journal of dairy science, 104(7), 7572-7582.

Merino V. M., Balocchi O. A., & Rivero M. J. 2019. Milk production, milk quality, and behaviour of dairy cows grazing on swards with low and high water-soluble carbohydrates content in autumn: A pilot trial. Animals, 9(12), 1012.

Molle G., Gregorini P., Cabiddu A., Decandia M., Giovanetti V., Sitzia M., Dattena M., Porcu C., Pasciu V., Gonzalez-Bulnes A., Berlinguer F., Cannas A. 2022. Does the timing of pasture allocation affect rumen and plasma metabolites and ghrelin, insulin and cortisol profile in dairy ewes?. Frontiers in Veterinary Science, 9, 969950.

Niness K. R. 1999. Inulin and oligofructose: what are they?. The Journal of nutrition, 129(7), 1402S-1406S.

Noronha A. D. F., Freitas S. L. R., Rodrigues D. F., Mendes F. F., Miguel M. P., Cunha P. H. J., Fioravanti S., & Silva, L. A. F. (2019). Characterization of ruminal acidosis and initial phase of laminitis inducted by oligofructose in crossbred calves. Pesquisa Veterinária Brasileira, 39, 99-106.

Nudda A., McGuire M. A., Battacone G., & Pulina G. 2005. Seasonal variation in conjugated linoleic acid and vaccenic acid in milk fat of sheep and its transfer to cheese and ricotta. Journal of Dairy Science, 88(4), 1311-1319.

Nudda A., Palmquist D. L., Battacone G., Fancellu S., Rassu S. P. G., & Pulina G. 2008. Relationships between the contents of vaccenic acid, CLA and n− 3 fatty acids of goat milk and the muscle of their suckling kids. Livestock Science, 118(3), 195-203.

Olszewska M. 2021. Effects of cultivar, nitrogen rate and harvest time on the content of carbohydrates and protein in the biomass of perennial ryegrass. Agronomy, 11(3), 468.

Pollock C. J., & Cairns A. J. 1991. Fructan metabolism in grasses and cereals.

Pollitt C. C., & Milinovich G. J. 2017. Experimental models of laminitis: oligofructose overload. Equine Laminitis, 59-63.

Rech Â. F., de Arruda Cordoba U., Fávaro V. R., Flaresso J. A. 2022. Principais características de cultivares de azevém-anual utilizados no estado de Santa Catarina, Brasil. Agropecuária Catarinense, 35(1), 85-92.

Rivero M. J., Anrique R. 2015. Milk fat depression syndrome and the particular case of grazing cows: A review. Acta Agriculturae Scandinavica, Section A—Animal Science, 65(1), 42-54.

Rogers J. A., & Davis C. L. 1982. Rumen volatile fatty acid production and nutrient utilization in steers fed a diet supplemented with sodium bicarbonate and monensin. Journal of Dairy Science, 65(6), 944-952.

Russell J. B., & Chow J. M. 1993. Another theory for the action of ruminal buffer salts: decreased starch fermentation and propionate production. Journal of Dairy Science, 76(3), 826-830.

Sarwar M., Shahzad M. A., & Nisa M. U. 2007. Influence of varying level of sodium bicarbonate on milk yield and its composition in early lactating Nili Ravi buffaloes. Asian-Australasian journal of animal sciences, 20(12), 1858-1864.

Satta F., Porcu M.A., Ledda A., Carta S., Deiana L., Cannas A. 2023. The concentration of water-soluble carbohydrates in the pasture grazed by lactating

ewes might be a main cause of milk fat depression. ASPA Conference, Monopoli (Bari, Italy), 13-16 June 2023.

Siciliano P. D., Gill J. C., Bowman M. A. 2017. Effect of sward height on pasture nonstructural carbohydrate concentrations and blood glucose/insulin profiles in grazing horses. Journal of Equine Veterinary Science, 57, 29-34.

Sterk A., Johansson B. E. O., Taweel H. Z. H., Murphy M., Van Vuuren A. M., Hendriks W. H., & Dijkstra J. 2011. Effects of forage type, forage to concentrate ratio, and crushed linseed supplementation on milk fatty acid profile in lactating dairy cows. Journal of Dairy Science, 94(12), 6078-6091.

Sun X., Chen A., & Li J. 2023. Chemical, Physical, and Degradation Characteristics of Ryegrass Cultivars Grown in Autumn and Winter for Dairy Cows. Animals, 13(20), 3158.

Taweel H. Z., Tas B. M., Smit H. J., Elgersma A., Dijkstra J., & Tamminga S. 2005. Effects of feeding perennial ryegrass with an elevated concentration of water-soluble carbohydrates on intake, rumen function and performance of dairy cows. Animal Feed Science and Technology, 121(3-4), 243-256.

Tayeb M. A. M., Al-Zeadan O. A. G., & Basher N. M. 2020. Effect of using sodium bicarbonate and mixture of vitamin e and selenium in production performance of awassi ewes. The Iraqi Journal of Agricultural Science, 51(1), 311-316.

Thoefner M. B., Pollitt C. C., Van Eps A. W., Milinovich G. J., Trott D. J., Wattle O., & Andersen P. H. 2004. Acute bovine laminitis: a new induction model using alimentary oligofructose overload. Journal of dairy science, 87(9), 2932-2940.

Toral P. G., Hervás G., Della Badia A., Gervais R., & Frutos P. 2020. Effect of dietary lipids and other nutrients on milk odd-and branched-chain fatty acid composition in dairy ewes. Journal of dairy science, 103(12), 11413-11423.

Tripathi M.K., Santra A., Chaturvedi O.H., Karim S.A. 2004. Effect of sodium bicarbonate supplementation on ruminal fluid pH, feed intake, nutrient utilization and growth of lambs fed high concentrate diets. Animal Feed Science and Technology 111, 27-39.

Van Eps A. W., & Pollitt C. C. 2006. Equine laminitis induced with oligofructose. Equine veterinary journal, 38(3), 203-208.

Vicente A. C. S., Carlis M. S. D. P., Santos I. J. D., Silva A. L. A. D., Dias P. C. G., Eckermann N. R., Teixeira de Souza T., Polizel D. M., Baggio M., Socolovski Biava J., Vaz Pires A., & Ferreira E. M. 2023. Intake and apparent nutrient digestibility, ruminal parameters, and nitrogen balance of lambs fed with corn and forage-free diets with sodium bicarbonate. Ciência Rural, 53(9), e20220406.

Vlaeminck B., Fievez V., Cabrita A. R. J., Fonseca A. J. M., & Dewhurst R. J. 2006a. Factors affecting odd-and branched-chain fatty acids in milk: A review. Animal feed science and technology, 131(3-4), 389-417.

Vlaeminck B., Fievez V., Tamminga S., Dewhurst R. J., Van Vuuren A., De Brabander D., & Demeyer D. 2006b. Milk odd-and branched-chain fatty acids in relation to the rumen fermentation pattern. Journal of Dairy Science, 89(10), 3954-3964.

Watts K. A. 2005. A review of unlikely sources of excess carbohydrate in equine diets. Journal of Equine Veterinary Science, 25(8), 338-344.

Watts, K. 2008. The influence of solar radiation and temperature on the diurnal fluctuation of NSC in grass. Copyright 2008. Rocky Mountain Research & Consulting, Inc. https://static1.squarespace.com/static/5e7fddf934de306c2b8cc313/t/5e8c82935a255f19a8a2cb9f/1586266771795/DiurnalFluctNSC.pdf

Westwood C. T., Bramley E., Lean I. J. 2003. Review of the relationship between nutrition and lameness in pasture-fed dairy cattle. New Zealand Veterinary Journal, 51(5), 208-218.

Westwood C. T., & Lean I. J. 2001. Nutrition and lameness in pasture-fed dairy cattle. In PROCEEDINGS-NEW ZEALAND SOCIETY OF ANIMAL PRODUCTION (Vol. 61, pp. 128-134). New Zealand Society of Animal Production; 1999.

Wittayakun S., Innaree S., Innaree W., & Chainetr W. 2015. Supplement of sodium bicarbonate, calcium carbonate and rice straw in lactating dairy cows fed pineapple peel as main roughage. Slovak Journal of Animal Science, 48(2), 71-78.

Woolpert M. E., Dann H. M., Cotanch K. W., Melilli C., Chase L. E., Grant R. J., & Barbano D. M. 2016. Management, nutrition, and lactation performance are related to bulk tank milk de novo fatty acid concentration on northeastern US dairy farms. Journal of dairy science, 99(10), 8486-8497.

Woolpert M. E., Dann H. M., Cotanch K. W., Melilli C., Chase L. E., Grant R. J., & Barbano D. M. 2017. Management practices, physically effective fibre, and ether extract are related to bulk tank milk de novo fatty acid concentration on Holstein dairy farms. Journal of dairy science, 100(6), 5097-5106.

Appendix Chapter 3

Supplementary Table 1. Complete milk fatty acids profile (total fatty acid methyl esters, FAME; g/100 g of total FAME).

Supplementary Table 1. Complete milk fatty acids profile (total fatty acid methyl esters, FAME; g/100 g of total FAME).

Item	Treatment		SEM	P-value
	CNT	BIC		
C4:0	2.37	2.39	0.04	**0.741**
C6:0	2.23	2.22	0.06	**0.861**
C7:0	0.02	0.02	0.002	**0.498**
C8:0	2.30	2.30	0.10	**0.955**
C9:0	0.23	0.22	0.006	**0.461**
C10:0	7.46	7.88	0.333	**0.390**
C10:1	0.01	0.01	0.0003	**0.142**
C11:0	0.37	0.36	0.02	**0.795**
C12:0	4.29	4.59	0.187	**0.282**
iso C13:0	0.03	0.02	0.002	**0.876**
anteisoC13:0	0.05	0.05	0.003	**0.694**
iso C14:0	0.16	0.14	0.01	**0.224**
C14:0	11.4	11.2	0.21	**0.433**
iso C15:0	0.35	0.33	0.01	**0.281**
anteisoC15:0	0.51	0.48	0.02	**0.294**
C14:1c9	0.19	0.18	0.01	**0.532**
C15:0	1.17	1.09	0.04	**0.163**
C15:1 c9	0.06	0.06	0.002	**0.452**
iso C16:0	0.37	0.37	0.01	**0.714**
C16:0	25.3	26	0.63	**0.445**
C16:1t4	0.01	0.01	0.002	**0.06**
C16:1t5	0.03	0.01	0.005	**0.07**
C16:1t6-7	0.04	0.02	0.006	**0.133**
isoC17:0	0.04	0.04	0.002	**0.342**
C16:1t9	0.58	0.53	0.01	**0.022**
C16:1t10	0.01	0.01	0.001	**0.678**
C16:1t11-t12	0.05	0.05	0.002	**0.692**
C16:1c7	0.31	0.32	0.01	**0.872**
anteisoC17:0	0.51	0.45	0.01	**0.003**
C16:1 cis-9	0.78	0.82	0.04	**0.489**
C16:1c10	0.03	0.02	0.002	**0.313**
C16:1c11	0.01	0.01	0.001	**0.70**
3,7,11,15-Tetramethyl-16:0	0.03	0.03	0.001	**0.336**
C17:0	0.90	0.82	0.02	**0.038**

Supplementary Table S1. Continuation.

Item	Treatment		SEM	P-value
	CNT	BIC		
iso C18:0	0.07	0.06	0.004	**0.065**
C17:1 c6-7	0.02	0.02	0.02	**0.463**
C17:1c8	0.06	0.06	0.002	**0.203**
C17:1 cis 9	0.29	0.25	0.01	**0.075**
C18:0 (SA)	9.55	10.1	0.25	**0.105**
C18:1t4	0.01	0.01	0.001	**0.096**
C18:1t5	0.01	0.01	0.001	**0.033**
C18:1 trans 6-8	0.19	0.21	0.007	**0.045**
C18:1t9	0.17	0.18	0.006	**0.265**
C18:1 trans 10	0.28	0.30	0.02	**0.482**
C18:1 trans 11 (VA)	0.80	0.80	0.04	**0.973**
C18:1 trans 12	0.23	0.25	0.01	**0.245**
C18:1t13-t14	0.63	0.67	0.04	**0.495**
C18:1 cis-9	19.6	18.7	0.69	**0.274**
C18:1 cis-11	0.38	0.36	0.01	**0.232**
C18:1 cis-12	0.16	0.17	0.008	**0.542**
C18:1c13	0.05	0.05	0.002	**0.315**
C18:1t16-c14	0.33	0.34	0.01	**0.403**
C19:0/C18:1c15	0.25	0.24	0.01	**0.56**
C18:2t10t14	0.02	0.03	0.002	**< 0.001**
C18:2t11t15	0.01	0.01	0.002	**0.499**
C18:2t9t12	0.01	0.01	0.001	**0.599**
C18:2c9t13	0.30	0.28	0.01	**0.388**
C17cyclo	0.11	0.13	0.01	**0.087**
C18:2t8c13	0.14	0.13	0.005	**0.67**
C18:2c9t12	0.09	0.09	0.002	**0.498**
C18:1c16	0.02	0.02	0.001	**0.987**
C18:2t9c12	0.02	0.02	0.001	**0.45**
C18:2t11c15	0.15	0.13	0.01	**0.362**
C18:2 n-6 (LA)	2.18	1.90	0.07	**0.023**
C18:2t12c15	0.04	0.04	0.001	**0.903**
C18:2c12c15	0.01	0.01	0.001	**0.579**
C20:0	0.27	0.27	0.007	**0.407**

Supplementary Table S1. Continuation.

Item	Treatment		SEM	P-value
	CNT	BIC		
Δ7,9 17:2	0.02	0.02	0.001	**0.38**
C18:3n6	0.06	0.08	0.004	**0.01**
C20:1c9	0.01	0.01	0.0003	**0.319**
C20:1c11	0.04	0.04	0.002	**0.361**
C18:3 n-3 (LNA)	0.77	0.59	0.02	**< 0.001**
CLA cis-9, trans-11 (RA)	0.45	0.43	0.02	**0.326**
C20:1c15	0.01	0.01	0.001	**0.655**
CLAt9c11/C21:0	0.09	0.09	0.004	**0.946**
CLA C18:2 trans 10, cis-12	0.007	0.008	0.0007	**0.672**
CLAt12t14	0.01	0.01	0.001	**0.099**
CLAt11t13	0.02	0.02	0.001	**0.15**
C20:2n9	0.02	0.02	0.001	**0.515**
CLAt9t11*	0.04	0.05	0.003	**0.053**
C18:4n3	0.01	0.01	0.001	**0.899**
C20:2n6	0.02	0.02	0.001	**0.447**
C20:3n9	0.05	0.05	0.003	**0.519**
C22:0	0.14	0.14	0.01	**0.986**
C20:3n6	0.03	0.03	0.001	**0.797**
10,14,17 C20:3*	0.01	0.01	0.001	**0.264**
C22:1n9	0.01	0.01	0.001	**0.519**
C20:3n3	0.01	0.01	0.0005	**0.715**
C20:4n6	0.17	0.15	0.01	**0.112**
C23:0	0.07	0.07	0.04	**0.789**
C20:4n3	0.01	0.01	0.0002	**0.323**
C22:2n6	0.13	0.12	0.01	**0.11**
C22:5n-3 (EPA)	0.07	0.06	0.003	**0.074**
C24:0	0.06	0.06	0.003	**0.451**
C22:3n6	0.01	0.01	0.0003	**0.256**
C24:1c15	0.03	0.02	0.002	**0.47**
C22:4n6	0.01	0.01	0.001	**0.388**
C25:0	0.01	0.01	0.001	**0.865**
C26:0	0.04	0.04	0.003	**0.585**
C22:5n-3 (DPA)	0.11	0.09	0.003	**0.005**
C22:6n-3 (DHA)	0.02	0.02	0.002	**0.522**

Abbreviations: EPA = eicosapentaenoic acid; DPA = docosapentaenoic acid; DHA = docosahexaenoic acid.

CHAPTER 4

Relationship between diet composition, grazed herbage and milk composition: a survey on five Sardinian dairy sheep farms

Abstract

In the last decade, a decrease in milk fat content has been observed in Sardinia during winter and spring months, leading to frequent inversions of the fat to protein ratio and a subsequent decrease in cheese yields. This study aimed to investigate the relationships between diet composition, grazed herbage, and milk composition of dairy ewes during these months in Sardinia. A survey was conducted on five dairy sheep farms in Sardinia, three of which located in Central-Northern Sardinia in the countries of Orune (800 m asl; 40°25'33.1"N 9°18'36.6"E), Nule (700 m asl; 40°27'22.5"N 9°13'47.5"E), and Oschiri (200 m asl; 40°43'42.7"N 9°04'37.5"E), and two located in Southern Sardinia in the countries of Villamassargia (120 m asl; 39°16'18.2"N 8°41'44.3"E) and Iglesias (200 m asl; 39°16'06.0"N 8°34'38.5"E). The survey was carried out from January to April 2024. Bulk milk samples, grazed herbage samples and data on the feed management were collected twice a month. The general monthly trend of grazed forage crops composition showed high crude protein (CP) content, although decreasing, and low water-soluble carbohydrates (WSC) from January to April. The milk composition trend indicated low milk fat concentration throughout all months of the survey; however, there was no inversion of the fat to protein ratio on any of the five farms. The relationships between ration components and milk composition were determined using linear regression analysis. The percentage of total forage in the ration was positively correlated, while the percentage of concentrates negatively correlated, with the milk fat content ($P = 0.007$). The percentage of starch in the ration was negatively correlated with both milk fat content ($P = 0.01$) and the milk fat to protein ratio ($P = 0.002$). Additionally, the percentage of non-fibrous carbohydrates (NFC) in the ration was inversely correlated with both milk fat content

(P = 0.006) and the milk fat to protein ratio (P = 0.001). In contrast, the neutral detergent fibre (NDF) of the ration was positively associated with the milk fat to protein ratio (P = 0.01). There was no correlation between the NDF content of the herbage and the milk fat and protein contents, while the amount of NFC (P = 0.056) of the herbage was inversely related to milk fat content. The relationship between herbage fat content and milk fat to protein ratio was also negative (P = 0.04). The grazed forage crops CP (P < 0.001) and dietary CP (P = 0.056) were positively associated with milk urea. In conclusion, the forage to concentrate ratio is confirmed as a parameter strongly and positively associated with milk fat content, whereas a diet rich in starch and NFC may negatively affect milk fat content and, consequently, cheese yields. Regarding grazed herbage, negative relationships were observed between milk fat content and herbage NFC, whereas the NDF content of herbage did not influence this milk component.

Keywords

milk fat content, grazing dairy ewes, precision feeding, feeding techniques, grazing nutritional unbalances.

Introduction

Dairy sheep farming systems in Sardinia are semi-extensive, and herbage from pastures is managed to optimize its utilization with the traditional scheme based on grazing during the day, and in certain season during the night, supplementation of concentrates at milking and of hay at night. In recent years, many dairy sheep farms have adopted a mixed system that combines the use of total mixed rations (TMR) prepared by mixed wagons with pasture use. Weather conditions significantly influence these farming systems, affecting both the quantity and chemical composition of herbage, which subsequently impacts milk production and composition of grazing ewes. During the winter and spring months, when lactating ewes have high nutritional requirements and pastures are in maximum qualitative development, nutritional imbalances related to grazing often occur and can negatively affect milk yield and composition, especially the milk fat content, which is particularly sensible to nutritional changes (Atalay, 2019), as well as the milk fat to protein ratio. In the last decade, a decrease in milk fat content has been observed in Sardinia during these months, leading to frequent inversions of the fat to protein ratio and a subsequent decrease in cheese yields. During these months, herbage is characterized by low NDF and physically effective NDF (peNDF), high CP or high NFC and/or WSC, which can lead to nutritional imbalances if not well managed, affecting negatively milk production and composition. Improper use of these pastures can cause a problem related to excess dietary protein or to WSC overload, leading to elevated milk urea levels (Cannas et al., 1998; Cannas, 2004; Molle et al., 2008) or decreased milk fat content (Chapter 2 of this Thesis), respectively. Indeed, the use of pasture in early stages of growth can increase DM intake (DMI) and energy intake, milk yield and milk

protein concentration, but it can also increase the risks of rumen pH reduction due to shortage of fibre able to stimulate rumination (Morand-Fehr et al., 2007). Additionally, variation in milk composition is greater when pastures consist of a single species compared to mixed species (Morand-Fehr et al., 2007); in Sardinia the use of pasture of pure annual ryegrass, especially tetraploid types, or oats are commonly used. Pastures of mixed species provide a better nutrients balance, whereas single species pastures can lead to specific nutrient overloads and deficiency of others. For instance, during the winter and spring months, characterized by low temperatures and sunny days grazing on grasses, especially tetraploid annual ryegrass, can lead to a reduction in milk fat content due to WSC overload in dairy sheep (Porcu et al., 2023). Additionally, incorrect grazing times or improper concentrate and fibre rich feeds supplementation (in terms of type or dose) prior to grazing can exacerbate these issues. For example, high levels of starch may be risky for grazing sheep in winter and spring when herbage can be low in NDF and high in WSC (Molle et al., 2008). A common feeding practice to limit these problems consists in a supply of an extra meal just before grazing to synchronize nutrient utilization from the herbage (Cannas, 2004). The most common supplements used for this practice (often called “third meal”) are beet pulp, rich in pectins and digestible fibre, useful to provide energy and fibre without risks of acidosis, both in case of pasture rich in CP or in WSC. Additionally feeding sequences, i.e the timing of supply of each ration component, are another important aspect. Estimating appropriate feeding time is difficult, as most diet balancing systems are based on static models and assume a regular feeding pattern during the whole day (Molle et al., 2008). Proper rations balancing is necessary for adequate feeding (Cannas, 2004) and becomes more complex when herbage is included in the ewe’s

diet. The forage to concentrate ratio is another important rationing parameter that influences milk yield and composition, particularly milk fat content. A higher forage to concentrate ratio is associated with increased milk fat content in dairy sheep (Morand-Fehr et al., 2007; Dong et al., 2013; Atalay, 2019; Angeles-Hernandez et al., 2020). In fact, this ratio affects the acetate to propionate ratio in the rumen, which increases with higher forage content and decreases as concentrate levels increase (Pulina et al., 2006; Kooman et al., 2018; Angeles-Hernandez et al., 2020). These nutritional parameters significantly influence milk composition, particularly milk fat content, and consequently affect cheese yields, the primary destination for sheep milk produced in Sardinia. Thus, this study aims to investigate the relationships between diet composition, grazed herbage, and milk composition, particularly the milk fat percentage of dairy ewes during the winter and spring months in Sardinia. With this goal, a survey was conducted from January to April 2024 on five dairy sheep farms in Sardinia.

Materials and methods

Data, sampling, and analyses

This survey was conducted from January to April 2024 on five dairy sheep farms (1, 2, 3, 4, 5) raising Sarda breed sheep in Sardinia (Italy). Farms 1, 2 and 3 are located in the north center of Sardinia (Orune, Nule, Oschiri, respectively: 200-800 m a.s.l.), while farms 4 and 5 are situated in the south of Sardinia (Villamassargia and Iglesias, respectively: 100-200 m a.s.l.). On each farm, the sheep were milked twice a day (evening and morning) using an automatic milking machine. The feeding system on

each farm is mixed, consisting of the use of TMR prepared by mixed wagon, concentrate supplied at milking and the utilization of cultivated forage crops for grazing.

The bulk milk samples and grazed forage crops samples on each farm were collected twice a month. On each sampling day, the following data were recorded: number of lactating sheep, number of sheep in suckling and milking period, flock milk yield, feeding management (type and quantity of forage, concentrate, and supplements), forage crops used for grazing (species, variety, sowing date, fertilization date, type of fertilization), grazing access time per day and start time, and feeding sequence (time of administration of each feed). Animal diet composition was estimated based on the intake of forage (hay, silage), concentrate and of grazed herbage of the forage crops. Herbage intake was estimated by calculating the difference between the expected intake of the sheep, on the different milk sampling days, and the total amount of other feed supplied with the rest of the ration. The expected total intake was estimated according to the flock characteristics (body weight, mean milk yield and composition) using the Small Ruminant Nutrition System (SRNS) software (Tedeschi et al., 2010). The milk yield of the flock was calculated by considering the total amount of milk produced and the number of lactating ewes on each sampling day. The flocks comprised lactating ewes and ewes in simultaneous suckling and milking period; thus, the total yield was corrected by adding the estimated milk consumed by the lambs. The estimation of total milk yield was calculated by adding a quantity of 877 g/d per lamb for each ewe in simultaneous suckling and milking period, as estimated by Pulina et al. (1985). On each sampling day, a daily bulk milk sample (a mixture of both milk of morning and evening milkings) was collected and analyzed for fat, protein, lactose,

and urea using a Milkoscan 6000 instrument (Foss Electric, Hillerød, Denmark), and for somatic cell count (SCC) using a Fossomatic 360 instrument (Foss Electric) in LAORE Laboratories (Oristano, Italy). The samples of grazed herbage were collected on the same day as the milk sampling. Dry matter (DM), neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL), crude protein (CP), water soluble carbohydrates (WSC), soluble protein, ash and ether extract (EE) were determined by near-infrared reflectance spectroscopy (NIRS) in Cargill Laboratories (Fiorenzuola D'Arda, Italy). Non-fibre carbohydrates (NFC) were calculated as follows: 100 − (NDF + CP + ash + EE).

Statistical Analysis

Statistical analyses were performed using the statistical software Minitab 21.4.1 (© 2023 Minitab, LLC). Data on milk yield, milk composition, and diet composition were analyzed using one-way analysis of variance (ANOVA), with a P-value < 0.05 considered significant. Data on grazed forage crops chemical composition was analyzed with a General Linear Model (GLM), with an ANOVA which included the fixed effects of months and botanical composition.

The statistical model used was as follow:

$$\boldsymbol{Yij = \mu + month_i + fam_j + month_i\, x\, fam_j + Error_{ij}}$$

where: μ is the mean, $month_i$ is the month of samples collection (i = January; February, March, April), fam_j is the botanical composition of samples (j = Grasses, Mix of grasses and legumes), $month_i$ x fam_j is the interaction, and $Error_{ij}$ is the experimental error.

The relationships between diet composition, grazed forage crops, and milk chemical composition were analyzed using simple linear regression analysis.

Results

Chemical composition of grazed forage crops, diet composition and milk composition

The chemical composition of grazed forage crops from January to April divided for botanical composition is reported in Table 1.

The farms had several forage crops utilized for grazing. On each sampling day, samples were collected from the forage crops used that date (a total of 70 samples). The grazed forage crops have been divided based on botanical composition into grasses (G; 14 samples) and a mix of grasses and legumes (M; 56 samples). The grasses species included tetraploid annual ryegrass (*Lolium multiflorum* Lam.) of different varieties (10 samples of westervoldicum variety and 1 sample of Italicum variety), oat (*Avena sativa* L.), and a mix of annual ryegrass and oat (1 sample). The mix of grasses and legumes, which represented the majority of samples, included different combinations of annual ryegrass, oats and different clover species (*Trifolium michelianum*, *Trifolium incarnatum, Trifolium alexandrinum, Trifolium repens, Trifolium squarrosum*). Due to the type of investigation, the specific botanical composition of the mixes, in terms of single contribution of every species present, could not be assessed. The mean of dry matter (DM) content showed increasing values from January to April ($P < 0.001$) in both grasses (from 10.2 to 17.3% DM) and mix (from 11 to 16.8). The CP values decreased from January to April ($P = 0.006$) in grasses (from 24.2 to 11.9% DM) and in mix (from 24.8 to 20.5 %DM), although less

markedly. However, the CP content did not differ between two botanical families. The soluble CP (i.e. non protein nitrogen plus soluble true protein) significantly differs between the months (P = 0.01), with a decreasing trend both in grasses (from 13.8 to 8.8%) and in a mix (from 12.2 to 11.7% DM) but remaining high during whole period (Table 1). In fact, the soluble CP expressed as a percentage of total CP content showed values from 57.3 to 74.2 % CP from January to April in grasses and from 49.7 to 58.3% CP in mix, significantly differ between botanical composition (P = 0.004), indicating that the forage crops were still young. The EE content was significantly affected by the month (P < 0.001), with values decreasing from January to April both in grasses (from 5.3 to 4.1% DM) and in a mix (from 5.1 to 3.9% DM), reaching a maximum value of 6.5% DM in January in a mix of forage crops. The NDF content was in tendency affected by the botanical composition (P = 0.06), with increasing values from January to April in grasses (from 39.4 to 49.4% DM), and stable values in mix (from 37.3 to 36.8% DM). The ADF content tends to be higher in grasses (P = 0.08) than in mix and did not differ between months. The ADL content differed significantly between months (P = 0.004), with increasing values from January to April in grasses (from 0.8 to 2.7% DM) and more stable values in mix (from 0.7 to 0.9% DM). The NFC content was significantly affected by the month (P = 0.002) and showed increasing values from January to April in grasses (from 18.8 to 26.1% DM), with maximum value of 35.4% DM recorded in March, and in mix (from 21 to 28.7% DM) with a maximum value of 35.7% DM recorded in February. The WSC content was not significantly affected by the botanical composition, probably because also the mix forage crops contain grasses. The WSC values remained low below in all months (P =

0.06), except in March for grasses (14.1 %DM) with a maximum value of 22.9% DM in an oat forage crop.

Table 2 shows the average composition of ration for each month. There is high variability in diet composition among farms, as shown in Table 3, as each had a specific ration to meet specific requirements and different feeding plane. However, the average diet composition remained stable during the months, with no significant differences, indicating well-balanced rations despite changes in grazed forage crops composition. The forage to concentrate ratio averaged around 60 to 40, an optimum value, in each month analyzed; however, the minimum and maximum values showed high variability among farms (Table 3). In fact, the minimum forage concentration was 35% of the ration recorded in January on Farm 3, while the maximum value was 75% of ration in February on Farm 2. For concentrates, the minimum inclusion was 25% of ration, recorded in February in Farm 2, and the maximum was 65%, recorded in January in Farm 3. The estimated herbage inclusion in diet was numerically higher in April, with a mean value of 26.8% of the ration, with maximum recorded value of 39% in the same month in Farm 3. The dietary CP (% DM) was numerically higher in April at 20% DM; however, there was high variability (Table 2 and 3) with a minimum of 13.7% DM recorded in March on Farm 3 and a maximum of 24.4% DM in February on Farm 2. The dietary NDF (% DM) was similar across all months, with a minimum value of 31.2% DM recorded in April on Farm 1, and maximum of 43.3% DM in March on Farm 5. The value of dietary NFC (% DM) was also consistent from January to April, with minimum value of 25.6% DM recorded in March, and maximum of 38.9% DM in February in Farm 3. Dietary starch (% DM) was numerically higher in

January at 14.5% DM, with a minimum value of 6.2% DM recorded in February on Farm 2, and a maximum of 19.5% DM in January on Farm 3.

The farm milk yield and milk chemical composition from January to April 2024 is reported in Table 4. In this study, the month did not significantly influence the farm milk yield or the chemical composition of the milk. The farm milk yield showed a numerical decrease from January to April (1.80 vs. 1.60 kg/ewe per day). The standard deviation, as well as the minimum and maximum values, indicated higher variability in production levels among the farms analyzed. The milk fat content was low across all months, with the numerically highest mean of 5.89% recorded in March, with minimum value of 5.40% in January and maximum of 6.35% in March. Despite this, the milk fat to protein ratio was consistently higher than 1, with no inversion of the ratio in any of the months considered. The highest average milk fat to protein ratio was 1.12, recorded in March, with a minimum value of 0.99 recorded in February and a maximum of 1.23 in February. The average urea concentration was below the threshold value of 45 mg/dL in each month, except for April, with a mean of 49.2 mg/dL. However, considering the minimum and maximum values recorded in each month, there were farms with values lower than 25-30 mg/dl, associated with inadequate dietary protein, and higher than 40-50 mg/d, indicating an excess of dietary protein (Cannas, 2004). It is important to consider that in April, milk was sampled only on farms 1, 2 and 3.

Table 1. General monthly trends in the chemical composition (% DM) of grazed forage crops from January to April 2024 on five dairy sheep farms (1, 2, 3, 4, 5), divided by botanic composition: grasses (G) and mix of grasses and legumes (M).

Variable	Botanic composition	Months																Months *P- value*	Botanic composition *P-value*
		January				February				March				April*					
		Mean	SD	Min	Max	**Mean**	SD	Min	Max	**Mean**	SD	Min	Max	**Mean**	SD	Min	Max		
DM, %	**G**	**10.2**	1.6	7.9	11.9	**9.3**	-	-	-	**15.6**	2.9	12.2	20.6	**17.3**	-	-	-	***<0.001***	***0.84***
	M	**11**	1.8	7.8	14	**11.9**	2.5	8.3	16.8	**13.4**	1.9	10.5	18	**16.8**	2.3	14.3	18.9		
CP, % DM	**G**	**24.2**	5.5	17.5	32.7	**22.3**	-	-	-	**17.7**	5.7	11.1	28.2	**11.9**	-	-	-	***0.006***	***0.16***
	M	**24.8**	3.7	17.2	32.5	**21.8**	4.9	14.6	28.3	**20.8**	5.1	12.9	30.1	**20.5**	8.1	11.3	26.4		
Soluble CP, % DM	**G**	**13.8**	2.7	10.2	17.3	**13.4**	-	-	-	**10.8**	2.7	8	14.7	**8.8**	-	-	-	***0.01***	***0.78***
	M	**12.2**	1.9	9.1	15.7	**11.4**	1.7	8.4	14.3	**10.4**	2.4	4.6	13.7	**11.7**	4.1	7.1	14.9		
Soluble CP, % CP	**G**	**57.3**	5.2	51.8	64.9	**60.2**	-	-	-	**62.4**	8.2	52.2	75.2	**74.2**	-	-	-	***0.11***	***0.004***
	M	**49.7**	7.1	34.5	64.3	**53.5**	9	42.4	71.3	**50.5**	9.2	30.1	66.2	**58.3**	4.2	55.4	63.2		
EE, % DM	**G**	**5.3**	0.6	4.6	6.1	**5.8**	-	-	-	**4.1**	0.7	3.1	5	**4.1**	-	-	-	***<0.001***	***0.13***
	M	**5.1**	0.6	4	6.5	**4.8**	0.6	4.1	5.8	**4.1**	0.5	3.4	4.9	**3.9**	0.5	3.3	4.3		
NDF, % DM	**G**	**39.4**	5.5	33.8	47.7	**38.6**	-	-	-	**39.6**	4	31.3	43.4	**49.4**	-	-	-	***0.34***	***0.06***
	M	**37.3**	3.6	30	42.3	**36.9**	4.2	30	44.1	**40.5**	5.7	29.2	49.5	**36.8**	7	29.5	43.6		
ADF, % DM	**G**	**21.3**	3.2	17.8	25.6	**21.6**	-	-	-	**21.8**	3.1	15.2	24.5	**26.8**	-	-	-	***0.17***	***0.08***
	M	**19.9**	2.1	16.1	23.2	**20.9**	2	17.7	24.7	**22.6**	2.8	16.6	26.1	**20.4**	3.9	16.3	24		
ADL, % DM	**G**	**0.8**	0.8	0	2.1	**0.9**	-	-	-	**2.2**	1	0.1	3.5	**2.7**	-	-	-	***0.004***	***0.25***
	M	**0.7**	0.6	0	2.2	**1.3**	1.1	0	3.1	**1.8**	0.9	0.2	3.6	**0.9**	1.6	0	2.8		
NFC, % DM	**G**	**18.8**	2.9	15.2	23.4	**21.6**	-	-	-	**27.5**	5.7	18.6	35.4	**26.1**	-	-	-	***0.002***	***0.55***
	M	**21**	4.3	14.2	29.8	**25.3**	5.5	17.2	35.7	**23.4**	3.2	18.1	28.7	**28.7**	4.2	24.2	32.4		
WSC, % DM	**G**	**7.9**	2.3	5.5	11.5	**7.1**	-	-	-	**14.1**	6.2	7.2	22.9	**9.6**	-	-	-	***0.06***	***0.54***
	M	**8.5**	3	5.2	14.9	**9**	4.5	3.8	17.4	**8.7**	3	5.2	16.5	**8.6**	3.3	6	12.3		
Ash, % DM	**G**	**12.2**	0.9	10.6	12.9	**11.6**	-	-	-	**11.2**	2.7	8.8	16.3	**8.5**	-	-	-	***0.02***	***0.75***
	M	**11.8**	1.6	9.3	16.2	**11.2**	1.1	10.1	14.3	**11.2**	0.7	9.9	12.4	**10.1**	0.6	9.4	10.6		

*Samples collected only on farms 1, 2, 3.

Table 2. Average composition of rations from January to April 2024 of the five dairy sheep farms (1, 2, 3, 4, 5).

Variable	Months																
	January				February				March				April*				Months
	Mean	SD	Min	Max	**Mean**	SD	Min	Max	**Mean**	SD	Min	Max	**Mean**	SD	Min	Max	***P- value***
Forage (% ration)[1]	59.5	12.4	35	72.3	61.2	12.1	37.5	75	56.7	7.2	40.7	67.9	55.7	2.3	52.7	57.4	0.73
Concentrate (% ration)	40.5	12.4	27.7	65	38.8	12.1	25	62.5	43.2	7.2	32	59.3	44.3	2.6	42.6	47.3	0.73
Herbage (% ration)	18.9	4.04	14	25.4	21.9	6	13.1	32.7	25	8.15	9	37.4	26.8	11.8	15.8	39.3	0.27
Dietary CP (% DM)	19	2.51	16	22.5	19.8	3.4	14.8	24.4	18.4	3.3	13.7	23.6	20	5.1	14.1	23.2	0.78
Dietary NDF (% DM)	35.7	3.2	32	40.6	35.5	3.4	31.7	39.7	37.6	3.1	31.6	43.3	35.5	4.75	31.2	40.6	0.42
Dietary NFC (% DM)	33.2	2.8	28	36.2	33	3.8	29	38.9	32.2	3.8	25.6	36.3	33	1.46	31.3	34	0.91
Dietary WSC (% DM)	5.1	1.25	3.62	7.3	5.75	1.3	4.13	7.5	5.56	1.16	4	8	5.48	0.55	4.95	6.05	0.70
Dietary starch (% DM)	14.5	3.3	9.9	19.5	12.1	4.51	6.2	17.9	12.8	3.64	6.7	17.7	12.5	1.56	10.9	14	0.55
Dietary EE (% DM)	3.1	0.6	2.3	4.1	2.83	0.44	2.41	3.6	2.78	0.70	2.15	4.7	2.6	0.32	2.17	3.2	0.56

[1] Forage = total of forages included in the diet (herbage, hay, haylage, silage).

*Samples collected only on farms 1, 2, 3.

Table 3. Average composition of the rations of five dairy sheep farms (1, 2, 3, 4, 5) from January to April 2024.

Variable	Farms																				*Farm P-value*
	1				2				3				4				5				
	Mean	SD	Min	Max	**Mean**	SD	Min	Max	**Mean**	SD	Min	Max	**Mean**	SD	Min	Max	**Mean**	SD	Min	Max	
Forage (% ration)[1]	**59.3**	4.3	55.5	68	**65.9**	8.3	56.6	75	**44.1**	7.4	35	52.7	**58.9**	2.2	55.9	61.4	**62.9**	2	59.4	64	<0.001
Concentrate (% ration)	**40.6**	4.3	32.1	44.5	**34.1**	8.3	25	43.4	**55.9**	7.4	47.3	65	**41.1**	2.2	38.6	44.1	**37.1**	2	36	40.6	<0.001
Herbage (% ration)	**18.9**	5	13.1	25.4	**17.8**	4.5	9	22.8	**30.1**	7.8	20.7	39.3	**20.1**	1.8	18.4	22.4	**30.7**	4.2	25.4	35.8	<0.001
Dietary CP (% DM)	**22.3**	0.6	21.6	23.2	**22.5**	1.2	20.8	24.4	**15.1**	1.06	13.7	16.8	**17.3**	0.8	16.5	18.6	**17.5**	0.5	17	18.3	<0.001
Dietary NDF (% DM)	**32.5**	1.2	31.2	34.9	**34.6**	2.6	31.7	39.3	**37.2**	1.9	34.8	40.6	**37.5**	1.2	36	39.7	**40.9**	1.3	39.5	43.3	<0.001
Dietary NFC (% DM)	**33.9**	1.2	31.2	34.8	**30.5**	1.2	29.1	32.1	**35.8**	1.7	33.6	38.9	**35.5**	0.7	34.1	36.3	**27.9**	1.4	25.6	29.6	<0.001
Dietary WSC (% DM)	**5.3**	0.6	4.7	6.7	**6.5**	0.9	5.3	7.4	**6.3**	1.1	5	8	**4.4**	0.4	4	5	**4.5**	0.7	3.6	5.5	<0.001
Dietary starch (% DM)	**13.4**	1.3	10.8	14.6	**9.1**	1.9	6.2	10.9	**16.9**	2.3	12.7	19.5	**16.1**	0.7	15.1	17	**10.1**	2.3	6.7	13.5	<0.001
Dietary EE (% DM)	**2.4**	0.1	2.3	2.5	**3.3**	0.4	2.6	3.7	**2.4**	0.2	2.1	2.7	**2.9**	0.6	2.4	4.1	**3.4**	0.7	3	4.7	<0.001

[1] Forage = total of forages includes in the diet (hay, herbage, haylage, silage).

Table 4. General monthly trends in the chemical composition of milk from January to April 2024 on the five dairy sheep farms (1, 2, 3, 4, 5).

Variable	Months																
	January				February				March				April*				Months
	Mean	SD	Min	Max	**Mean**	SD	Min	Max	**Mean**	SD	Min	Max	**Mean**	SD	Min	Max	***P- value***
Farm milk yield (kg/ewe per d)	1.80	0.30	1.26	2.23	1.63	0.40	1.06	2.13	1.62	0.32	1.00	2.06	1.60	0.47	1.07	1.98	0.62
Milk fat,%	5.66	0.20	5.40	5.83	5.83	0.24	5.46	6.15	5.89	0.29	5.51	6.35	5.64	0.17	5.50	5.83	0.28
Milk protein,%	5.19	0.26	4.95	5.54	5.34	0.28	5.01	5.77	5.26	0.19	4.98	5.63	5.19	0.39	4.80	5.58	0.74
Milk fat to protein ratio	1.09	0.07	1.01	1.17	1.09	0.08	0.99	1.23	1.12	0.05	1.03	1.20	1.09	0.08	1.00	1.15	0.73
Lactose,%	4.93	0.10	4.79	5.01	4.80	0.30	4.32	5.11	4.91	0.07	4.79	5.00	4.90	0.08	4.82	4.98	0.47
Urea, mg/dl	42	7.95	34.4	50.7	39.5	16.2	18.6	55.2	38.9	13.1	18.4	57.4	49.2	8.52	39.4	54.2	0.65
SCC, log10	2.93	0.16	2.8	3.2	2.86	0.25	2.5	3.2	2.75	0.2	2.4	3	2.8	0.3	2.5	3.1	0.49

*Samples collected only on farms 1, 2, 3.

Relationship between diet composition, grazed forage crops and milk composition

The relationship between dietary components, grazed forage crops, and milk composition has been studied by examining possible associations. Figure 1 shows a negative correlation between the percentage of concentrates in a ration and milk fat content (%), and a positive correlation with the percentage of forage (P = 0.007). Therefore, the forage to concentrate ratio of the ration was positively correlated with milk fat content. Figure 2 shows a significant negative correlation between dietary starch (% DM) and milk fat percentage (P = 0.01). Additionally, dietary starch was found to be negatively related also to the milk fat to protein ratio (P =0.002). The dietary NFC (% DM) resulted negatively correlated with milk fat content (P = 0.006), as shown in Figure 3, and with milk fat to protein ratio (P = 0.001). Conversely, Figure 4 shows a positive relationship between dietary NDF (% DM) and milk fat to protein ratio (P = 0.01). Figure 5 shows a strong positive correlation between dietary CP (% DM) and milk urea (mg/dl). Regarding the composition of grazed forage crops, Figure 6 shows a negative correlation between grazed forage crops NFC (% DM) and milk fat content (P = 0.056). Additionally, the NFC content of grazed forage crops was also negatively correlated with the milk fat to protein ratio (P = 0.001), as well as the fat content of grazed forage crops (% DM; P = 0.04), as reported in Figure 7. Figure 8 shows a close positive correlation between grazed forage crops CP content (% DM) and milk urea (mg/dL).

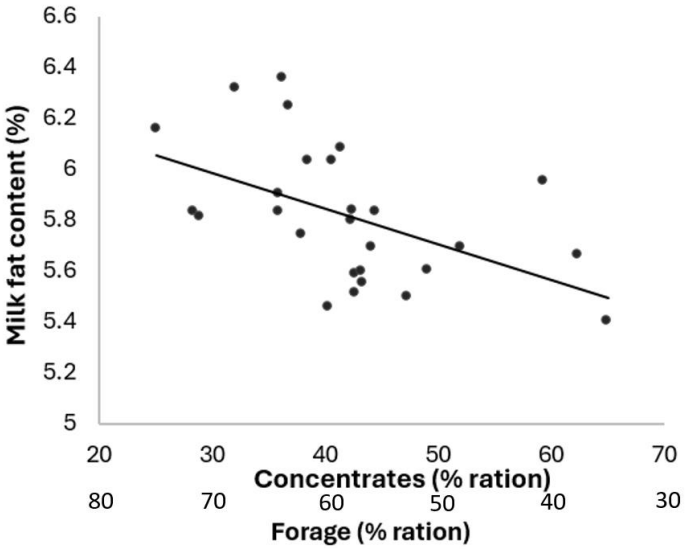

Figure 1. Regression analysis of forage to concentrate ratio and milk fat content (%; y = 6.407 - 0.01405 x, P = 0.007, R^2 = 26%).

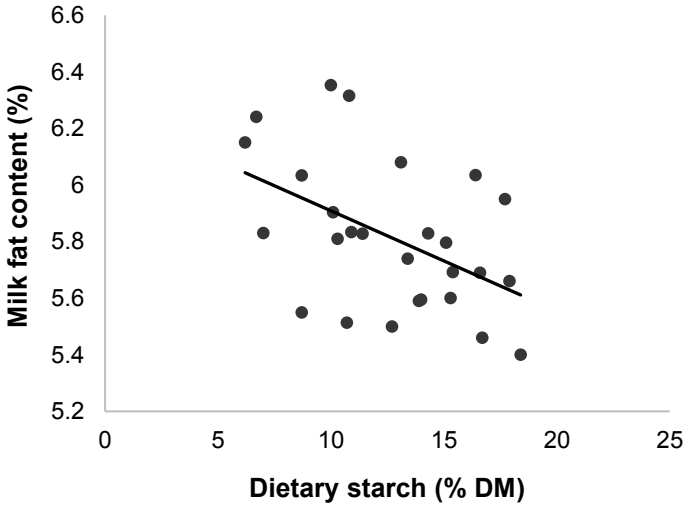

Figure 2. Regression analysis of dietary starch (% DM) and milk fat percentage (y = 6.263 - 0.03541 x, P = 0.01, R^2 = 23%).

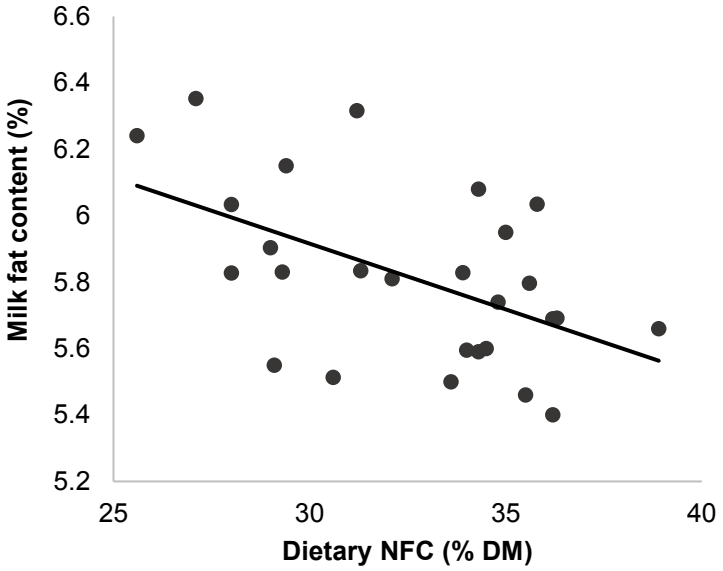

Figure 3. Regression analysis of dietary NFC (% DM) and milk fat content (%; y = 7.105 - 0.03962 x, P = 0.006, R^2 = 27%).

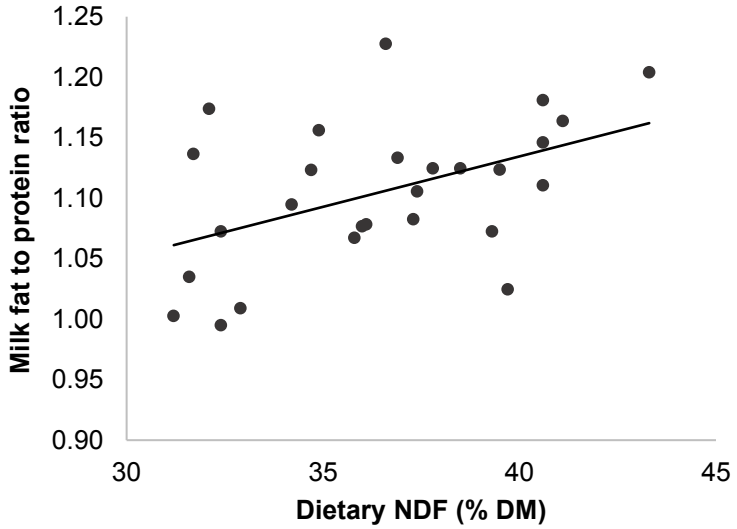

Figure 4. Regression analysis of dietary NDF (% DM) and milk fat to protein ratio (y = 0.8012 + 0.008332 x, P = 0.01, R^2 = 22.3%).

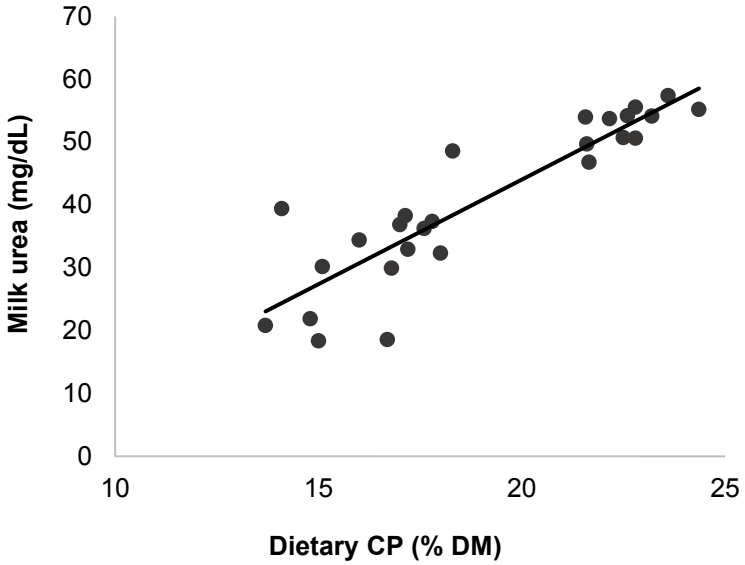

Figura 5. Regression analysis of dietary CP (% DM) and milk urea (mg/dl; y = 22.52 + 3.327 x, P = < 0.001, R^2 = 80%).

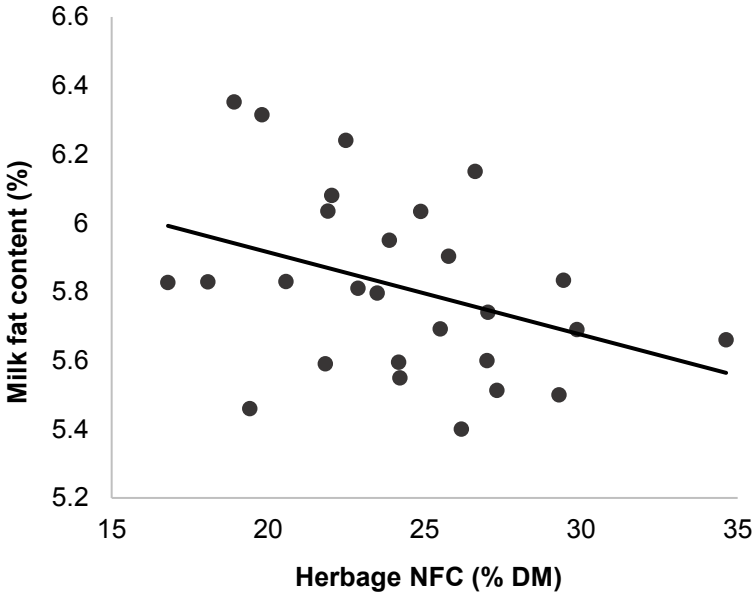

Figure 6. Regression analysis of grazed forage crops NFC (% DM) and milk fat content (%; y = 6.395 - 0.02400 x, P = 0.056, R^2 = 14%).

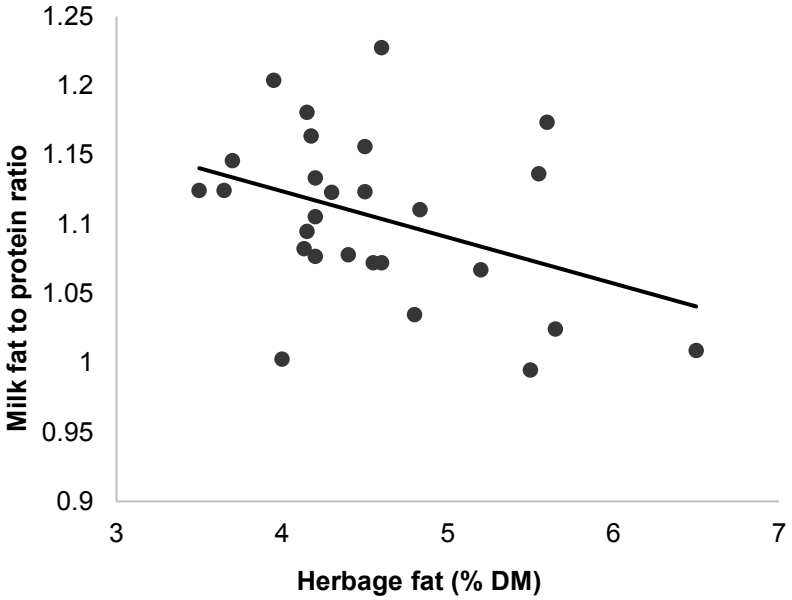

Figure 7. Regression analysis of grazed forage crop fat (% DM) and milk fat to protein ratio (y = 1.257 - 0.03327 x, P = 0.04, R^2 = 15%).

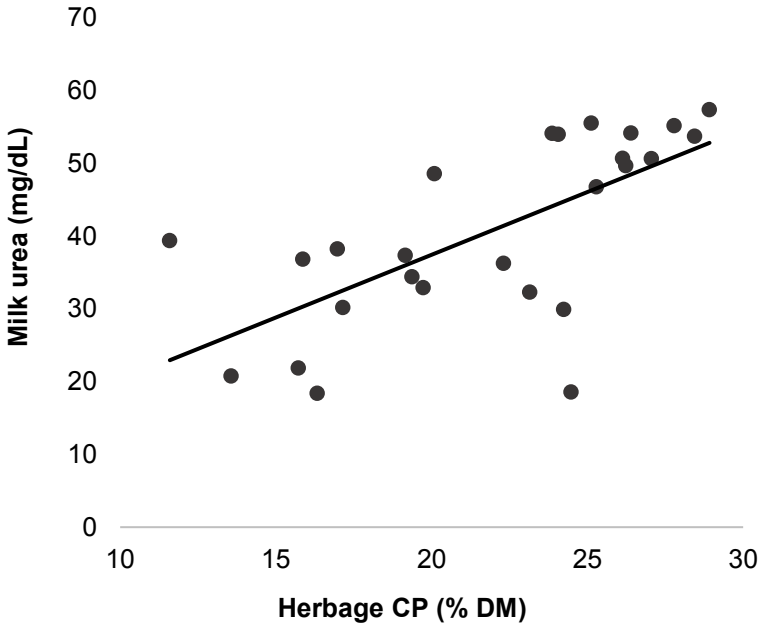

Figure 8. Regression analysis of grazed forage crop CP (% DM) and milk urea (mg/dl; y = 2.961 + 1.724 x, P = < 0.001, R^2 = 45.1%).

Discussion

In this study, the forage to concentrate ratio is confirmed to be a parameter positively associated with milk fat content, as shown in Figure 1, consistent with findings by Morand-Fehr et al. (2007); Dong et al. (2013); Atalay (2019); Angeles-Hernandez et al. (2020). In fact, this ratio is positively related to the acetate to propionate ratio in the rumen (Kooman et al., 2018; Liu et al., 2019; Angeles-Hernandez et al., 2020). High concentrate diets lead to increased propionate production (Maxin et al., 2011) which limits the flow of precursors toward the mammary gland for milk fat synthesis due to elevated levels of circulating insulin (Cannas, 2004; Koch and Lascano, 2018; Angeles-Hernandez et al., 2020). An unbalanced forage to concentrate ratio may result in acidosis (Mele et al., 2006; Kara 2020) and milk fat depression (Peterson et al., 2003), and this ratio is the main factor of the diet involved in the variation of biohydrogenation (Sterk et al., 2011). The C18:1 trans-10 fatty acid (FA), which is involved in milk fat depression (Fievez et al., 2012), is a potent inhibitor of de novo FA synthesis in the udder and tend to increase in low forage diet than in high forage diet (Mele et al., 2006). In fact, in high concentrate diets rumen biohydrogenation pathways shift towards the production and accumulation of C18:1 trans-10 and CLA trans 10 cis-12, along with an increased duodenal flow of unsaturated FA (Pulina et al., 2006; Mele et al., 2006), inclusive of these isomers. Thus, a diet with excessive amount of concentrate can depress milk fat and protein because of acidosis (Caja and Bocquier, 2000; Dong et al., 2013; Kitkas et al., 2019). This study showed that also dietary NFC is negatively related to milk fat concentration (Figure 3). NFC has different effects at different stages of lactation (Cannas, 2004). Indeed, using large amounts of cereal grains in early lactation is beneficial for producing more milk during

a period of physiological negative energy balance, but in second part of lactation, it stimulates fat deposition and increases body weight, which negatively affects milk production (Cannas, 2004). At this physiological stage, the energy tends to convert into body fat deposition at the expense of milk fat yield and milk yield, when concentrate rich in starch are used, due to their stimulation of gluconeogenesis and insulin response (Cannas et al., 1998; Molle et al., 2008). Regarding milk fat synthesis, the negative correlation between dietary NFC and milk fat percentage are related to starch and sugars present in NFC, which can lead to sub-acidosis, and consequently, milk fat depression, while the pectin's included in the NFC group are not fermented to lactate and have very high cation exchange capacity (Van Soest et al., 1991). This is supported by the negative relationship found between dietary starch and milk fat content (Figure 2), as high starch diet is associated with a decrease in rumen pH and lower acetate to propionate ratio in rumen (Vlaeminck et al., 2006; Kara, 2020). Nudda et al. (2004b) showed that higher NFC levels increase the concentration of the isomer C18:1 trans-10 in sheep milk, an indicator of rumen acidosis (Fievez et al., 2012), resulting from slower biohydrogenation in the rumen and higher escape from the rumen. In this study, the NFC content of grazed forage crops was also negatively associated with milk fat concentrations (Figure 6), due to sugars and starch. In the study of Satta et al. (2023) and in Chapter 2 of this Thesis, a negative correlation between the WSC of herbage and milk fat was reported. content. However, in this study the WSC of grazed forage crops were very low from January to April but tended to differ between the months ($P = 0.06$) with high mean value recorded in March in grasses (14.1% DM), and a maximum value of 22.9% DM, recorded in the same month on oat herbage (*Avena sativa* L.). The WSC content did not differ between botanical

families, probably because grasses were compared with a mix of grasses and legumes not with legumes, and the presence of grasses in the mix did not allow to highlight the differences. In fact, the maximum values recorded in the mix (17.4 and 16.5% DM, in February and March respectively; Table 1) are not typical of legumes, which have very low WSC content (Porcu et al., 2023). The majority of grasses collected during this study were the most commonly used in Sardinian dairy sheep farms in terms of species, variety, and ploidy (*Lolium multiflorum* Lam. ssp. westervoldicum, tetraploid type), which in recent years reached very high levels of WSC during winter and spring (>25-30% DM; Chapter 2 of this Thesis). The CP and soluble CP contents were high in each month, except for the grasses sample in April (CP: 11.9 %DM on oat pasture), and both were significantly different between months (P = 0.006, P = 0.01; for CP and soluble CP, respectively) but did not differ between the botanical families. These, along with relatively low and consistent fibre values, indicate that the pastures were young and in the vegetative stage (King et al., 2012; Jafari, 2012), indicating favorable growth conditions. The conditions that favor plant growth involve the consumption of WSC, as sugars are utilized by the plant for growth and development (Watts, 2010). However, unfavorable conditions for plant growth, but not for photosynthesis, and stress conditions lead to WSC accumulation (Geor, 2009; Jensen et al., 2014). Additionally, Satta et al., (2023) and Chapter 2 of this thesis reported inverse relationship between CP and WSC contents in plants. This study was conducted in a year characterized by winter and spring with higher mean temperatures and minimum temperatures compared to last years (ARPAS, 2024). These weather conditions probably favored plant growth and consequently sugar use, leading to lower WSC content. In fact, low temperatures are essential for WSC accumulation (Watts, 2004;

Watts, 2008). Indeed, in this study the relationship between WSC content of grazed forage crops and milk fat content did not occur. In the current study, dietary NDF was found to be positively associated with the milk fat to protein ratio (Figure 4), as the fermentation of fibre produces acetate which favors milk fat synthesis. However, no relationship was found between the NDF content of grazed forage crops and both fat content and the fat to protein ratio in milk. This is likely because the level of peNDF in herbage was low, and the NDF of grazed forage crops did not effectively stimulate rumination and thus did not sufficiently buffer ruminal pH, favouring the synthesis of precursors of milk fat. In contrast, the fat content of grazed forage crops was negatively related to the milk fat to protein ratio (Figure 7). The herbage fat is rich in polyunsaturated FA (PUFAs), which can interfere with microbial activity in rumen, altering biohydrogenation processes and consequently affecting milk fat synthesis (Bauman et al., 2011; Delavaud et al., 2022). Furthermore, high levels of fat in the ration, especially if rich in PUFA, can reduce milk fat content due to their depressive effect on microbial activity (Nudda et al., 2004a). The dietary protein was found strongly and positively associated with milk urea (Figure 5), in accordance with Cannas et al. (1998). In this study, the coefficient of determination of regression was very high at 0.80, compared to 0.82 in the study by Cannas et al. (1998). Considering that this is a field study conducted on bulk milk samples and that the dietary concentrations were calculated on a flock basis and on grazing sheep this is very high. Additionally, the CP content of grazed forage crops was found to be positively related to milk urea (Figure 8), also if with a lower precision. Milk urea is confirmed as a very efficient nutritional indicator of protein balance, even when used under field conditions, as well as in sheep farms that use pasture in their feeding plans.

Conclusions

In conclusion, diet composition and grazed herbage significantly influenced milk composition, particularly milk fat concentration. The forage to concentrate ratio is confirmed to be as a parameter strongly and positively associated with milk fat content, whereas diets high in starch and NFC may negatively affect milk fat content and, consequently, cheese yield. Negative relationships were found between milk fat content and herbage NFC, whereas the NDF content of herbage did not influence this milk component. The use of forage crops of mixed species for grazing is preferred than single grasses species, for a better balance of nutrients and to avoid possible single nutrient overload. Based on the results of this study, we can conclude that the observed decrease in milk fat percentage in Sardinia over the past decade, especially during winter and spring months, represents a multifactorial nutritional issue, in addition to genetic factors. Therefore, further studies should focus on feeding strategies and techniques, including pasture management, to mitigate the issue of milk fat depression in sheep during winter and spring season in Sardinia.

References

Angeles-Hernandez J. C., Alberto R. V., Kebreab E., Appuhamy J. A. R. N., Dougherty H. C., Castelan-Ortega O., & Gonzalez-Ronquillo M. 2020. Effect of forage to concentrate ratio and fat supplementation on milk composition in dairy sheep: A meta-analysis. Livestock Science, 238, 104069.

Atalay H. 2019. Milk fat/protein ratio in ketosis and acidosis. Balıkesir Sağlık Bilimleri Dergisi, 8(3), 143-146.

Arpas (Agenzia Regionale per la Protezione dell'Ambiente della Sardegna). 2024. Riepiloghi mensili meteorologici ed agrometeorologici. Consulted in September 2024. https://www.sar.sardegna.it/pubblicazioni/riepiloghimensili/mensili.asp

Bauman D. E., Harvatine K. J., & Lock A. L. 2011. Nutrigenomics, rumen-derived bioactive fatty acids, and the regulation of milk fat synthesis. *Annual review of nutrition*, *31*(1), 299-319.

Cannas A., Pes A., Mancuso R., Vodret B., Nudda A. 1998. Effect of dietary energy and protein concentration on the concentration of milk urea nitrogen in dairy ewes. *Journal of Dairy science*, *81*(2), 499-508.

Cannas A. 2004. Feeding of lactating ewes. In *Dairy sheep nutrition* (pp. 79-108). Wallingford UK: CABI Publishing.

Caja G., Bocquier F. 2000. Effects of nutrition on the composition of sheep's milk. Cahiers Options Méditerranéennes, 52(59-74).

Delavaud C., Fougère H., Bertrand-Michel J., & Bernard L. 2022. Milk fat depression and plasma lipids in dairy cows and goats. *animal*, *16*(12), 100635.

Dong, H., Wang, S., Jia, Y., Ni, Y., Zhang, Y., Zhuang, S., Shen X., & Zhao, R. 2013. Long-term effects of subacute ruminal acidosis (SARA) on milk quality and hepatic gene expression in lactating goats fed a high-concentrate diet. PloS one, 8(12), e82850.

Fievez V., Colman E., Castro-Montoya, J. M., Stefanov I., & Vlaeminck B. 2012. Milk odd-and branched-chain fatty acids as biomarkers of rumen function—An update. *Animal Feed Science and Technology*, *172*(1-2), 51-65.

Geor R. J. 2009. Pasture-associated laminitis. Veterinary Clinics of North America: Equine Practice, 25(1), 39-50.

Jafari, A. A. 2012. Environmental and genetic variation for water soluble carbohydrate content in cool season forage grasses. *Carbohydrates—Comprehensive Studies on Glycobiology and Glycotechnology; IntechOpen: Rijeka, Croatia*, 373-388.

Jensen K. B., Harrison P., Chatterton N. J., Bushman B. S., Creech J. E. 2014. Seasonal trends in nonstructural carbohydrates in cool-and warm-season grasses. Crop Science, 54(5), 2328-2340.

Kara K. 2020. Milk urea nitrogen and milk fatty acid compositions in dairy cows with subacute ruminal acidosis. Veterinární medicína, 65(9).

King C., McEniry J., Richardson M., & O'kiely P. 2012. Yield and chemical composition of five common grassland species in response to nitrogen fertiliser application and phenological growth stage. *Acta Agriculturae Scandinavica, Section B–Soil & Plant Science*, *62*(7), 644-658.

Kitkas G. C., Valergakis G. E., Kritsepi-Konstantinou M., Gelasakis A. I., Arsenos G., Kalaitzakis E., Panousis N. 2019. Effects of ruminal pH and subacute ruminal

acidosis on milk yield and composition of Holstein cows in different stages of lactation. *Journal of the Hellenic Veterinary Medical Society*, *70*(2), 1551-1560.

Koch L. E., Lascano G. J. 2018. Milk fat depression: etiology, theories, and soluble carbohydrate interactions. Journal of Animal Research and Nutrition, 3(2), 2.

Kooman H. M. A. 2018. Subacute ruminal acidosis and the relationship with the detection of ruminal acidosis in milk based on milk-fat content, other milk production parameters and nutritional aspects in dairy cows (Master's thesis).

Liu H., Xu T., Xu S., Ma L., Han X., Wang X., Zhang ., Hu L., Zhao N., Chen Y., Pi L., Zhao X., & Zhao, X. 2019. Effect of dietary concentrate to forage ratio on growth performance, rumen fermentation and bacterial diversity of Tibetan sheep under barn feeding on the Qinghai-Tibetan plateau. *PeerJ*, *7*, e7462.

Maxin G., Glasser F., Hurtaud C., Peyraud J. L., & Rulquin H. 2011. Combined effects of trans-10, cis-12 conjugated linoleic acid, propionate, and acetate on milk fat yield and composition in dairy cows. *Journal of dairy science*, *94*(4), 2051-2059.

Mele M., Buccioni A., Petacchi F., Serra A., Banni S., Antongiovanni M., Secchiari P. 2006. Effect of forage/concentrate ratio and soybean oil supplementation on milk yield, and composition from Sarda ewes. *Animal Research*, *55*(4), 273-285.

Molle G., Decandia M., Cabiddu A., Landau S.Y., Cannas A. 2008. An update on the nutrition of dairy sheep grazing Mediterranean pastures. Small Ruminant Research, 77(2-3), 93-112.

Morand-Fehr P., Fedele V., Decandia M., & Le Frileux Y. 2007. Influence of farming and feeding systems on composition and quality of goat and sheep milk. Small Ruminant Research, 68(1-2), 20-34.

Nudda A., Battacone G., Bencini R., Pulina G. 2004a. Nutrition and milk quality. In *Dairy sheep nutrition* (pp. 129-149). Wallingford UK: CABI Publishing.

Nudda A., Fancellu S., Porcu F., Boe F., Cannas A., 2004b. Responses of milk fat composition to dietary non-fiber carbohydrates in Sarda dairy sheep. J. Dairy Sci. 87 (Suppl. 1), 310 (Abstract).

Peterson D. G., Matitashvili E. A., & Bauman D. E. 2003. Diet-induced milk fat depression in dairy cows results in increased trans-10, cis-12 CLA in milk fat and coordinate suppression of mRNA abundance for mammary enzymes involved in milk fat synthesis. *The Journal of nutrition*, *133*(10), 3098-3102.

Porcu M.A., Ledda A., Mazza A., Fenu A., Cannas A. 2023. Causes of milk fat depression of lactating dairy ewes fed fresh annual ryegrass herbage. Animal - science proceedings, Volume 14, Issue 4, 2023, Page 566, ISSN 2772-283X. (abstract)

Pulina G., Rossi G., Brandano P. 1985. Stima della produzione lattea della pecora in base al ritmo di accrescimento dell'agnello. Estratto da "Studi Sassaresi" Sezione III – Annali della Facoltà di Agraria dell'Università di Sassari, Vol. XXXII – 1985/1986.

Pulina G., Nudda A., Battacone G., & Cannas A. 2006. Effects of nutrition on the contents of fat, protein, somatic cells, aromatic compounds, and undesirable substances in sheep milk. *Animal Feed Science and Technology*, *131*(3-4), 255-291.

Satta F., Porcu M.A., Ledda A., Carta S., Deiana L., Cannas A. 2023. The concentration of water-soluble carbohydrates in the pasture grazed by lactating

ewes might be a main cause of milk fat depression. ASPA Conference, Monopoli (Bari, Italy), 13-16 June 2023.

Sterk A., Johansson B. E. O., Taweel H. Z. H., Murphy M., Van Vuuren A. M., Hendriks W. H., & Dijkstra J. 2011. Effects of forage type, forage to concentrate ratio, and crushed linseed supplementation on milk fatty acid profile in lactating dairy cows. *Journal of Dairy Science*, *94*(12), 6078-6091.

Tedeschi L.O., Cannas A., Fox D.G. 2010. A nutrition mathematical model to account for dietary supply and requirements of energy and other nutrients for domesticated small ruminants: The development and evaluation of the Small Ruminant Nutrition System. Small Ruminant Research, 89(2-3), 174-184.

Van Soest P. J., Robertson J. B., & Lewis B. A. 1991. Symposium: carbohydrate methodology, metabolism, and nutritional implications in dairy cattle. *Journal of dairy science*, *74*(10), 3583-3597.

Vlaeminck B., Fievez V., Tamminga S., Dewhurst R. J., Van Vuuren A., De Brabander D., & Demeyer D. 2006. Milk odd-and branched-chain fatty acids in relation to the rumen fermentation pattern. *Journal of Dairy Science*, *89*(10), 3954-3964.

Watts K. A. 2004. Forage and pasture management for laminitic horses. Clinical techniques in equine practice, 3(1), 88-95.

Watts K. 2008. The influence of solar radiation and temperature on the diurnal fluctuation of NSC in grass. Copyright 2008. Rocky Mountain Research & Consulting,Inc.

https://static1.squarespace.com/static/5e7fddf934de306c2b8cc313/t/5e8c82935a255f19a8a2cb9f/1586266771795/DiurnalFluctNSC.pdf

Watts K. 2010. Pasture management to minimize the risk of equine laminitis. Veterinary Clinics: Equine Practice, 26(2), 361-369.

CHAPTER 5

General conclusions

The decrease in milk fat content observed in dairy sheep farms in Sardinia (Italy) is an important issue of the last decades, particularly during winter and spring months. This may be attributed to the increased milk yield of ewes, due to genetic selection and improved nutrition, to the effect of PUFA of grass, and to SARA. In grazing conditions, the common use of modern tetraploid annual ryegrass varieties with high WSC content may be a cause of SARA.

The present thesis: i) investigated if pasture WSC might affect milk fat synthesis and concentration, ii) tested a nutritional technique (bicarbonate supplementation) to mitigate the negative effects of WSC overload on milk fat content, and iii) conducted a field survey to investigate the relationships between diet, grazed herbage, and milk composition, particularly the milk fat content of dairy ewes during the winter and spring months in Sardinia.

The findings of first study, in which milk fat content of ewes fed unshaded grass with higher WSC content (SUN) was lower compared to those fed shaded grass with lower WSC content (SHADE), especially during afternoon milking (three hours after grass feeding), and there was a tendency for higher levels of C18:1 trans-10 in the SUN group, suggested that WSC overload from grasses may be a cause of milk fat depression (MFD) observed in dairy ewes grazing during the winter and spring.

The results of the second study indicated that sodium bicarbonate may be used to prevent ruminal subacidosis and milk fat depression from WSC overload in lactating grazing ewes, because of improved total dry matter intake, herbage intake, milk fat percentage, and milk fat yield, especially in afternoon milking when this buffer was supplemented.

The third study showed that diet composition and grazed herbage influenced milk composition, and particularly milk fat concentration. The forage to concentrate ratio is confirmed to be as a parameter strongly and positively associated with milk fat content, whereas diets high in starch and NFC may negatively affect milk fat content and, consequently, cheese yield. Negative relationships were found between milk fat content and herbage NFC, whereas the NDF content of herbage did not influence this milk component.

The present thesis shows that WSC overload from grasses in grazing dairy ewes represents a new nutritional issue that may contribute to milk fat depression. Therefore, WSC content is an important herbage quality parameter to consider when formulating rations for grazing dairy sheep. This study indicates that the problem of WSC accumulation in grasses is closely linked to weather conditions and does not occur every year. Specifically, sunny days with low temperatures, and stress conditions for plants are necessary for WSC accumulation, even in species and varieties selected for high WSC content. Additionally, it is important to consider that the trials of this thesis were conducted indoors, and the feeding behaviour of grazing ewes may differ in terms of species and plant selection, grass intake rate, and be influenced by time-restricted access to pasture. Thus, WSC intake of grazing ewes, in terms of both quantity and hourly intake rate, and the negative effects of WSC overload, could be exacerbated in grazing ewes. Additionally, the relationship found between milk fat content and other dietary and herbage compounds indicates that the decrease in milk fat percentage in Sardinia over the last decade, especially during winter and spring months, represents a multifactorial nutritional issue, in addition to genetic factors.