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CORSO DI DOTTORATO DI RICERCA
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Ciclo XXX

Permanent effects of starch and fiber supplied during uterine and postnatal life on first lactation performance of dairy sheep

dr. Antonello Ledda

Coordinatore del Corso	Prof. Antonello Cannas
Referente di Curriculum	Dott. Gianni Battacone
Docente Guida	Dott. Alberto Stanislao Atzori
Tutor	Prof. Antonello Cannas

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ABBREVIATIONS

ADF	acid detergent fiber
ADL	acid detergent lignin
ATP	adenosine triphosphate
AUC	area under the curve
BCS	body condition score
BW	body weight
BHB	β-hydroxybutyrate
CLA	conjugated linoleic acid
CP	crude protein
DBP	days before parturition
DHA	docosahexaenoic acid
DIM	days in milk
DM	dry matter
DPA	docosapentaenoic acid
EE	ether extract
EDTA	ethylenediaminotetraacetate
EL	early lactation
ELISA	enzyme-linked immunosorbent assay
EPA	eicosapentaenoic acid
F	diet rich in soluble fiber
FA	fatty acids
FAME	fatty acid methyl esters
FID	flame ionization detector
FPCMY	fat protein corrected milk yield
GLUT	glucose transporter

GTT	glucose tolerance test
GH	growh hormone
HOMA	homeostasis model assessment
HPLC	high-performance liquid chromatographic
IGF	insulin-like growth factor
iNDF	indigestible NDF
ITT	insulin tolerance test
LACT	lactating animals
LCFA	long chain fatty acids
Ldiet	lactation diet
MFA	multivariate factor analysis
MCFA	medium chain fatty acids
MUFA	monounsaturated fatty acids
n	number of samples
NDF	neutral detergent fiber
NDF-d	degradability of NDF
NDSF	neutral detergent soluble fiber
NEB	negative energy balance
NEFA	non-esterified fatty acids
NFC	nonfiber carbohydrate
NSC	nonstructural carbohydrates
OBCFA	odd and branched chain fatty acids
peNDF	physically effective NDF
PGdiet	prenatal and growing diet
PMSG	pregnant mare's serum gonadotropin
PPAR-γ	peroxisome proliferator-activated receptor-gamma
PREG	pregnant animals
PUFA	polyunsaturated fatty acids
QUICKI	quantitative insulin check index

RA	rumenic acid
RIA	radio immuno assay
S	diet rich in starch
SCC	somatic cell count
SCFA	short chain fatty acids
SEM	standard error of the mean
SFA	saturated fatty acids
SRNS	small ruminant nutrition system
TAG	livertriacylglycerides
TFA	trans fatty acids
TMR	total mixed ration
TNF-α	adipokines tumor necrosis factor
UFA	unsaturated fatty acids
UM	units of measurements
VA	vaccenic acid
VFA	volatile fatty acid

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GENERAL ABSTRACT

This thesis contributes to advance knowledge on sheep production response to dietary carbohydrates. The thesis presents the main results of first lactation performances of sheep exposed to different dietary carbohydrate sources (starch from cereal grains or soluble fiber from soyhulls and beet pulp) during uterine life, growing and mid lactation.

The first Chapter described an overview of the literature on the relationship among dietary carbohydrate and sheep performances, the mechanism regulation of insulin resistance in ruminants and some evidence from literature that related fetal programming to insulin resistance and impaired glucose metabolism in ruminants.

The literature review evidences the experimental hypothesis that exposure to different carbohydrate sources (starch or fiber) might induce permanent changes in glucose metabolism of the animals which might affect the response to carbohydrates in the first lactation, especially after the lactation peak when peripheral tissue are in completion with the mammary gland for the glucose use.

The second Chapter described the experimental design in details.

The third Chapter investigated the lactation performances of primiparous sheep fed with different carbohydrate sources supplied after the lactation peak. The results showed no significant differences in animal performance or rumen parameters when part of the dietary starch is substituted with soluble fiber.

The fourth Chapter described the effects of the exposure to different sequences of dietary starch and fiber during: 1) uterine life, 2) postnatal growing and 3) mid lactation on the lactation performance of primiparous dairy sheep. The observed results did not show a clear effect of carbohydrate type on the animal response in mid lactation due to extreme individual variability. Observed patterns suggested that carbohydrates might have a mediated or subordinated role, in respect to dietary and environmental factors, which regulates the animal response to carbohydrates in the short term.

The fifth Chapter described the results of glucose tolerance test (GTT) and insulin tolerance test (ITT) executed during pregnancy and lactation to test the animal response to a glucose of insulin bolus in respect to dietary treatments. The results showed that sequences of dietary carbohydrates affected basal insulin and glucose of the experimental animals. In addition correlations among milk production, body reserves, basal glucose and basal insulin in mid lactation varied depending on the carbohydrate exposure received by the animals early in life and in mid-lactation.

Finally, a brief paragraph of general conclusion closes the dissertation.

CHAPTER 1

1. INTRODUCTION AND LITERATURE REVIEW

Recent investigation on small ruminants evidenced that the carbohydrate type might have a particular role in modulating the performance of lactating sheep. A specific challenge of research in animal nutrition applied to dairy sheep is to define the optimum levels and the type of carbohydrates that can maximize animal performance. Pursuing this objective, research was oriented to review and investigate how animal response and metabolism are affected by dietary carbohydrates (amilaceous vs. fibrous). In particular, several studies were carried out to test animal responses to carbohydrates at different stages of pregnancy and lactation (Cannas et al., 2002; Bovera et al., 2004; Cannas et al; 2013) and on small ruminant species such as sheep and goats (Cannas et al., 2004;Cannas et al., 2007; Lunesu, 2016). On the other hand, the most recent research in human and livestock nutrition (both monogastric and ruminants)is trying to increase the basis of the nutritional programming of animal performances. Nutritional strategies capable to allow metabolic programming will be highly beneficial to improve management, feed efficiency in livestock and diffusion of healthy diets in humans. Several studies were carried out on mammals to investigate and quantify the animal response to different nutritional programs previously provided. It has been demonstrated that nutritional conditions and environment to which animals are exposed either in uterine lifeor early in postnatal life (birth, suckling and weaning phases, pre-pubertalphases, pregnancy)might impair, or favor, physiological status, production and performances of adult animals (Duque-Guimaraes and Ozanne, 2013). This focus, on which more research need to be addressed, also involves complex mechanisms of epigenetic modifications that can affect gene expression, during the life of an individual, or permanently involving transgenerational changes(Feeney et al., 2014). This review will investigate the sheep response to different carbohydrate sources and the most relevant aspects of nutritional fetal programming for sheep production.

1.1. Dietary substrates

Carbohydrates are the first organic product derived from vegetal photosynthesis starting from carbon dioxide and water. They are composed by carbon, hydrogen and oxygen. Considering organic chemistry, carbohydrates can be classified in monosaccharide, disaccharides, oligosaccharides and polysaccharides (Van Soest, 1994). The most abundant carbohydrates in nature are polysaccharides such as starches which have their important role within vegetal organisms,as major reserves constituents (White, 1973) and other polysaccharides, particularly cellulose, which consist of structural component of the vegetal cell. In practice, carbohydrate fractions are defined by the chemical or enzymatic methods used for their analysis.

Reserve carbohydrates including organic acids, mono-oligosaccharides, starches and fructans, are usually stored in the cell content whereas the structural carbohydrates such as cellulose, hemicellulose, pectins, galactans and glucans, belong to the cell wall (Figure 1). The cellulose is the most abundant among the structural carbohydrates, comprising over 50% of all the carbon vegetation (Choct, 1997). All the reserve carbohydrates have important roles in the physiological processes of the plant life including growth and respiration processes.

Chemists are trying to classify reserve carbohydrates for their nutritional relevance since 1800. Their synthesis is the basis for the production of direct food for humans and monogastric species, representing the most relevant component of grains (Capper et al., 2013) whereas structural carbohydrates are the basis of the mechanical function of the vegetable cells and of the whole plants. They are associated to non-carbohydrate compounds like lignin and other substances that enhance the mechanical and physical properties of the plant (Van Soest, 1994). Structural carbohydrates represent the important part of non edible substrates by humans that can be fermented by ruminants in order to produce indirect human food (Capper et al., 2013). Classification in simple sugars, reserve carbohydrates and structural carbohydrates could provide a further first classification (Van Soest et al., 1994).

An additional chemical separation can be made among the structural carbohydrates included in the analytical part of the neutral detergent fiber (NDF; Van Sorest, 1994), which is composed by insoluble fiber cellulose and hemicelluloses, lignin and cutin (Hall, 2003) and thenon fiber carbohydrates (NFC). The latter consist of organic acid, starch

and soluble fiber, mono and oligosaccharides (Hall, 2003). The so-called NFC is usually obtainable by difference as: NFC = 100 – (Crude Protein + NDF + Ashes + Fat) in a given feed and they are not measured by a specific analysis.The distinction between NFC and NDF is not always satisfying from a nutritional point of view. Neutral detergent fiber is recognized as one of the most important variables related with animal intake and performances. In diets for dairy animals, Mertens (1997) suggested that NDF could be a valuable tool for identifying the maximum intake level, the ratio between forage and concentrate (F:C). In addition, considering its particle size distribution, it is strongly and positively related with rumination activity, ruminal pH and milk fat content (Mertens, 1997).

On the other hand, NDF also constitutes the most relevant part of low degradable nutrients in animal diets. In fact, NDF consists of different fractions of chemical compounds. One fraction is potentially degraded and fermented at ruminal level (pdNDF), mainly represented by cellulose and hemicelluloses which degradability mainly depends on their chemical structure, physical form and level of intake and other feed and animal characteristics (Robinson et al., 1986).Undegradable NDF(INDF), which includes lignin and other compounds, such as Maillard, is not degraded even within 10 days of permanence in the ruminal environment. In addition, they can incrust and reduce the degradability of fiber carbohydrates in a proportion equal to approximately 2.4 times their weight (Van Soest, 1994).Separation of the NFC into more nutritionally relevant fractions through analyses for its components, allows better diet formulation (Hall, 2003). In particular, pectins, fructans, galanctansand β-glucans are the components of the neutral detergent soluble fiber (NDSF) which includes carbohydrates from the lamella mediana of the cell walls and from cell content. Soluble fiber comprehends the non-starch, non-NDF polysaccharides including pectic substances, (1 →3)(1 →4)-β-glucans, fructans, and gums. NDSF + NDF represent the non-starch polysaccharides. From a nutritional point of view NDFS and NDF carbohydrates differ in term of degradation rate in rumen, being the components of soluble fiber those with high degradation rate. NDSF are abundant in forages in early vegetative stages and agro-industrial byproducts such as beet pulp, citrus pulp and soyhulls which are often defined as non forage fiber sources (Grant, 1997). The remaining part of NFC consists of organic acids, mono- and oligosaccharides and starches that are abundant in grains and cereals. Analytically they are identified as the fraction of non structural carbohydrates (NSC).

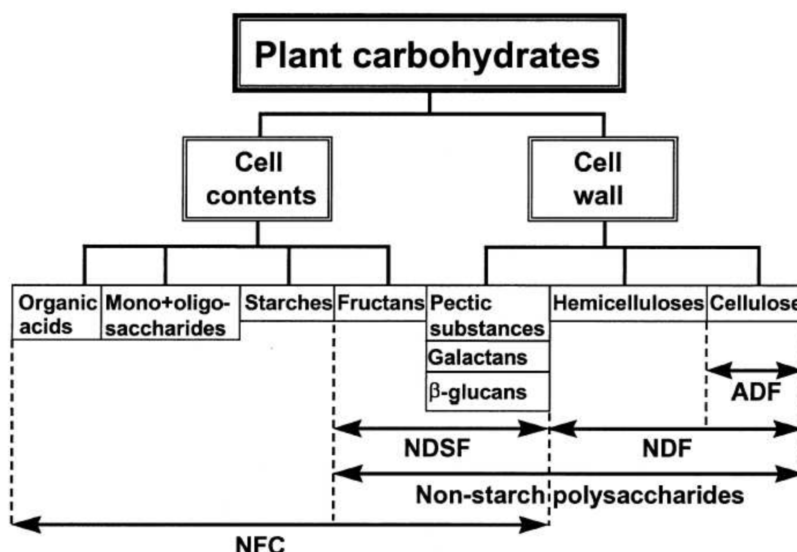


Figure 1. Plant carbohydrate fraction classification as proposed by Hall (2003).ADF = acid detergent fiber, β -glucans = (1 $\rightarrow$ 3) (1 $\rightarrow$ 4)- β -D-glucans, NDF = neutral detergent fiber, NDSF = neutral detergent-soluble fiber (includes all non-starch polysaccharides not present in NDF), NFC = non-NDF carbohydrates (includes organic acid, starch and soluble fiber, mono and oligosaccharides).

1.2. Carbohydrate source: lipogenic vs. glucogenic substrates

Carbohydrates play a principal role in the diet of ruminants, and both NFC and NDF are very important for the ruminal and metabolic efficiency of animals (Allen, 1997). Fermentation in the rumen is by far the most important process whereby ruminants digest carbohydrates. For most diets, the rumen accounts for over 0.9 of the digestion of all carbohydrates (Sutton, 1979), although this value can be altered when high ruminal passage rate allows that a certain amount of carbohydrates is digested in the intestine or fermented in the lower tract (Van Soest, 1994). Rumen fermentation occurs in anaerobic conditions for intermediation of microbial species which role has been well established in the past for the most relevant processes in quantitative terms (Eldsen, 1945).

Products of rumen fermentations account for more than 70% of the ruminant energetic supply which mainly depends on the amount volatile fatty acids (VFA) produced with microbial fermentation of substrates. Carbohydrates fermented at ruminal level principally end in acetic acid – C2 - (60-70%), propionic acid - C3 - (20-30%), butyric

acid – C4 – (about 10%) and, in minor extent, lactate (Van Soest, 1994). Other end products of microbial fermentations include principally CO_2, CH_4which present energetic losses of the fermentation process eliminated with eructation.

Fermentation of different carbohydrate sources determines different proportion of VFA. Fiber compounds are likely to produce higher proportions of acetic acids than simple sugars and starches, which are likely to be fermented in propionic acid in sheep (Eldsen, 1945) as well as in other ruminants (Van Soest, 1994). On the other hand, acetic and propionate VFA, although resulting from two different metabolic patterns, both represent important sources of metabolic fuel (Van Soest, 1994; Aguggini et al., 1998).VFAare absorbed from the rumen walls and then used to produce energetic substrates in different organs.

Acetic acid is transported to the liver and converted in the Krebs's cycle to acetil coenzyme – A and then used either as precursor of lipogenesis and as source of ATP (Church, 1988; Aguggini et al., 1998). Acetic acid is the most important precursor of *de novo* milk fatty acids synthesized in the mammary gland (Church, 1988).

Propionic acid is transported to the liver and involved in the gluconeogenic processes to produce glucose (Church, 1988). Propionate is considered the most important glucogenic precursor and provides about 60 - 74 % of total glucose to the ruminant (Achenbach et al., 2010).

Butyric acid, similarly to acetic acid, is used in the liver and mammary gland to produce ATP and milk fatty acids of *de novo* synthesis (Church, 1988).

Lactate is beneficial to the organism if it is produced in low amounts from microbial fermentations in the rumen, it can provide about 16-26 % of total glucose from gluconeogenesis in the ruminant (Aschenbach et al., 2010). In this way, through the change in the dietary ratio of starch:fiber compounds it is possible to influence ruminal fermentation and blood metabolites: diets rich in starch favor production of propionic acid, while fiber rich diets favor acetic acid and butyric (Eldsen, 1945; Khorasani and Kennelly, 2001).Modulating dietary starch and fiber it is also possible to provide glucogenic or lipogenic substrates respectively (Van Knegels et al., 2005; 2007). In this sense changes in metabolism are expected when animals are exposed to different carbohydrate sources, either dairy cows (Van Knegels et al., 2007), sheep (Bovera et al., 2004; Cannas et al., 2013) or goats (Lunesu 2016) in the short or long term.

Glucogenic dietary ingredients are either fermented in the rumen to produce highamounts of propionate or are digested in the small intestine and absorbed as glucose. The use of dietary substrates embraces, at tissue level, all the metabolic processes that involve the intake, absorption of nutrients, and tissue utilization of absorbed nutrients for maintenance, conceptus growth and milk production (Bauman and Currie, 1980; Figure 2). It also includes the variation in body reserves to store excesses of nutrients or to provide metabolic fuels during nutrient shortages.

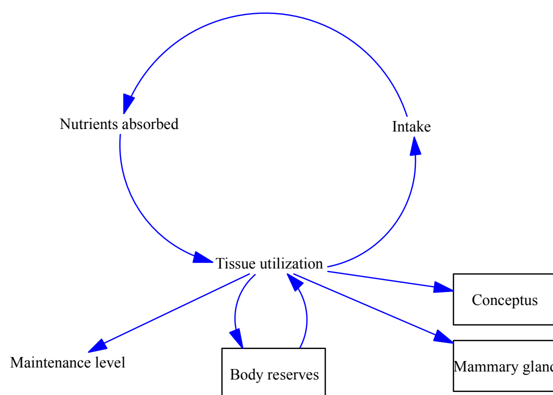


Figure 2. Animal metabolism. Adapted from Bauman and Currie (1980).

1.3. Nutrient use in lactation

The regulation of nutrients partitioning in various body tissues involves homeostatic and homeorhetic changes (Bauman and Currie, 1980). *Homeostasis* indicates the maintenance of physiological equilibrium or constant conditions in the internal environmental (Bauman and Currie, 1980) whereas *Homeorhesis* relates to the ability of the animal to adjust biological processes in a dominant physiological state (Bauman, 2000). Lactation and pregnancy represent an impressive example of Homeorhesis for milk production and conceptus growth with an extraordinary proportion of nutrients used for milk production at peak of lactation and conceptus growth in late pregnancy (Bauman and Currie 1980; Bell and Bauman, 1997). Many physiological situations have been described with homeorhetic regulations (Bauman, 2000).

In ruminants, acetic acid accounts for the most important dietary product quantitatively absorbed in the gastrointestinal tract and used for metabolic processes as above described. In particular, dairy sheep, showing high demand of milk fat precursors to support the high

milk fat content, demonstrated to have particular exigencies of acetate from diet fermentation (Cannas et al., 2002). On the other hand, glucose, beside being absorbed in limited amount in the gastrointestinal tract, is also involved in a strong homeorhetic regulation of supply and removal of glucogenic precursors in the blood, quantitatively modulated by different hormones. The primary organ producing glucose is the liver (Brockman and Laarveld, 1986). Glucose, produced with gluconeogenic processes from dietary sources or from depletion of body reserves,is the primary nutrient supportingconceptus growth and milk synthesis(Bauman and Currie, 1980; De Koster and Opsomer, 2013; Figure 3) and needs to be permanently available (Aschenbach et al., 2010)

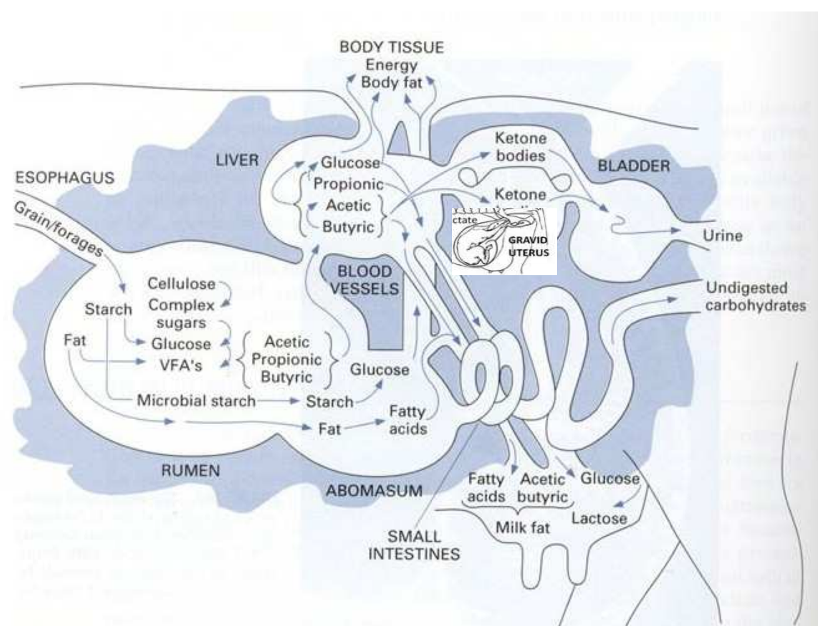


Figure 3. Energy pathways in ruminants. (Adapted from Bryant and Moss, Montana State University, USA).

1.4. Energy partitioning in lactation and carbohydrate metabolism

As well known, milk production curve of dairy ruminants increases in early lactation until the peak (first 1-2 months from parturition), then decreases progressively in mid to late lactation (for the next 6 to 8 months in dairy sheep) until the dry period. This shape results from a well-controlled nutrient partitioning to the mammary gland and body reserves that progressively change during lactation under hormonal control. Many authors

reviewed the adaptations of animals to milk production in comparison to non lactating state and the endocrine control of organs and tissues in different stages of lactation and pregnancy. The whole process is briefly summarized in Figure 4 in terms of performance and physiological aspects.

Early lactation

After parturition, proliferation of active cells in the mammary gland increases the glucose demand for lactose synthesis and milk production until the lactation peak (Knight, 2005).Glucose demand of mammary gland can account up to 80% of total glucose turnover (Bauman and Currie, 1980; Huntington, 1997). This enormous glucose demand increases glucogenic rates in liver and stimulate glycogen mobilization from liver and muscles (Reynolds et al., 1988). Hammon et al., (2010) have found that during this phase of high glucose demand for milk production, glucose utilization is primarily reduced in non mammary tissues such as muscle and adipose tissue. Intake also increases but often animals experience negative energy balance (nutrient intake is not sufficient to meet energy requirements) thus body reserves are mobilized for glucogenic supply. High demand of nutrients requires that large amount of milk produced in early lactation is produced by gluconeogenic processes and using body reserves (Moe, 1975). Lactation yield in dairy cows have been found to be related to the magnitude of body reserve mobilization (Bauman, 1985). In early lactation, high levels of growth hormone (GH) stimulate milk production and seem to drive the whole homeorhetic regulation (Bauman, 2000). Bell and Bauman (1997) stated that only GH can be assigned a clear and powerful role in the homeorhetic regulation of adaptations in glucose production and disposal, coordinated with mammary and non-mammary tissues. Even if the GH secretion from the pituitary is pulsatile (Wallace et al., 1972; Laurentie et al., 1987), growth hormone does not seem to be involved in the minute-to-minute regulation of metabolism, but it can alter the homeorhetic sensitivity of tissues to insulin with respect to glucose metabolism (Brockman and Laarveld, 1986). Moreover GH appears to be antagonistic to the lipogenic effects of insulin (Wallace and Basset, 1966; Troike et al., 2017).The majority of information about GH effects on milk production came from studies on exogenous somatotropin infusion in lactating ruminants which showed increases in milk yield and a series of coordinated adaptations in body tissues to support milk yield. In addition to GH effects, utilization of glucose by mammary gland is enhanced by low blood levels of

insulin and a state of insulin resistance of the adipose tissue. In particular, insulin favors uptake of glucose by peripheral tissues (adipose tissues and muscle) but it is not active in the mammary gland (Laarveld et al., 1981). The main reason for this difference is that glucose transporters at cellular levels (GLUT) are of two types: insulin dependent (GLUT 4, the only one)and insulin independent (being GLUT 1, 2, 3, 4, 5 and 8 the most important). In the adipose tissue GLUT 1 (especially for uptake of basal glucose) and GLUT 4 are the most active (Sasaki, 2002), whereas in the mammary gland the insulin dependent GLUT 4 is absent (Komatsu et al., 2005) and glucose uptake is mainly due to GLUT 1 and GLUT 8 (Bell et al., 1990; Sasaki, 2002; Komatsu et al., 2005). Low concentrations of insulin can also have a catabolic effect (Brockman and Laarveld, 1986). The state of insulin resistance starts in late pregnancy and continue in early lactation(Bell and Bauman, 1997; De Koster and Opsomer, 2013). Adipose tissue in early lactation appears not sensitive to insulin action and shows reduced glucose uptake and lipogenesis (Wilson et al., 1996; Sasaki et al., 1994; De Koster and Opsomer, 2013).

The GH effect on reducing insulin sensitivity of adipose tissue was attributed to modification of signal transduction at post-receptorial level more than to insulin binding (Wilson et al., 1996; Sasaki, 2002; Troike et al., 2017). Van Knegsel et al. (2005) hypothesized that part of the metabolic effect of the negative energy balance (NEB) experienced by early lactating animals is due to an imbalance in C2:C3 nutrient ratio. Negative energy status promotes mobilization of body fat (Tamminga et al., 1997) and increases the availability of C2 compounds. The C2:C3 imbalance might be induced by the excess of C2 nutrients (in turn stimulated by body fat mobilization) that cannot be oxidized completely as a result of a deficiency in C3 nutrients and causing alternatively: i) increase in plasma ketone body concentration; ii)increase in livertriacylglycerides (TAG), iii)increases in milk fat production (Palmquist and Mattos, 1978) (Van Knegsel et al. 2005).Lipogenic dietary ingredients such as dietary fat, or forages, also stimulate the ruminal production of acetate and butyrate and the availability of C2 nutrients, promoting the first two metabolic patterns. However, in early lactation, due to high metabolic requirements, diets high in lipogenic nutrients increase the risk of ketosis and fatty liver by increasing the C2:C3 nutrient ratio (Drackley, 1999; van Knegsel et al., 2005). In contrast, glucogenic dietary ingredients are either fermented in the rumen to produce highamounts of propionate or digested in the small intestine and absorbed as glucose, reducing the unbalance of C2:C3 nutrients.

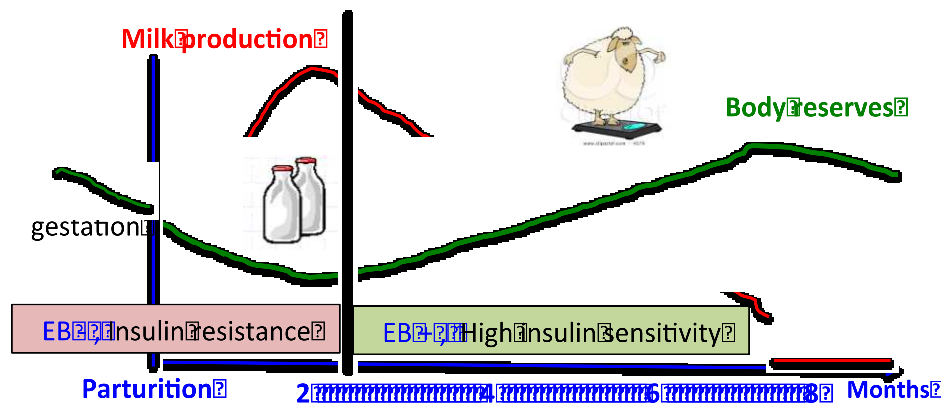
In dairy sheep the use of high starch or high NFC diets (glucogenic substrates) in early lactation has been related to increased production of propionate at rumen level, reduction of negative energy balance and higher milk production compared to diets with high NDF or low NFC (lipogenic substrates) (Cannas et al., 2002;Bovera et al., 2004). It confirms that glucogenic substrates should be used to support milk production in early lactation for dairy sheep and to favor nutrient utilization by the mammary gland (Cannas et al., 2002; Cannas et al., 2013; Lunesu, 2016). These findings were also observed in dairy cows (Van Knegsel et al., 2007) and goats (Cannas et al., 2007; Lunesu, 2016).

Mid lactation to late lactation

After the lactation peak, the milk production declines while the feed intake is still increasing thus the animal tends to reach a positive energy balance. This phase is characterized by a strong competition for nutrients among mammary gland and adipose tissue. Growth hormone concentration decreases as lactation advances, insulin levels raise and the adipose tissue became more sensitive to insulin, favoring the glucose uptake from adipocytes and lipogenesis (Bauman, 2000; De Koster and Opsomer, 2013). Insulin is the primary hormonal regulator of metabolism in the resting animal. When energy intake is high, insulin concentrations are high and growth and/or body gain is promoted (Brockman and Laarveld, 1986).In mid to late lactation the homeorhetic regulation seems oriented to recover the body reserves lost in early lactation.In this phase, this adaptation is more evident in sheep than in dairy cows, probably because sheep are generally less selected for milk production than cows. In fact, sheep vs. cows show lower persistency of GH after lactation peak and higher insulin sensitivity of peripheral tissues (Cannas et al., 2002). The hormonal regulation in mid-to late lactation is also controlled by the insulin-like growth factor-1 (IGF-1)that is low in early lactation and high in late lactation (Zulu et al., 2002; Taylor et al., 2004). This growth factor is dependent from GH and is negatively correlated with parity, milk yield (Taylor et al., 2004) and genetic merit (Verkaamp et al., 2003) in dairy cows. IGF was also positively correlated with body condition score (BCS) (Pulina et al., 2012), with blood non-esterified fatty acids (NEFA) and with fibrous carbohydrates in dairy ewes (Cannas et al., 2004). It can be considered a good marker of energy balance that, oppositely to GH, increases insulin sensitivity and it is positively associated to glucose and insulin concentrations (Zulu et al., 2002). Levels of IGF-1 and insulin and increases of body reserves are associated with increased blood

levels of leptin, an hormone secreted by adipocytes, which showed high importance in regulating the feed intake when energy balance is positive (Chilliard et al., 2001). Leptin plays an anorexigenic role of increasing energy expenditure and decreasing feed intake (Troike et al., 2017).

As highlighted by Cannas et al. (2002), in mid to late lactation the use of glucogenic carbohydrates (fermented in propionate at ruminal level and then transformed to glucose in the liver) are likely to stimulate insulin action in peripheral tissues and also to favor the nutrient partitioning versus lipogenesis and accumulationof body reserves. Conversely, the use of lipogenic carbohydrates (fermented in acetic acid and do not stimulate insulin secretion and action),is likely to limit the insulin response and lipogenesis sparing glucose that can be used by the mammary gland for lactose synthesis.



Lactation phase	Early	Mid to late
Milk production	Increases - high	Decrease
Milk fat	Low	High
Mammary cells	proliferation	involution, death
	fatty acid uptake from body reserves (> C16)	Synthesis of de novo fatty acids (< C10)
Feed intake	Increase	Stable or Decrease
Body reserves	Depletion	Increase
Prolactin	High	Low
GH	High	Low
Insulin	resistance	high sensitivity
IGF-1	Low	High
Leptin	Low	High
Liver	High activity	Low activity
Adipose tissue	Lipolysis	Lipogenesis
	Insulin resistance	Insulin sensitivity
Muscle	Glycogen mobilization	Glycogen accumulation

Figure 4. Homeorethic control of lactation in ruminants. The figure summarize the main aspects related with nutrient partitioning in early and mid-lactating ewes.

In addition, the high availability of fat precursors could allow high milk solids and metabolic fuel in form of ATP. Following the C2:C3 balance of nutrients (Van Knegels et al., 2007), it can be argued that excesses of C2 nutrients in mid lactation, when animal is experiencing positive energy balance and low risk of ketosis or fatty liver can result increasing milk fat and nutrient partitioning of dietary energy to the mammary gland. Supporting this theory, Lunesu (2016) reported a list of studies observing significant or numeric increases of milk yield and milk solids, higher or constant dry matter (DM) intake and lower or similar body reserve accumulation, when corn or barley grains in diets of mid lactating sheep were substituted by high digestible fibrous substrates. Literature data and experimental findings reported by Lunesu (2016) were summarized in this review (Figure 5). Data showed that reduction of dietary NFC concentration in mid lactating sheep was associated with increases in fat and protein corrected milk yield (FPCMY) (Figure 5). Within trial the difference between groups receiving diets with high and low NFC was statistically significant (P <0.05) for the majority of the studies (Lunesu, 2016).

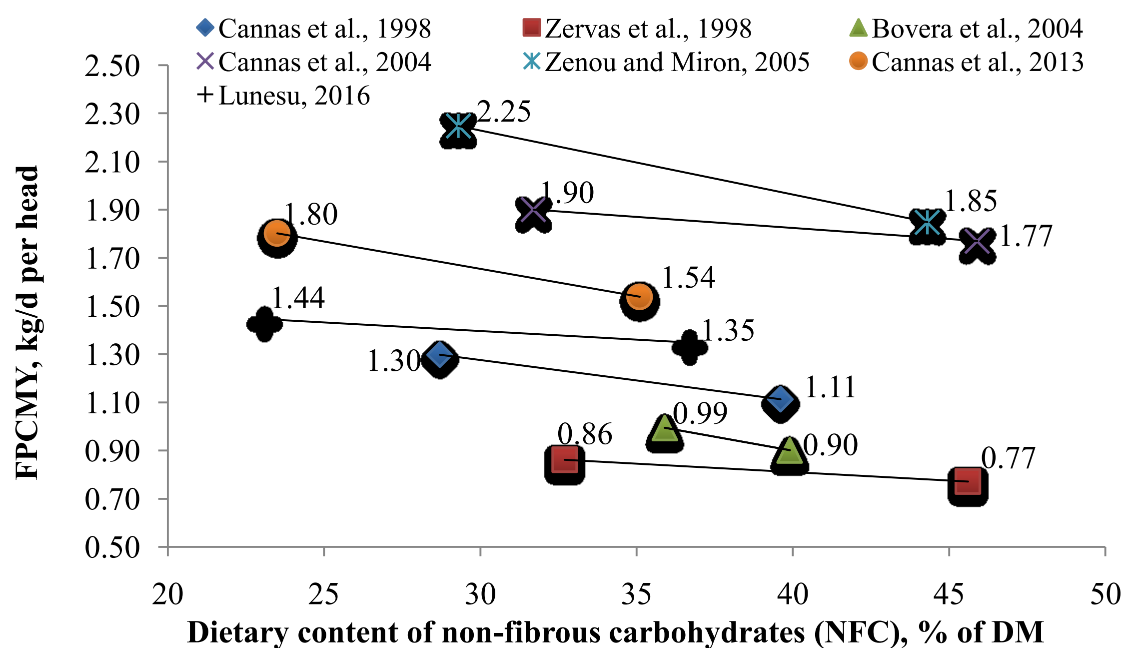


Figure 5. Effects of dietary NFC concentration on fat and protein corrected milk yield (FPCMY) in 7 studies from literature carried out with mid-lactating sheep. Within study, the higher NFC content was reached using glucogenic carbohydrates from cereal grains whereas the lower NFC content was obtained substituting part of the grains with soluble fiber or high digestible NDF. Data from original papers were reported by Lunesu (2016).

Based on the data reported in Figure 5, on average, a mean reduction of 10.8 ± 4.4 % (ranging from -4% to -15%) of NFC allowed an increase of 0.170 ± 0.11 kg/d (ranging from 0.09 to 0.260 kg/d) of milk per head. Despite this general positive effect those data did not allow to define an average optimum level of NFC for mid-lactating sheep in order to maximize FPCMY by increasing lactation persistency.

Different studies showed that dairy goats might reach higher milk yield, with high starch and high NFC diets both in early and mid lactation in respect to low starch and low NFC diets (Lunesu, 2016).

On the best of our knowledge, no effects on GH stimulation by fiber carbohydrates were documented or hypothesized in literature and, on the other hand, insulin action is heavily stimulated by diet and has been demonstrated that is strongly involved in the hormonal control of the mid lactation phase and milk production decline after the lactation peak. In addition the evident switch among high and low states of insulin resistance of peripheral tissue, from early to late lactation suggests that lactation persistency might be modulated with nutritional strategies and moreover by varying the carbohydrate source of the diet. In this sense glucose metabolism and insulin resistance need to be deeply focused in order to improve understanding ofdietary nutrient partitioning to mammary gland.

1.5. Insulin resistance and lactation persistency

Defining insulin resistance

Generally insulin increases the glucose utilization in muscle and adipose tissue as much as 5 fold (Brockman, 1993). Insulin in the liver reduces glucose output and gluconeogenesis whereas inhibit lipolysis in adipose tissue favoring the re-esterification of non-esterified fatty acids (NEFA). The concepts of insulin resistance need to be better defined in order to describe the tissue response. Insulin resistance is defined as a state whereby a normal concentration of insulin induces a decreased biological response in sensitive tissues and in isolated adipocytes with a sigmoidal dose-response curve (Kahn, 1978; Baumgard et al., 2017). It can be separated into two components: insulin insensitivity and responsiveness. The maximal effect of insulin defines the responsiveness whereas the concentration of insulin allowing half-maximun response indicates the insulin sensitivity (De Koster and Opsomer, 2013; Figure 6). Sensitivity

affects the cells at receptorial level and moves the curves to the right, whereas responsiveness is due to disruption of glucose metabolism inside the cell, and depresses the upper asymptote (Figure 6).

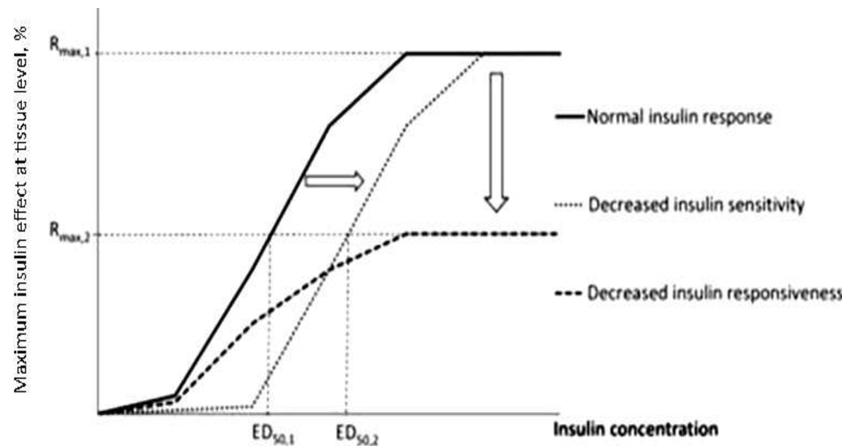


Figure 6. Insulin sensitivity and responsiveness. The normal insulin response is characterized by a maximal biological effect ($R_{max,1}$), and an insulin concentration to elicit a half maximal effect ($ED_{50,1}$). A decreased insulin sensitivity is characterized by a normal maximal biological effect ($R_{max,1}$),while an increased insulin concentration is needed to elicit half of the maximal effect ($ED_{50,2}$). A decreased insulin responsiveness is characterized by a decreased maximal biological effect ($R_{max,2}$), while a normal insulin concentration is needed to elicit a half of the maximal effect ($ED_{50,1}$). Adapted from (Khan, 1978).

Insulin resistance (well known in human medicine for causing diabetes mellitus of type 2) is something different and independent from insulin secretion deficiency of pancreatic β cells (causing diabetes of type 1), even thought diabetes 2 might end in diabetes 1. Insulin resistance is commonly associated to obesity and negative effects of overweight, hyperglycemia and metabolic disorders in humans. Nevertheless, human literature often focuses the pathological aspect related to irreversible insulin resistance, hyperglycemia and clinical obesity. In dairy ruminants, status of hyperglycemia is documented but very rare because high glucose requirements of mammary gland cause enormous uptake of glucose not allowing hyperglycemia except in some stages of pregnancy (De Koster and Opsomer, 2013).

Otherwise, insulin resistance is an important evolutionary process that allows the physiological prioritization of nutrients to the most important physiological processes for life (Soeters and Soeters, 2012). Insulin resistance is a mechanism that is activated in conditions of stress and of nutrient shortages in order to optimize the nutrient use and to succor life in the short term (Soeters and Soeters, 2012).
Sheep have a general lower responsiveness to insulin than humans and rats (Sasaki et al., 2002). In the majority of the cases, insulin resistance of ruminants is a reversible condition and can be considered a non permanent adaptation to a certain metabolic condition. Thus we will refer to insulin resistance in terms of reduced insulin sensitivity or as combined effect of sensitivity and responsiveness. Insulin resistance has been documented in pregnancy and lactation in dairy cows (Kerestes et al., 2009; Chagas et al.., 2009; Ji et al., 2012), sheep (Vernon et al., 1990; Petterson et al., 1993) and goats (Debras et al., 1989).

GH and insulin resistance

A recent work of Troike et al. (2017) extensively reviewed the role of GH in regulation of adipose tissue in knockout mice and confirmed that GH acts inhibiting insulin action, decreasing glucose uptake, stimulating lipolysis and inhibiting lipogenesis. In addition, the same authors confirmed that GH regulates several properties of the adipose tissue including endocrine and immune cell functions. These effects are more evident in visceral than in subcutaneous tissue. A specific role of GH in this phase has been identified on reducing the ability of insulin to stimulate lipogenesis and reduce the activity of insulin dependent glucose transporters (GLUT 4) at post-receptorial level (Bell and Bauman, 1997; Sasaki, 2002).Insulin promotes body gain by stimulating fat and protein synthesis, whereas GH promotes lean growth (Brockman and Laarveld, 1986). Modulation of GH is barely controlled by several balancing feedback loops acting on the pituitary and involving (among others): i) high GH blood levels in local regulation (Muller et al., 1999); ii) serum levels of IGF-I in systemic regulations (Muller et al., 1999); iii) other circulating molecules such as adipokines and sex hormones (Troike et al., 2017).It is quite clear to the scientific community that GH modulates lipolysis and inhibiting lipogenesis but basic mechanisms and molecular players remains unclear (Troike et al., 2017). A relevant role has to be attributed to the same adipose tissue and involves its main hormones, the adipokines of which leptin and adiponectin are the most prominent.

In particular adiponectin is strongly involved in regulation of insulin resistance having high insulin-sensitizing properties and it is used as proxy to predict insulin resistance in humans (Hara et al., 2006).

The adipose tissue plays an important role in the determination and modulation of insulin sensitivity. On the other hand, from a nutritional point of view, insulin resistance or sensitivity appears as one of the most important mechanisms related to changes in nutrient partitioning and lactation persistency and it could be interconnected to dietary carbohydrates supplied to the animal.

Mechanism involved in the regulation of insulin resistance at cellular level

Even if not all mechanism of insulin action and insulin resistance are well known in ruminants, a general mechanism of insulin action in the sheep adipose tissue was hypothesized and explained by Sasaki et al. (2002). In adipocytes and muscles, insulin binding to receptors at membrane level is followed by a cascade of signals that stimulates the translocation of the insulin-sensitive glucose transporter GLUT 4from an intracellular membrane pool to the plasma membrane. The translocation is activated through the intracellular signaling pathway of insulin phosphatidylinositol 3-kinase (PI3-kinase) and a specific role of the insulin receptor substrate (IRS-1). Starting from this point, Sasaki et al. (2002) suggested that the lower sensitivity to insulin might be due to lower capacity of insulin signal transduction and lower glucose transport activity. They also suggested that the impairment of insulin signaling during lactation is at a post receptorial step and is probably downstream of PI3- kinaseconsidering that insulin receptors seems to remain unaltered during lactation (Vernon et al., 1985). It would confirm that not all aspects of insulin action in adipose tissue are impaired during lactation (Vernon 1989).As highlighted by De Koster and Opsomer (2013) NEFA concentration in blood can act inhibiting insulin secretion (Bossaert et al., 2008) and it is likely to be involved in the development of insulin resistance in early lactating ruminants. In fact, high NEFA oxidation of fatty acids can inhibit the use of glucose as substrate for cellular metabolism (Randle cycle; Hue and Taegtmeyer, 2009) and reduces the translocation of GLUT 4 to the membrane acting directly on the IRS-1 phosphorylation and signaling activation (Le Marchand-Brustel et al., 2003). It is in agreement with the C2:C3 equilibrium previously described and suggested by Van Knegsel et al. (2007) and also with an important role of the energy balance on the maintenance of insulin resistance from early to mid lactation.

This pathway can be considered part of the physiological adaptation to changes in energy balance.
Other mechanism activating insulin resistance encompass an immune regulation and an inflammatory state of adipose tissue caused by the production of pro-inflammatory adipokines tumor necrosis factor (TNF-α) and the reduction of adiponectin secretion, also demonstrated in dairy cows (Ingvartsen andBoisclair, 2001). De Koster and Opsomer(2013) reviewed several works observing overconditioneddairy cows with high TNF-α levels in plasma and showing a low-grade inflammatory status that can induce insulin resistance and high inflammatory reaction to infections. Pro-inflammatory adipokines such as TNF-αand interleukine-6, and PPAR-γ are involved in the inflammatory processes causing insulin resistance. It has to be considered that dairy ruminants often experience inflammatory status in periparturient period (Trevisi et al., 2015). Undoubtedly, physiological and pathological pathways can contribute to insulin resistance in late pregnancy and early lactation.
Insulin resistance is influenced by many other factors that are related with short-term and long-term epigenetic modifications causing a sort of metabolic memories including insulin resistance (Milagro et al., 2013). Several cases of insulin resistance related with maternal diet and inflammations mediated by TNF-α, Interleukin-6and PPAR-γ (Soeters and Soeters, 2012)have been demonstrated in ruminants (Paliy et al., 2014), fetal and suckling age(Duque-Guimaraes and Ozanne, 2013).Several genes seem to be involved with epigenetic changes related with insulin resistance (adiponectin, leptin, insulin, insulin-stimulated glucose transporter - GLUT4, insulin receptor signal - IRS-1) (Fuston and Summer, 2013; Milagro et al., 2013).
Specific insulin resistance is also a characteristic that we enhanced with selection. In fact cows with high genetic merit are more insulin resistant in early lactation (Chagas et al., 2009). In these terms, insulin resistance acts only on reserve organs and allows defense against starvation, disease and trauma and promote metabolic efforts for lactation growth and pregnancy in borderline conditions (Soeters and Soeters, 2012). It should be considered an evolutionary skill developed to overcome metabolic barriers. A particular aspect regards the nutritional factors related with diet and carbohydrate sources. Several evidences in literature highlighted that nutritional strategies can influence insulin resistance of adipose tissue. It has been demonstrated that concentrate diets give a positive effect on glucose clearance in glucose tolerance test and high sensitivity to

insulin (Xing et al., 1991). Chagas et al. (2009) observed that cows with high genetic merit showed increases of glucose clearance when fed with 3 or 6 kg of concentrate to pasture integration. In particular, the relation was positive and linear with the concentrate intake and the authors suggested a possible threshold level of NSC to increase peripheral tissue sensitivity to insulin.

1.6. Nutrient partitioning towards fetus, insulin resistance

Metabolic regulation during pregnancy

In the pregnant mother, many metabolic changes drive the nutrient partitioning to the gravid uterus and body reserves. In the first stage of pregnancy, the animal metabolism is mainly anabolic in order to accumulate more reserves to be used in late pregnancy and sequent lactation (Svennersten-Sjaunja and Olsson, 2005). Changes in appetite, body composition, energy consumption and metabolism occur to ensure an adequate flow of substrates to the developing fetus in early and late pregnancy and in preparation for parturition and milk production (Owens 1991). These changes can occur directly or indirectly via endocrine mediated mechanisms (Bauman and Currie, 1980) and with strong metabolic adaptation including the insulin sensitivity in peripheral tissues (Vernon et al., 1981). Furthermore, metabolic adaptations in pregnancy are driven by the endocrine system with the aim to distribute the nutrients in the maternal body and then, finally, to provide simple elements such as glucose and amino acids to the growing fetus (Gluckman, 1997). Several hormones play predominant roles in mediating the altered responses to insulin and other homeostatic regulations in pregnancy including, among others, progesterone in mid-pregnancy and placental lactogen and estrogens in late-pregnancy (Bauman and Currie 1980). A very important hormone for the intake regulation and caloric expenditure in anabolic conditions, including appetite and metabolism, is leptin, which is produced by adipose cells (Masuzaki et al., 1995). In a generic view, the homeorhetic switch from deposition of body reserves in early pregnancyto fat mobilization in late pregnancy has been studied and deeply investigated in the 80' (Vernon et al., 1981). The same authors also investigated the relationship among insulin, adipocyte insulin receptors and circulating hormones and blood metabolites.

Insulin resistance in pregnancy and metabolic programming

Metabolic adaptations of late pregnant sheep, including increased gluconeogenesis from metabolites and mobilization of fatty acids from adipose tissues(Vernonet al., 1981), have been recently related with the development of gestational insulin resistance (Husted et al., 2008). Rohads et al. (2004) studying dairy cows in late gestation, observed development of insulin resistance, whichoriginates from negative energy balance and low growth hormone levels. It has to be noticed that before reaching negative balance, pregnancy and dry off are considered the only physiological states on which a ruminant can be found in an obese state. Cases of obesity have been studied in cows (Agenäs and Holtenius, 2003) and sheep (Zhu et al., 2009; Long et al., 2010; Zhang et al., 2011). Obesevs. lean sheep, in response to an infusion of insulin glucose, showed hyperinsulinemia and moderate hyperglycemia (Bergman and Corlett, 1989). Cows that were overfeed during the dry period,showed high NEFA, high levels of TNF-α, low glucose clearance and high insulin-resistance in the successive lactation (Agenäs and Holtenius, 2003).Other studies demonstrated that obese cows showed high plasma TNF-α levels, which then led to a inflammatory status generating insulin-resistance(O'Boyle et al., 2006). These evidences highlight both the physiological and pathological status that might induce metabolic adaptation mediated by insulin resistance.

As demonstrated and reviewed in many studies, and previously described in the above section the insulin resistance in dairy animals start in late pregnancy and continue in transition and sequent lactation (De Koster and Opsomer, 2013; Baumgard et al., 2017).

In addition to the homeorhetic regulation of the pregnant mother, fundamental consequences of developing insulin resistance in pregnancy are the risk to transmit metabolic imprinting to the fetus in the mechanism called metabolic or fetal programming (Hanley et al., 2010; Milagro et al., 2013). It involves many complex interactions and evolutionary strategies that have its roots in epigenetic modifications (Milagro et al., 2013;Duque-Guimaraes and Ozanne, 2013). Godfrey et al. (2011) observed that the altered maternal environment could influence epigenetic processes and developmental changes associated with cardiovascular disease, hypertension, obesity and type II diabetes of the offsprings. In fact, metabolic programming and metabolic imprinting describe early life events that impact upon on later physiological outcomes (Hanley et al., 2010). They can impact the future progeny for many generations and/or happen early in postnatal life

and involve the same individual for short or long term (Milagro et al., 2013). However, the two mechanisms of programming and imprinting are distinct: metabolic programming can be defined as a dynamic process whose effects are dependent upon a critical window whereas metabolic imprinting is more related to imprinting at the genomic level (Hanley et al., 2010).In these sense metabolic diseases, including low glucose tolerance can be directly influenced by epigenetic mechanisms. Intense research activity is actually focusing on these mechanism in humans, to overcome and prevent obesity problems and encompassing multidisciplinary approaches including social aspects related to income and dietary habits (Duque-Guimaraes and Ozanne, 2013), microbiological environment (Clemente et al., 2012; Shen et al, 2013), genetics (Milagro et al., 2013),physiological and health features (Lawrence et al., 2008; Catalano et al., 2009).

Metabolic programming from dietary effects

During fetal growth, rapid cell division in the various tissues occurs by stimulating metabolic signaling. It is also induced by exposure to biochemical inputs and environment, which may have later-life consequences. This mechanisms generically is called “fetal programming”(Godfrey and Robinson, 1998).Literature reports many studies that related dietary factors with metabolic programming of the offsprings either in humans (Elahi et al, 2009; Catalano et al., 2009), rats (Samuelsson et al., 2008) and ruminants (Poore et al., 2013). In particular studies in humans and rats have showed that environmental of pre-natal diet can induce diseases in later life (Zeisel, 2009).

Relatively to the focus of this thesis two types of relevant studies are reported in literature evidencing changes in glucose and lipid metabolism caused by diets: those investigating effects of dietary component andthose which focused on the effects of the level of energy supply. Evidences from literature indicate that exposure in fetal life, or in the first phases of postnatal life, to excesses of dietary fat, undernutrition and overnutriton are commonly related to higher risk to develop insulin resistance, lower glucose tolerance, altered lipid and glucose metabolism in humans, monogastric and ruminant species (Parlee et al., 2014).

Considering the dietary components, studies that focused effect of different carbohydrate source on fetal programming in pregnancy and early growing in dairy ruminants are very scarce. At the opposite the effects of the inclusion of high and low content of fats in the diets of pregnant ruminants have been deeply investigated and literature evidences

showed that mother diets rich in fat are likely to be associated with altered glucose and lipid metabolism, glucose resistance and insulin sensitivity of the offsprings (Khanal et al., 2015).

Reduced nutrient supply to the fetus negatively influences the growth and metabolism in adult life (Phillips, 1996). Poor fetal nutrition leads to negative permanent changes of the structure and function of tissues and organs (Hales and Barker, 1992). Ewe undernutrition during early and mid gestation reduced postnatal growth and glucose tolerance and increased adiposity in male offspring (Ford et al., 2007). In ruminants, shortage of nutrients during mid gestation decreased muscle fiber size and the number of intramuscular adipocytes of the offspring (Du et al., 2010).Tygesen et al. (2007) observed that ewes exposed to restrict feeding during the last week of pregnancy, reduced birth weight and induced low growth rate of the offspring after birth. Ford et al. (2007) also observed in underfed ewes during mid-gestation that their offspring increased BW and fat deposition during puberty more than those of ewes fed adequately to requirements. On the other hand, overfed pregnant cows presented increased percentage of live calves at weaning and with greater weight in respect to adequate feeding(Stalker et al., 2006).

In humans, maternal undernutrition with postnatal hypernutritional diet, can cause adipocyte metabolism and fat mass, which lead to later obesity (Budge et al., 2005).Ewe undernutrition, during late fetal life disrupts the adaptation to glucose-insulin homeostasis to physiological changes in the adult animal, like late gestation and lactation (Husted et al, 2008). The same authors found lower basal level of insulin in animals that experienced undernutrition in comparison to adequate feeding in late prenatal life. Undernutrition also impaired pancreatic insulin secretion capacity during adult life and reduced plasticity of down-regulation of insulin, with general impaired glucose tolerance in adult sheep (Gardner et al., 2005; Husted et al., 2007;Husted et al., 2008). Rhind and Brooks (2001) observed that also reproductive performance is clearly influenced by prenatal factors including undernutrition and overnutrition. These mechanisms seems to target a thrifty metabolic phenotype (Neel., 1962; Parlee and Dougald, 2014) that could be very beneficial with sequent undernutrition (either in uterine life and/or in postnatal life) but can have detrimental effects with adequate (or overnutrition) in postnatal and adult life (Duque-Guimaraes et al., 2013). It has to be noticed that poor maternal nutrition has to be evaluated on the basis of the level and the length of restriction (Symonds, et al., 2004). Not only undernutrition, but also maternal obesity increase offspring disease, diabetes,

obesity and cardiovascular problems and moreover increase the risk of neonatal mortality (Nohr, et al., 2007). Prenatal overnutrition can lead to development of obesity in the offspring, whether the diet fat feeding is changed or continued (Taylor and Poston, 2007).Overfeeding or underfeeding pregnant ewes altered colostrum quality and quantity and reduced offspring birth weight (Swanson, et al., 2008).

Several authors agree on the fact that more nutritional studies are needed to quantify the degree of impact of maternal nutritional disturbance during gestation.

Parlee and Dougald (2014) reviewed the main factors that might cause lower glucose tolerance in several species highlighting that high fat diets and global-nutrient or protein restricted diets in pre-conception periods and pregnant phases cause increases in the adipose tissue and adipocyte hypertrophy, in both male and female offsprings. The same authors explained a possible physiological mechanism: when restricted or high fat diets are supplied, they induce placenta inflammation that in turn modifies the nutrient supply to the fetus reducing the pancreatic cell development and impairing the leptin regulation, leading to hyperfagia. Similarly happens after birth, when high fat milk is ingested by the offspring.

Otherwise, in dairy sheep, overweight mothers favored milk yield in the first lactation of their offspring than mothers underweight (Van Der Linden et al., 2009). Nevertheless, the effect was only appreciable in first lactation whereas it was not evident in mature ewes of five years of age(Patten et al.,2017). Bach (2012), studying dairy cows, suggested that pre-natal and post-natal diets play a main role in long term of the offspring milk production and moreover in first lactation performances. Similarly, Blair et al., (2010), observed that prenatal diet can affect milk performance of offspring but not of grand-offspring. Recent studies in cows showed that heifers born from dams supplied with fatty acid tended to produce additional milk in their first lactation (Garcia et al., 2016).

From our point of view it is very interesting that mechanisms of metabolic programming involving maternal and postnatal life can affect metabolic adaptation later in life, since they represent a possible way to improve animal efficiency acting on environmental and non-genetic factors in animal husbandry.

1.7. General considerations and research approaches

Beside the pathological and clinic consequences, the most interesting aspect of insulin resistance regards the reversible adaptation of dairy animals in order to favor milk production in early lactation. It stimulates to investigate eventual nutritional strategies aimed to program and manage the insulin resistance status in order to positively influence the second part of lactation and the milk production persistency.

Literature evidences demonstrated that insulin resistance starting in mid-late gestation is maintained in early lactation to favor milk production. Then, after lactation peak, in some undefined point of the lactation curve, insulin resistance decreases while fattening increases. Strategic managerial aspects, able to delay reduction of insulin resistance in lactating sheep should be identified in order to improve lactation persistency and dairy efficiency. On the other hand, previous studies reported in literature compared glucogenic with lipogenic diet usually using amilaceous vs. high fat diets, respectively. From a nutritional point of view, it could be very interesting to investigate if different glucogenic and lipogenic carbohydrate sources (such as starch and fiber, respectively), supplied in prenatal life, growing and lactation, and could induce changes in the metabolism of glucose, insulin and lipids. In addition, the most part of literature studying effects of carbohydrates from different sources, such as cereal grains or agro-industrial by-product (beet pulp, citrus pulp, soyhulls, high digestible forages) were focused on dairy cattle and investigated aspects mainly related to early lactation phases. Otherwise, previous studies in sheep and goats compared different dietary carbohydrates focusing short-term effects in early and mid lactation. On the best of our knowledge there is very scarce information on the effects of exposure to different carbohydrate early in life on insulin resistance and on metabolic programming on small ruminants performances.

From these initial considerations and aiming to partially cover this lack of knowledge providing new research findings on these aspects this research aimed: i) to improve the understanding of the nutritional impact on the basic mechanism of the insulin regulation and nutrient partitioning to the mammary gland in small ruminants; ii) to discover nutritional opportunities to increase lactation persistency in dairy sheep farms.

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CHAPTER 2

1. EXPERIMENTAL DESIGN, ANIMALS AND TREATMENTS

This work has been carried out within the project "*Permanent effects of starch and fiber, supplied during uterine and postnatal life of dairy sheep, on gastrointestinal microbiota and energy partitioning between milk production and fat deposition*" funded by the Italian Ministry of University and Research in the Program "Futuro in Ricerca" FIR, 2013 (Linea intervento 1 - cod. RBFR13V9JE).

The experiment consisted of three main periods as summarized in Figure 1:

- Period 1: **Pre-natal life,** for the last 75 days of uterine life (pregnant mothers);
- Period 2: **Post-natal growing**, from birth to first lambing (growth and pregnancy);
- Period 3: **First lactation,** from parturition to end of lactation (primiparous ewes).

In the trial, two groups of sheep were fed from their uterine life to the end of their first lactation with different combinations of two main diets, one being rich in starch (diet S), and the other with low starch content, but high soluble fiber (diet F) from soybean hulls or beet pulp.

1.1 General objective of the research

The main objective of the project was to assess differences in mid lactation performances and lactation persistency induced by the exposure to different carbohydrates amilaceous or fibrous. Exposure to diet S or F was tested on the same animal groups early in life on 1) prenatal phases and 2) growing, expecting that the carbohydrate source might have long term effects on future lactation performances, and then was tested in 3) mid lactation, expecting that S or F might have short term effects in the same phase.

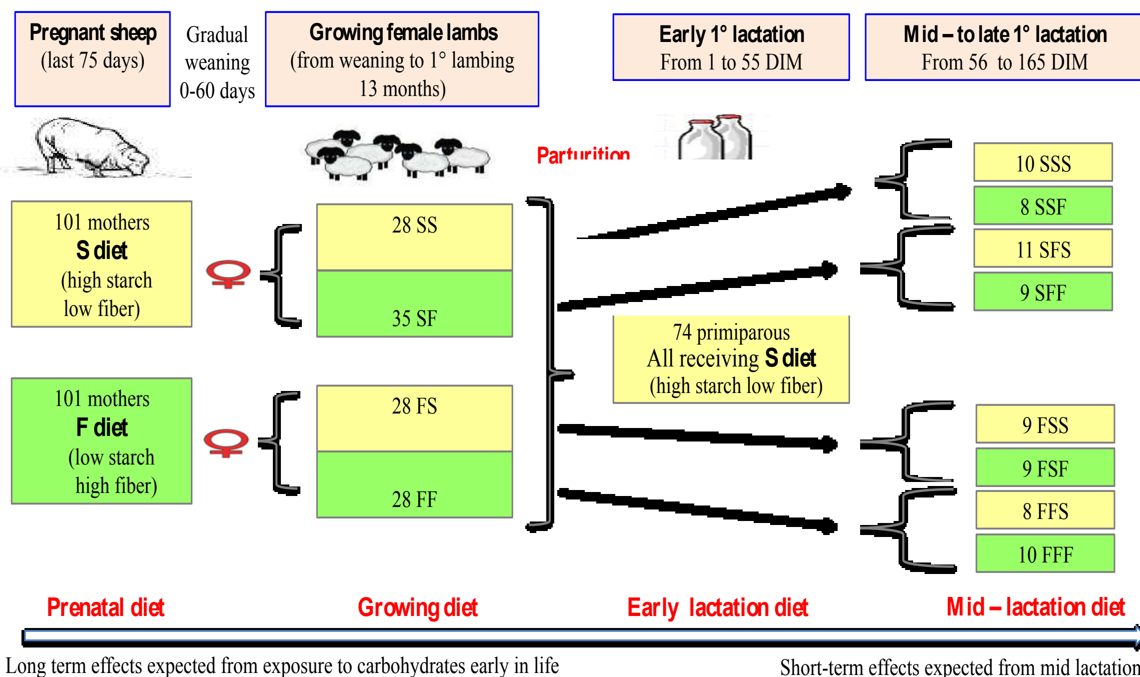

Figure 1. Experimental design of the research project. The sequence of letters indicates the nutritional plan received by each group in the whole experiment. Letters sequences (S, F; SS, SF, FS, FF; SSS, SSF, FSS, FSF, SFS, SFF, FFS and FFF) referred to animals or groups: the first letter (S or F) indicates the diet (starch or fiber) fed during last 75 days of uterine life, the second letter the diet (starch or fiber) fed during the growing phase and the third letter the diet (starch or fiber) fed in mid lactation after 56 days in milk (DIM). All the females were kept in each phase but only 74 primiparous sheep were milked until the end of the trail without showing health problems in any phase.

1.2 Experimental groups

A complete list of the acronyms used for the names of the groups and the offered diets is below reported:

Relatively to the nutritional period 1 (prenatal phase):

1. S = diet S rich in starch received in the last 75 days of uterine life;
2. F = diet F rich in soluble fiber received in the last 75 days of uterine life.

Before the experimental period all the mothers received a diet based on ryegrass hay and a commercial mix similar to that of diet S.

Relatively to the nutritional period 2 (growing phase):

1. SS = Diet S received either during uterine life and growing;
2. SF = Diet S received during uterine life and diet F received during growing;
3. FS = Diet F received during uterine life and diet S received during growing;
4. FF = Diet F received either during uterine life and growing.

From birth to 25 days all the sheep were fed with mother milk while the mothers during this suckling period were fed with a mix of diets S and F. Lambs were then gradually weaned until 60 days of age, and then were separated from their mothers. During the gradual weaning, lambs started to have access to the concentrate used for the growing diets of period 2.

Relatively to the nutritional period 3 (from 56 to 165 DIM):

1. SSS = Diet S received either during uterine life, growing and during mid to late lactation;
2. SSF = Diet S received either during uterine life and growing and diet F in mid to late lactation;
3. SFS = Diet S received during uterine life, diet F during growing and diet S in mid to late lactation;
4. SFF = Diet S received during uterine life, diet F during growing and diet F in mid to late lactation;
5. FSS = Diet F received during uterine life, diet S during growing and diet S in mid to late lactation;
6. FSF = Diet F received during uterine life, diet S during growing and diet F in mid to late lactation;
7. FFS = Diet F received either during uterine life and growing and diet S in mid to late lactation;
8. FFF = Diet F received both during uterine life and growing and during mid to late lactation.

In period 3, from the day after lambing to 55 DIM all the ewes were fed with a diet rich in starch, similar to diet S. Their offspring were removed at birth and fed with milk replacer in separate pens.

1.3 General principles used in diet formulation

The S and F diets were based on different carbohydrate sources: one glucogenic, being rich in starch (diet S), the other lipogenic with low starch and high content of soluble fiber (diet F). The glucogenic diets (S) were formulated in all the periods with high amounts of corn and barley grains. Whereas in the lipogenic diets (F) were formulates similarly to diet S but substituting the most part of the corn grain with digestible fiber from soyhulls and beet pulps. The two experimental diets were isoproteic within periods whereas between periods crude protein varied in order to meet protein requirements of the physiological stages. The offered amount in each phase and the ration balancing were carried out using the small ruminant nutrition system (SRNS) model (Tedeschi et al., 2010). Crude protein was lower (about 13% of CP) in pregnancy phase and higher (16-17% of CP on average) in growing and lactation phases.

1.4 Detailed description of the experimental design

The experiment was conducted in a private farm located in Sassari, in the North of Sardinia, Italy. The work started with a metabolic and reproductive screening of the farm flock. In the pre-experimental period a flock of pregnant Sarda dairy sheep was monitored with ultrasound vaginal probe, identifying the stage of pregnancy by measuring the bi-parietal distance of cranial bones (Greenwood et al., 2002). The metabolic profile of serum blood at fasting was then analyzed by Istituto Zooprofilattico Sperimentale della Sardegna. Animals were selected in order to get 202 multiparous sheep on good health conditions and similar stage of pregnancy. The number of animals was considered sufficient to get about 10 animals per group in the final phase of the period 3. All activities were performed in accordance with DPR 27/1/1992 (Animal Protection Regulations of Italy) in conformity with European Community regulation 86/609 on animal welfare. All animals during the experimental period had enough space availability and free access to clean and fresh water through watering place.

1.4.1 Period 1: Prenatal phase

The 202 pregnant sheep were randomly allocated to 2 different groups (101 animals for group) homogeneous for body conditions score (BCS), body weight (BW) and gestational age. When the groups were created all the sheep were, at least, at 75 days from expected day of lambing: the groups were housed in separated pens, indoor and managed with straw as bedding material. Each group was then randomly assigned to one of the experimental dietary treatments either with high content of starch (diet S) or soluble fiber (diet F), as before described. During this prenatal phase all the animals were fed a diet with mixed hay (clover and ryegrass) and with a pellet that consisted on the experimental concentrate (Table 1). The diets were supplied twice a day (8:00 a.m. and 7:00 p.m.); the pellets were supplied each time in individual headlocks in order to offer the same amount of diet to each animal. The mixed hay was supplied in a separate bunk and left available for all day in a restricted amount in order to respect the forage to concentrate ratio of experimental treatments. The offered amount was estimated proportional to animal requirements. The lambing period ranged between November 15 and December 10, 2014.

Table 1. Experimental feeds supplied in prenatal life (last 75 days of pregnancy), and growing (from weaning to first lambing).

Offered feeds	Hay	Prenatal concentrate		Growing concentrate	
Item		Diet S	Diet F	Diet S	Diet F
Dry matter, % of as fed	90.0	87,5	86.4	87.2	87.9
Crude protein, % of DM	11.0	17.6	17.9	20.1	19.1
NDF, % of DM	63.1	15.7	32.9	20.2	40.2
ADF, % of DM	49.9	6.2	27.9	8.6	26.9
ADL, % of DM	3.9	1.2	1.7	0.9	1.6
Ether extract, % of DM	1.1	2.9	3.2	2.8	3.0
Ashes, % of DM	11.3	8.5	10.3	4.4	5.9
NFC, % of DM	26.7	55.3	35.7	52.6	31.8
Starch, % of DM	-	44.5	15.4	42.3	13.7
Forage:concentrate ratio		45/55	45/55	40/60	40/60

*TMR was fed ad libitum; during milking each ewe received 160 gr/d of DM of pelleted mix (17% of CP, 24% of NDF, 4% of EE and 9% of ashes).

Measurements and samplings in period 1.

Feed samples were collected every 20 days for subsequent chemical analysis. The offered feeds and orts were weighted daily. Body weight (BW) and body condition score (BCS) of each animal were monitored every 20 days. The BW was measured by using a professional electronic scale (Teobil 6, Electronic Weighing System, Sondrio, Italy). The BCS was estimated manually by 3 trained operators with manual check of the lumbar fat depots, using a score from 0 to 5 point scale (Russel et al., 1969), approximating 1/8 point.

1.4.2 Period 2: Post-natal growing

At parturition, the mothers were moved in a different pen and fed a diet that included the same hay and an average mix of the S and F concentrates. At birth all lambs were weighted and individually identified with hear tags reporting the same number of their mothers. Mothers and lambs from the S and F groups remained in the same pen for the first 25 days and lambs were fed with the mothers' milk. Thus, mothers and lambs proceeding from S and F prenatal groups were split in two groups and reassigned to the diet, S and F, obtaining 4 groups each one with a specific nutritional history plan (SS, n = 28; SF, n = 35; FS, n = 28; and FF, n = 28; Total, n = 119) (Figure 1). In particular from 25 to 60 days of age, lambs were fed a starter that consisted of a small pellet with formulation of concentrates S and F (Table 1). Only the lambs had access to the starter concentrate whereas the mothers followed with the same post-partum diet in the same pen. Male lambs were slaughtered approximately at 25-30 days, whereas female lambs were kept with the mothers until 60 days of age. At about 2 months of age female lambs were separated in 4 different pens to continue the experimental trial (Figure 1). From 4 to 11 months, lambs were kept in outdoor pens without grass cover. The diets were supplied twice a day (7:30 a.m. and 6:30 p.m.), pellets and hay were supplied in different mangers ensuring enough bunch space for each head. The diet was offered in a restricted amount in order to respect the forage to concentrate ratio of calculated diet and the offered amount was calculated taking into account animal requirements and orts. The onset of puberty was estimated as the appearance of the first corpus luteum with ultrasound scan performed with transrectal probe. The estrous cycle was synchronized using intravaginal progestagen sponges for 12 days and pregnant mare's serum gonadotropin (PMSG) at the

time of sponge removal. The sheep were naturally mated with 8 different rams (ratio 1/15). The majority of sheep were mated in the first estrus cycle and the remaining part in the following two cycles without significant differences among groups. A group of 23 lambs were not mated and were excluded from the trial. The parturition occurred between mid-March and the end of April 2016.

Measurements and samplings in period 2

Samples of feeds were collected each month for subsequent chemical analyses. The offered feeds and orts were weighted twice per week. Body weight (BW) and body condition score (BCS) of lambs were monitored weekly until 4 months of age and then every 21 days. During the last 4 months of pregnancy, the sheep were kept indoor and BW and BCS were measured every 15 and 7 days respectively. BW was measured by using a professional electronic scale (Teobil 6, Electronic Weighing System, Sondrio, Italy). The BCS was estimated manually by 3 trained operators with manual check of the lumbar fat depots, using a score from 0 to 5-point scale (Russel et al., 1969), approximating 1/8 points.

1.4.3 Period 3: First lactation

After parturition, all the lambs were individually identified weighted and separated from their mothers. Lambs where then fed *ad libitum* with a milk replacer and excluded from the trial to avoid the effect of suckling from the ewe’s lactation performance. The ewes were milked with a milking machine two times a day (8:00 a.m. and 6:00 p.m.).

Only 74 sheep completed the period 3 of the trial. The majority of them (n = 56) were mated at the first estrus cycle and after parturition were milked for approximately 165 days of lactation. Unfortunately, a group of ewes suffered of mastitis problems and it was mandatory to exclude them from the trial and their data from the dataset. Other two ewes showed a constant milk production lower than 0.5 kg/d from the beginning to the end of lactation and were also excluded as outliers.

Lactation diets

For the first 15 days from lambing: all the sheep were fed with a transition diet with a proportion of 50:50 concentrate to forage ratio. The forage consisted of alfalfa hay and the concentrate was a mixture of the S and F concentrates supplied in pregnancy (Table 1).

From 15 to 55 DIM: the whole group of sheep was fed with a dry total mixed ration (TMR) based on ryegrass hay, wheat straw, soybean meal and cereals grain such as corn and barley (Table 2). During milking a commercial mix was also offered in the doses of 160 g/d per head (Table 2). Both the TMR and the mix were characterized by a high starch content assuming that glucogenic substrates in this phase are needed to support metabolic efforts of the mammary gland until the lactation peak.

From 56 to 165 DIM: the sheep were assigned to two different groups: 36 fed diet F and 38 fed diet S in the mid to late lactation. In this phase, the animals were kept indoor and housed in two pens corresponding to their lactation diet. Almost an equal number of animals with glucogenic and lipogenic nutritional background was included in the lactation groups. In fact, taking into account the diet of prenatal life (period 1) and growing phase (period 2), ewes of the groups SS, SF, FS and FF, were randomly assigned either to S, or F, mid lactation diet (Figure 1; Table 2). From the split of the 4 groups SS, SF, FS and FF, 8 groups of sheep (SSS, SSF, SFS, SFF, FSS, FSF, FFS and FFF, as reported in Figure 1) were obtained, homogeneous per milk yield, BW and BCS, within group of diet received from uterine life to first lactation.

The S and F diets consisted of a dry TMR offered *ad libitum* and based on different carbohydrates. Diet S was markedly glucogenic, being rich in starch; diet F was markedly lipogenic with low starch content (Table 2). Both diets S and F offered diets could be considered similar in protein content (16.1 and 16.8% of CP) and different for their starch content (25.8 and 15.2%, respectively) (Table 2).

Measurements and samplings of period 3

Samples of experimental unifeed were collected monthly to obtain an average value of composition of the offered TMR. Every 10 days, twice daily (8:00 a.m. and 6:00 p.m.), individual milk yield was measured and milk samples were collected for subsequent analyses. Body weight (BW) was measured every 15 days and body condition score

(BCS) was assessed each 7 days using the procedure explained for periods 1 and 2. Animal intake was measured individually at approximately 25, 45, 65, 100 and 140 DIM. For this particular measure, the animals were locked in headlocks 4 times a day, for 2 consecutive days (5.00 a.m., 10.00 a.m., 15.00 p.m. and 20.00 p.m.) and fed a precise amount of 1,000±5 g of TMR for 1 h. TMR was offered in separated individual square boxes to avoid interaction among animals and to keep orts. The average daily intake was calculated as difference from feed and orts recorded in the 8 feeding times of the two consecutive days.

Table 2. Diets of the period 3 offered to the experimental groups in the mid to late lactation phase from 56 DIM to the end of lactation.

Feed	UM	Milking mix	TMR EL	Offered EL diet	TMR S	TMR F	Offered S diet	Offered F diet
Ryegrass hay chopped	% of DM		27		26.7	26.0		
Wheat straw	% of DM		16.2		17.0	15.0		
Soybean meal, 44%	% of DM		19.5		19.1	19.0		
Corn flaked	% of DM		17.2		18.0	5.0		
Corn meal finely ground	% of DM		8.0		10.0	5.0		
Beet pulp	% of DM		6.1		6.0	28.0		
Molasses cane	% of DM		5.0		1.0	1.0		
Vit-min mix	% of DM		1.0		1.0	1.0		
Bicarbonate sodium	% of DM				1.2			
Offered amount	% of as fed	8.0	92.0		92.0	92.0	100	100
Composition								
Dry matter	% of as fed	88.3	89.3	89.2	89.8	89.9	89.7	89.8
Ashes	% of DM	8.6	8.4	8.4	10.0	10.2	9.9	10.1
Crude protein	% of DM	17.1	17.2	17.2	16.0	16.8	16.1	16.8
NDF	% of DM	23.8	41.3	39.9	40.4	44.9	39.1	43.2
ADF	% of DM	10.9	23.4	22.4	27.3	32.0	26.0	30.3
ADL	% of DM	4.7	3.8	3.9	5.6	4.9	5.5	4.9
Lipids	% of DM	3.9	2.7	2.8	1.6	1.5	1.8	1.7
Starch	% of DM	41.7	25.2	26.5	24.3	12.7	25.7	15.0
Non-starch soluble NDF	% of DM	4.9	5.2	5.2	7.7	13.9	7.5	13.2
NFC	% of DM	46.6	30.4	31.7	32.0	26.6	33.2	28.2

EL = in early lactation; S = starch; F = fiber; Non-starch soluble NDF = estimated as difference among NFC and starch;.

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CHAPTER 3

Lactation performances of primiparous dairy sheep fed with carbohydrates of different sources from mid to late lactation

ABSTRACT

The aim of the study was to investigate the effects of glucogenic versus lipogenic diet on the productive performances of primiparous dairy sheep in mid lactation. This work focused on lactation performances of the 72 primiparous Sarda sheep included in the trial described in Chapter 2. The 72 sheep were selected considering the animals which, after receiving the dietary treatments of period 1 and 2, completed the first lactation of period 3. Only productive performances of period 3 were presented in this work. The 72 sheep, after lambing until 55 DIM, received a glucogenic diet with high starch content (26.5% on DM basis). From 56 to 165 DIM, the sheep were assigned to two different diets (S; n=37, with high starch; F; n= 35, with high soluble fiber). During early, mid-late and end lactation the following parameters were measured: i) Feed intake (n= 72); ii) Body weight and BCS (n=72); iii) Volatile fatty acids from rumen samples (n=18); iv) Milk yield and chemical parameters (fat, protein, casein and lactose) (n= 72); v); Milk fatty acids (n =12). The results showed no significant differences in animal performance or rumen parameters due to diet or interactions among diet and days in milk. The fat and protein corrected milk yield at the end of lactation in the F group was only numerically higher than in the S group (1.1 vs. 1.0 kg/d per head, respectively). The S group showed higher fat content than F group in late lactation (P = 0.004) whereas casein content was significantly higher in the S group in mid to late lactation than in F group (P=0.042; P = 0.002, respectively). Milk fatty acids were largely influenced by the diet; MCFA, SFA, OBCFA, and the *de novo* were higher in F group than in S group (P < 0.0001), whereas, LCFA, UFA and MUFA were higher in S than F group (P < 0.01). PUFA, CLA, oleic, vaccenic, linoleic and linolenic acids were not influenced by diet (P > 0.05). The time largely influenced FA profile.

The general observed patterns were consistent with previous literature but did not show any significant difference. Previous literature not always highlighted significant

differences in nutrient partitioning and higher milk yield when lipogenic diets from soluble fiber were fed in substitution to glucogenic diets after lactation peak, to favor lactation persistency in dairy sheep. Nevertheless, numerical or significant increases were observed. An extreme individual variability was observed among animals, suggesting that other factors than diet are involved in the complex mechanism that impair glucose and lipid metabolism. Effects on milk fatty acids profile showed some inconsistence with the existent literature, whereas the evolution during lactation, related to the progression of lactation and the energy status of the animals, agrees with previous reports. Glucogenic diet positively affected fat and protein content but total milk yield tended to be lower compared to the lipogenic diet. This response to nutritional background on milk production and quality suggests investigating effects of different sources of carbohydrates on metabolic changes and energy Homeorhesis in mid-lactating dairy sheep.

1. INTRODUCTION

Increasing yearly production levels per head either achieving high lactation peaks and/or high lactation persistency is one of the most important goals to achieve high efficiency in dairy farms (Vandehaaret al., 2016). It could be highly beneficial for dairy sheep, because, even considering the smaller body size and the higher milk solid contents; they usually have much lower production levels than cows and goats (Cannas, 2007). In addition, increasing milk yields with the use of digestible fibrous substrates from agro-industrial by-products increases efficiency and reduces environmental impact of small ruminant productions (Capper et al., 2013). Previous studies of Cannas et al. (2002) highlighted that the shape of lactation curve and persistency could be influenced by nutritional strategies. In particular in early lactation, glucogenic diets with high starch contents help to support lactose production and milk yield; then in mid-late lactation, switching to lipogenic diets by substituting the most part of the starch with digestible fiber from soyhulls or beet pulp help to maintain lactation persistency (Cannas et al., 2004). This hypothesis arises from the fact that reducing the dietary glucogenic substrates, from mid to late lactation, should limit the peripheral insulin response, reducing the accumulation of body reserves and sparing glucose that can be oriented to the mammary gland, which glucose transporters are not insulin dependent (Sasaki, 2002). Several authors, using high digestible fiber vs. high starch diets in mid lactation, observed increase in milk production and reduction of body reserves deposition (Bovera et al., 2004; Cannas et al., 2004; Zenou and Miron, 2005; Lunesu et al., 2016; 2017). Following these evidences this works aimed to show the lactation performances of two groups of primiparous sheep which received the same glucogenic diet in early lactation and a different glycogenic or lipogenic diet from mid to late lactation. A more specific aim was to highlight the short-term animal response to carbohydrates of different sources, supplied to mid lactating primiparous sheep.

2. MATERIAL AND METHODS

Experimental design

This chapter will focus on the lactation performances of the 74 Sarda primiparous sheep that completed the period 3 of the whole trial described in Chapter 2. Two ewes showed a constant milk production lower than 0.5 kg/d from the beginning to the end of lactation and were excluded from this work as outliers. The majority of the considered animals (n = 56) lambed in the same estrus cycle, approximately from the 12th to 30th March 2016, and was milked for approximately 165 days of lactation.

Lactation diets

For the first 15 days from lambing: all sheep were fed with a transition diet with a proportion of 50:50 concentrate to forage ratio.

From 15 to 55 DIM: all sheep were fed with a dry total mixed ration (TMR) based on ryegrass hay, wheat straw, soybean meal and cereals grain such as corn and barley (Table 1). During milking a commercial mix was also offered in the doses of 160 g/d per head (Table 1). A high starch content characterized both the TMR and the mix assuming that glucogenic substrates in this phase are needed to support metabolic efforts of the mammary gland until the lactation peak.

From 56 to 165 DIM: the sheep were assigned to two different groups, receiving diet F (n = 36) and S (n = 38) in the mid to late lactation, respectively. The two groups resulted homogeneous per milk yield, BW and BCS. The S and F diets consisted of a dry TMR based on different carbohydrates offered *ad libitum*. Diet S was glucogenic, being rich in starch; diet F was lipogenic with low starch content (Table 1). The diet S was formulated with high amounts of corn grains whereas in the diet F most of the corn grain was substituted with digestible fiber from soyhulls and beet pulps to reduce starch and increase soluble fiber (Table 1).Diets S and F had both a similar protein content (16.1 and 16.8% of CP, respectively) and were different for starch content (25.7 and 15.0%, respectively) (Table 1).

In these phases, the animals were kept indoor with free access to water and TMR. Animals were housed in two pens corresponding to their lactation diet. In each pen, there was an equal number of animals with similar sequences of glucogenic and lipogenic

nutritional experiences as described in Chapter 2. Thus, the short term response was analyzed on groups that included a balanced number of animals with different exposure to carbohydrates early in life.

2.1. Measurements and samplings

Animal measurements

Samples of experimental unifeed were collected monthly to obtain an average value of composition of the offered TMR. Each 10 days twice daily (8:00 a.m. and 6:00 p.m.), individual milk yield was measured and milk samples were collected for subsequent analyses. Body weight (BW) was measured every 15 days and body condition score (BCS) was assessed every 7, following the procedure explained for period 1 and 2. Animal intake was measured individually at approximately 25, 45, 65, 100 and 140 DIM. For this particular measure, the animals were locked in headlocks 4 times a day, for 2 consecutive days (every day at 5.00 a.m., 10.00 a.m., 3.00 p.m. and 8.00 p.m.) and fed a precise amount of 1.000 ± 5 g of TMR for 1 h. TMR was offered in square boxes, in order to exclude interaction among animals and to keep orts. The average daily intake was calculated as the difference from feed and orts recorded in the 8 sampling points of the two consecutive days.

Rumen sample collection

Samples of rumen fluid were collected from 18 sheep (9 for each feeding group) with similar lambing date. Rumen content sampling was carried out twice in the last phase of pregnancy, once in early lactation at 45 DIM and twice in mid lactation at 75 and 135 DIM. Sampling was performed 3 hours after the morning feeding, but water was available to the animals until 1h before the sampling. Rumen liquid was extracted using a stomach tube and a manual evacuation pump. The first portion (about 30 mL) of the sample collected was discarded to reduce saliva contamination. After sampling, rumen fluid was immediately filtered by a sterile dressing to separate solid from rumen liquid. The pH was immediately measured by a pH meter (Orion 250A, Orion Research Inc., Boston, MA, USA), equipped with a glass electrode with Polysolve reference electrolyte (model 238405, Hamilton Company, Reno, NV, USA), and a thermometer. The samples

of rumen liquid of each animal were immediately frozen and then stored at −80°C until analysis.

Milk samples collection

Starting at day 10 ± 2 after lambing, for 6 months, individual milk samples of the morning milking were collected every 10 days from 12 sheep (6 per dietary treatment S and F) homogeneous per lambing date. After collection the milk was immediately refrigerated and stored at -20°C for the analysis of fatty acids (FA) composition.

2.2. Chemical analysis

Feed and milk chemical analysis

The whole sample of feed was dried (60°C in a ventilated oven until constant weight), ground through a 1 mm screen using a laboratory mill (Thomas-Wiley, Arthur H. Thomas Co., Philadelphia, PA, USA) and stored until analysis. All samples were assayed in duplicate. The DM was determined by gravimetric loss of free water by heating at 105°C for 3 h (AOAC, 1995, method 945.15), ash was determined as gravimetric residue after incineration at 550ºC for 2 h (AOAC, 1995, method 942.05) and ether extract (EE) was obtained following the method 920.29 of AOAC (1995). The crude protein (CP, N×6.25) was determined using the Kjeldahl method (AOAC, 1995, method 984.13). The NDF, ADF and ADL were determined using anAnkom 220 Fiber Analyser (Ankom^{TM} Technology Corporation, Fairport, NY, USA; Mertens et al., 2002). The NDF analyses utilized a neutral detergent solution containing sodium sulphite and a heat stable amylase (activity = 17,400 Liquef on units/mL, Ankom Technology). The NDF, ADF and ADL contents were corrected for the residual ash content. The neutral (*i.e.*, NDICP) and acid (*i.e.*, ADICP) detergent insoluble CP fractions were determined as residual nitrogen of Ankomfiber bags (Van Soestet al., 1991). Starch was measured by polarimetry (Polax 2L, Atago®, Tokyo, Japan) according to EC (1999). The non-starch carbohydrates included in the NFC were calculated as: 100 - (CP + ash + EE + NDF + Starch).

Individual milk samples from the morning milking were analyzed for fat, protein, casein and lactose using a Milkoscan 6000 instrument (Foss Electric, Hillerød, Denmark), for SCC using a Fossmatic 360 instrument (Foss Electric) and for pH.

Volatile fatty acids analysis

A high-performance liquid chromatographic (HPLC) method was used to determine the VFA in rumen liquid as described in Correddu et al. (2015). Briefly, a sample of approximately 2 mL was defrozen and centrifuged (15,000 x *g*, 10 min, 4°C); the supernatant was withdrawn by syringe and, after filtration (PTFE 0.45 µm, 13 mm), it was injected into a HPLC system (Varian Inc., Palo Alto, California, USA). The HPLC was equipped with an auto sampler (Varian 9300), a degasser (Varian 9012 Q), a UV detector (Varian 906P Polychrom) and an Aminex HPX 87H column (Biorad Laboratories, Hercules, CA, USA). The temperature of the column was set at 55°C, and H_2SO_4 0.008 N (at the flow rate of 0.6 mL/min) was used as eluent. Concentrations of VFA were estimated by comparison with a calibration curve obtained by injecting 5 µl of 5 standard solutions (5.6, 11.25, 22.5, 45 and 90 mmol/L of acetic acid, and 5, 10, 20, 40 and 80 mmol/L of propionic and butyric acid) obtained by appropriate dilutions of a standard mixture of VFA containing 5.40, 5.76 and 7.02 mg/mL of acetic, propionic and butyric acids, respectively, in H_2SO_4 0.1 N. The concentration of total and individual VFA was expressed as mmol/L and mol/100 mol (%) of total VFA, respectively.

2.3. Fatty acids determination

The milk fat extraction and the fatty acid methylation were carried out as described by Nudda et al. (2005).A 7890A gas chromatographic system, (Agilent Technologies, Santa Clara, CA, USA), equipped with a capillary column (CP-Sil 88, 100 m × 0.250 µmi.d., 0.25 µm film thickness, Agilent Technologies), an auto-sampler (7693,Agilent Technologies) and a flame ionization detector (FID) was used for the separation of methyl ester of fatty acids (FAME). Helium (1 mL/min flow rate) was used as carrier gas with a pressure of 28 psi and 1µLof sample was injected. The split ratio of the split/splitless injector was 1:80. The temperatures of injector and detector were 250°C. The oven temperature was programmed as follows: the initial temperature was set at 45°C for 4 min, increased at 13°C/min to 175°C, and held for 27 min; then it was increased at 4°C/min to 215°C, and held for 35 min. OpenLAB CDS GC ChemStation Upgrade software data system (Revision C.01.04, Agilent Technologies Inc., Santa Clara,

CA, USA) was used to compute retention time and area of each individual FAME, which were identified by comparing their retention times with those of standard methyl esters and published isomeric profiles, as detailed in Nudda et al. (2005). The FA concentration of individual FA and that of groups of FA were expressed as g/100 g of total FAME. Groups of FA were calculated as follow: short-chain fatty acids (SCFA), sum of the individual fatty acids from C4:0 to C10:0; medium-chain fatty acids (MCFA), sum of the individual fatty acids from C11:0 to C17:0; long-chain fatty acids (LCFA), sum of the individual fatty acids from C18:0 to DHA; saturated fatty acids (SFA), sum of the individual saturated fatty acids; monounsaturated fatty acids (MUFA), sum of the individual monounsaturated fatty acids; polyunsaturated fatty acids (PUFA), sum of the individual polyunsaturated fatty acids; trans fatty acids (TFA), sum of the individual trans fatty acids; CLA, sum of individual conjugated of linoleic acids; odd- and branched-chain fatty acids (OBCFA), sum of individual odd- and branched-chain fatty acids; *de novo*, sum of the fatty acids synthesized de novo in the mammary gland (from C6:0 to C16:0); ratios between some groups of FA were also calculated: SCFA:LCFA, SFA:UFA and *de novo*: LCFA.

2.4. Degradability estimation

The kd of B1 fraction was estimated with a seven hours starch degradability test, whereas the kd of B2 fraction was measured with *in situ* incubations. The digestibility of NDF (dNDF) was measured *in situ* by incubating nylon bags in the rumen of two cannulated dry cows during 12, 24, 48 and 288 h as detailed by Gallo et al. (2017). The undigestible fraction after 288 h of incubation was considered as the indigestible NDF (iNDF). All animal experiments were performed in accordance with DPR 27/1/1992 (Animal Protection Regulations of Italy) in conformity with European Community regulation 86/609. The cows were daily fed 10 kg DM of a total mixed ration consisting of alfalfa hay, ryegrass hay, corn silage and concentrate (*i.e.*, 300, 300, 300 and 100 g/kg DM; respectively) in two portions at 08.00 a.m.and 6.00 p.m..The diet contained 120 g CP and 550 g NDF per kg DM.

2.5. Statistical Analysis

Measures of BW and BCS of the experimental groups were compared using a T-test for a significant level of P <0.05. Data collected periodically, from parturition to end of lactation, were analyzed as follow:

- Data of milk production and milk composition were analyzed with the PROC MIXED procedure of SAS (SAS version 9.2 SAS Institute Inc., Cary, NC; 2002) with the following linear mixed model:

$$y_{ijklm} = \mu + diet_i + dim_j + diet \times dim_{(ij)k} + a_l + e_{ijklm}$$

where:

- y_{ijkl} is the observed trait (i.e, milk yield, fat corrected milk, intake,.etc);
- μ is the overall mean;
- $diet_i$ is the fixed effect of the ith diet (1: S for starch diet, 2: F for Fiber diet);
- dim_j is the fixed effect of the jth class of days in milking (DIM); (j=11 for milk production and milk composition; j=5, for intake)
- $diet \times dim_{(ij)k}$ is the fixed effect of the kth interaction between diet and time;
- a_l is the random effect of the animal (l = 72 for milk production milk composition, BW, BCS, intake);
- e_{ijklo} is the random residual term.

Means were compared using Tukey test and were considered significantly different for P < 0.05.

Data of rumen pH, VFA and milk FA composition were analyzed by the PROC MIXED procedure of SAS (version 9.2; SAS Institute Inc., Cary, NC). The model included the fixed effect of diet (D; 2 levels: S = starch diet, F = fiber diet), sampling date (T= 2 levels for rumen pH and VFA; S= 9 levels for milk FA) and the diet × sampling date interaction (D × T); moreover, to account for individual variability, the random effect of animal was nested within each treatment. The significance of group mean differences was assessed using Tukey Honestly Significant Difference (HSD; P < 0.05).

When designs were unbalanced, due to different numbers of animals per treatment, the inclusion of Kenward-Roger correction was tested in the statistical model using the SAS

statement "DDFM=KENWARDROGER". It should be noticed that for the umbalanced designs both the models(with or without the inclusion of the Kenward-Roger correction)were tested, giving no difference of statistical results, in terms of significance, for each considered variable.

3. RESULTS AND DISCUSSION

3.1. Diets

In the early lactation phase (from parturition to 55 DIM) all the animals were fed with an early lactation diet. Diet offered in early lactation (EL) was richer in glucogenic substrates and energy than S and F diets offered in mid-late lactation (Table 1). Furthermore, higher content of straw and bicarbonate was added in S diet offered in mid lactation in respect to EL (Table 1). It was a decision planned in the first 55 DIM considering that the levels of milk fat and protein in the early phase of lactation resulted very low (5.15% and 4.93%, respectively; Table 2)in comparison with standard milk of Sarda sheep (6.5% and 5.8% for milk fat and protein, respectively). It was attributed to the use of TMR with chopped forages. In fact, peNDF of the EL diet(21%) was very close to the lower limit of 20% suggested for adequate rumination in lactating sheep (Rossi et al., 1991; Cannas et al., 2001), thus straw was slightly increased to preserve animals for possible low effective fiber intake and stimulate rumination without providing high digestible fibrous compounds such as soluble fiber.

The S and F diets fed in mid lactation were very different for their NFC composition especially in terms of soluble fiber content (7.5% vs. 13.2% of DM, respectively) and starch (25.7% vs. 15.0% of DM, respectively), following the aims of the original design. Despite that, it has to be noticed that both S and F diets, showed lower levels of NFC (33.0% and 28.2% of DM respectively), than diets with "high NFC" reported in literature for similar trials (Lunesu, 2016; see figure 5 in Chapter 1).

Starch digestibility was similar among diets (73.6% vs. 73.9% of starch content for S and F offered diet, respectively; Table 1). It indicates that the starch source was very similar. On the other hand, as expected, the amount of digestible NDF daily supplied by S diet

was lower than F diet. The difference was about 4% of NDF on offered diet (24.2% vs. 27.8% of DM, respectively; Table 1). The S diet supplied a lower amount of indigestible NDF than F diet (7.2% vs. 9.9% of DM, respectively; Table 1). These differences were not so high in comparison with other studies with similar focus and resulted in a very small difference in diet energy content among S and F diet (1.52 vs. 1.43 Mcal NEL/kg of DM, respectively; Table 1)

3.2. Animal intake

Although all ewes were fed the same EL diet in early lactation, in this chapter, animal performances were shown separately for the two groups (S and F). It was preferred for clarity and completeness and to highlight that those animals proceeded from a previous part of the trial.

The two groups of animals showed similar performances in early lactation (from parturition to 55 DIM) and also in mid-late lactation (from 56 to 165 DIM) (Figure 1). The pattern of dry matter intake showed the expected trend of dairy animals with increasing values in the first two months from parturition (on average from 1.62 to 1.82 kg/d of dry matter per head at 25 and 45 DIM respectively). Similar dry matter intake was observed for the groups S and F from parturition to 55 DIM when the groups were fed with the same diet (Figure 1).Decreasing trends were observed in mid to late lactation (from 1.87vs. 1.83 kg/d of dry matter per head at 65 DIM to 1.59 vs. 1.66 kg/d of dry matter per head at 140 DIM for S and F groups, respectively). No differences were observed among groups except for the F group compared to the S group, showing higher average intake after 100 DIM for (Figure 1), even though the difference was not significant due to higher individual variability. In previous studies, low NFC diets (with lower energy content) were generally eaten significantly more than high NFC diets (with higher energy content), probably to compensate the daily energy intake (Cannas et al., 2004; Zenou and Miron, 2005). Otherwise in other studies no difference in intake was observed among dietary treatments comparing different carbohydrate sources (Lunesu, 2016). Different results were observed in lactating dairy cows, showing highest DMI in high starch diet compared to low starch diet, probably because of the lower palatability of

the former (47.9 vs. 32.2% of DM, respectively; Potts et al., 2017). In this case the energy content of the two diets might have caused the similar intake level.

3.3. Milk yield and composition

Average lactation performance was not significantly different between S and F groups both in early and in mid-late lactation (Table 2 and 3). The lactation pattern of the two groups followed the classical shape of the lactation curve of dairy sheep with average values of 1.7 kg/of milk per head from parturition to 55 DIM (Table 2; Figure 2). After the diet switch, we observed that milk yield of both groups remained quite stable from 56 DIM to approximately 100 DIM, then declined until 165 DIM (Figure 2). On average the milk production from 56 to 165 DIM was equal to 1.40 kg/d per head with significant DIM effects ($P < 0.001$) and no interaction between Diet and DIM ($P > 0.1$; Table 3). The milk yield observed in the last weeks of lactation was on average slightly higher for the F group but the difference was only numeric and slightly appreciable(Table 3; Figure 2). This is in contrast with previous studies that observed increases in milk yield comparing low vs. high NFC diets (35.9% vs. 39.9% of DM, respectively) in mid lactating primiparous Sarda sheep (Bovera et al., 2004). Dietary NFC in Bovera et al. (2004) was on average higher than in our research but with a small difference among groups. Contrasting effects on milk production when soluble fiber substituted starch from cereal grains were already highlighted in previous studies (Cannas et al., 2013; Lunesu et al., 2016).

Fat and protein corrected milk yield (FPCMY) showed a similar trend to milk production with average values of 1.41kg/d per head in early lactation and 1.28kg/d and 1.25kg/d per head in mid-late lactation for S and F respectively(Table 2 and 3). A significant effect of DIM was observed for milk yield in both early and mid to late lactation and only in mid to late lactation for FPCMY ($P < 0.01$; Table 2 and 3).

Significant but small differences were observed for milk fat in early lactation (5.25% and 5.04% between S and F groups respectively; Table 2 and Figure 3).Milk protein content also tended to be higher in S than in F group (4.98 vs. 4.88 %; $P = 0.09$; Table 2). The difference was quite small but indicated that the two groups were slightly different even before the diet switch at 56 DIM.

Nevertheless the milk fat and protein content resulted on average lower than that of standard milk (6.50% fat and 5.80% protein) conventionally used for the Sarda sheep, resulting in lower FPCMY than MY (Table 2 and 3). However, the observed values of milk quality were similar to those observed by Bovera et al. (2004).

The milk fat and protein content increased with the progress of lactation, as expected, with a significant effect of DIM (P< 0.01; Table 3). No significant difference were observed between S and F groups for the milk fat from 56 to 165 DIM (Table 3). Oppositely, at the end of the experimental trial (132-170 DIM) milk fat was significantly higher for F group than S group in late lactation (6.91% vs. 6.58%, respectivley; P < 0.05; Figure 3 and Table 3), in agreement with the high amount of precursors provided by the soluble fiber contained in the F diet fed in this phase.

In the whole mid-late lactation phase the milk protein content was significantly higher in F group than in S group (P= 0.01; Table 2 and 3; Figure 3). Close to the dry off, 165 DIM, the milk fat content of S and F groups were6.5% vs. 6.9% and the protein content 5.6% vs. 6.0% , respectively, with significant effect of diet (P< 0.01; Table 3).

These results are in contrast with other studies that observed high milk protein and lower milk yield in high NFC vs. low NFC groups of pluriparous sheep (Cannas et al., 2013; Zenou and Miron, 2005).On the other hand, trends of milk fat, protein and casein of our study agree with findings on primiparous sheep of Bovera et al. (2004). The F group also showed significant lower values of milk urea than S group in mid-late lactation (44.4mg/dl vs. 41.7 mg/dl; P= 0.01) despite the slightly higher CP content of the F vs. S diet (Table 1), indicating a possible higher ruminal fermentation efficiency.

Surprisingly the amount of milk solids (g/d) produced by both groups from mid to late lactation was not significantly affected by DIM (P= 0.75; Table 3). It indicated a constant effort at the mammary gland level for the synthesis of milk components throughout the lactation. Similar performances of the two S and F groups were not expected. Considering previous finding of Bovera et al. (2004; Figure 5 in Chapter 1) a higher lactation persistency was expected in the F group fed a diet with high soluble fiber in mid-late lactation. Nevertheless, it has to be noticed that the group showing higher lactation persistency in Bovera et al. (2004) was feda diet with high soluble fiber and low NFC for the whole experimental trial, from the last part of pregnancy until the end of the lactation.

3.4. Body weight and reserves

Body weight and BCS of the S and F groups were not statistically different at parturition (Table 4 and Figure 5). Post-partum BW (44.3 and 45.0 kg for S and F groups, respectively) and post-partum BCS (2.88 and 2.91 for S and F groups, respectively) at lambing and before the diet switch (50 DIM) were not statistically different between groups (P> 0.05; Table 5). The observed general trend showed a diminution of body weight in the first month of lactation and then an increase from early to late lactation in agreement with the body reserves depletion and accumulation cycles in lactating mammals, even if the changes were very small (Figure 5). Similar trend were observed for BCS (Figure 6).Body weight showed increasing trend from parturition to 55 DIM, probably for the effect of increased intake and gastrointestinal content after lambing; increasing trends of BW were observed also from 56 to 165 DIM but without effects of diet (Table 4; Figure 5). The cumulative increase in body weight from 56 to 165 DIM was not significantly different between groups and equal to 3.44 and 3.83 kg for S and F groups, respectively (Table 4). The average BCS was very similar among groups (2.80 vs. 2.83 at 55 DIM and 2.94 vs. 2.92 at 165 DIM for S and F groups, respectively; P <0.1) the differences were smaller than the detectable range of 0.125 points (Table 4).Furthermore the BCS variation from 56 to 165 DIM was affected by Diet (P < 0.001) and by Diet (P = 0.001) and by Diet × DIM (P = 0.005) showing a quite stable pattern for the F group and a significant increasing trend for the S group (Table 4 and Figure 6). It might be considered an indication of the tendency of the S group to increase fattening in mid to late lactation more than the F group. Previous studies observing higher milk yield substituting starch with high soluble fiber in mid lactation diets also observed a reduction in body reserves deposition in animals fed soluble fiber (Grum et al., 1996; Cannas et al., 2004; Bovera et al., 2004; Lunesu, 2016) indicating a different nutrient partitioning induced by the dietary carbohydrates. The differences observed in this study in terms of BCS appear significant only in terms of BCS change and very small in the BCS scale. It could be partially attributed to the fact that sheep often accumulate visceral fat that is not detectable by lumbar BCS scoring (Cannas, 2001). Similar results were observed by Cannas et al. (2013) in sheep and by Ferraretto et al., (2011) in dairy cows.

3.5. Rumen

In Table 5 and 6 the values of the ruminal pH, total and individual VFA in the 18 sheep sampled during pregnancy (Table 5; two sampling date: at 50and 8 days before lambing, respectively), during early lactation (Table 6; one sampling date: 35 DIM) and during mid lactation (Table 6; two sampling date: 76 and 127 DIM)are reported.The value of pH during pregnancy was not affected by the diets ($P > 0.05$). During lactation pH increased from the first to the second sampling date (6.36 to 6.42; $P < 0.05$);nor total neither individual VFA were affected by diet ($P > 0.05$). The effect of time was significant for total VFA, acetic acid ($P < 0.001$) and for butyric acid ($P < 0.05$) (78 to 97 mmol/L, 68% to 66% and 14% to 15%, respectively). The interaction Diet × DIM did not influence any variable ($P > 0.05$). These results were also quite unexpected, as it has been reported that glucogenic and lipogenic diets lead to different production and, consequently proportion, of ruminal VFA. In particular, the glucogenic diet should produce high level of propionate, whereas, lipogenic diet should produce high levels of acetate and butyrate. The lack of effect in the proportion of VFA in the present work is in agreement with several studies on sheep (Lunesu et al., 2016) and cows (Chagas et al., 2009) showing no dietary differences among total or individual VFA but differences in production performances attributable to the diet. Other possible explanation might be due to the method adopted for sampling of rumen content that only considered one time point 3 hours after the main meal. The value of pH was within the normal range of rumen pH (5.5-7.0; Dziuk, 1984) and was not influenced by the diet ($P > 0.05$). The reported results are indication of the VFA concentration in the rumen liquid and not the cumulative amount of produced and absorbed VFA during the day.

3.6. Milk fatty acids

In tables 7 and 8 are reported the means of the concentrations of individual FA and groups of FA, respectively, for the two groups of animals during the experimental period (after the diet-switch), and the effect of the diets, DIM and their interaction on these traits. In general, DIM was found to be the main effect that influenced the concentration of FA; this result was expected since the milk FA profile varies largely, during lactation,

due to the changes of physiological status of ewes and to the variations of the environmental conditions. Most of the individual FA and groups of FA were influenced by the Diet; however, the FA considered important from a nutritional point of view were not influenced: among individual FA, vaccenic acid (C18:1t11), rumenic acid (CLA c9,t11), oleic, linoleic and linolenic acids were not affected by Diet ($P > 0.05$). Considering the FA groups, PUFA, TFA and the n-6 to n-3 ratio were not influenced by Diet ($P > 0.05$). These FA have become very important in the food quality definition, and in particular that of animal derived foods. The presence of PUFA in dairy products is considered beneficial for human health, since a number of works have associated these FA to a positive effect in reduction of risk for cardiovascular diseases (Calder, 2004; Simopoulos, 2008). The rumenic acid, whose production largely depends on ruminal biohydrogenation of PUFA, has been associated to promising healthy effects, such as anti-carcinogenic and anti-inflammatory activity (Sofi et al., 2010). In the last decades, the increase in the concentration of PUFA, CLA and VA in dairy products has been the object of several researches; in particular, many studies focused on nutritional strategies to achieve increase of beneficial FA in milk and dairy products (Nudda et al., 2014), being the diet one of the main effect capable to influence milk FA profile. The lack of influence of the diet on the concentration of these beneficial FA was expected, as the two dietary treatments used in the present study were not designed to influence milk FA profile. This results partially agrees with the investigation of Philippeau et al. (2017), that found no difference in the concentration of PUFA, vaccenic, oleic, linoleic and linolenic acids in milk of cows fed high or low starch diets; differently from our findings, in that work the concentration of rumenic acid was affected by diet, with the milk from high starch diet fed cows exhibiting the highest concentration. The main effects related to the different dietary treatments were observed for medium and long chain FA (both individual and groups), saturated and monounsaturated FA, odd and branched chain FA, *de novo* FA and all ratios, except that of n-6 to n-3.

The concentration of MCFA was higher in F diet ($P < 0.0001$), mainly due to the higher concentration of C16:0, the major MCFA, in the same group ($P < 0.05$); on the contrary, C18:0 and LCFA were higher ($P < 0.05$ and $P < 0.01$, respectively) in milk of ewes fed S diet. The total concentration of OBCFA increased in F diet ($P < 0.01$), due to the increase of some individual iso, anteiso and linear-odd chain FA.

The OBCFA derive largely from the activity of rumen microorganisms, even if the *de novo* synthesis of some of them (linear-odd chain FA in particular) in the mammary gland cannot be excluded (Vlaeminck et al., 2006). The kind of forage in the diet, forage to concentrate ratio, lipid supplementation, and the presence of secondary plant metabolites are able to affect, directly or indirectly, the growth and the activities of ruminal microorganisms, thus affecting the concentration and the relative abundance of OBCFA in milk (Vlaeminck et al., 2006; Correddu et al., 2015). Shift in OBCFA concentration in milk has been proposed as a diagnostic tool to predict shifts in microbial population, mainly associated with the variation of diet composition (Vlaeminck et al., 2004). It has been reported that switch of dietary proportions of maize silage and forages, which determines the starch to NDF ratio, can affect the relative abundance of amilolytic and cellulolytic bacteria, resulting in shift of OBCFA concentration. In particular, high level of starch in the diet should increase the concentration of liner odd chain FA, whereas higher NDF should promote the cellulolytic activity and consequently the concentration of iso and anteiso FA (Cabrita et al., 2007). In the present work, no shift was observed among OBCFA and, together to the expected increase of iso and anteiso FA in F diet, also the linear-odd chain FA increased in the same group. This result could be explained with a higher activity of mammary gland FA synthesis in the F group. This hypothesis may be supported by the observed higher concentration of FA of *de novo* synthesis in milk of F group compared to that of S group ($P < 0.05$). These considerations are in accordance with Vlaemink et al. (2006) that partially explained the inconsistency of results among studies, with the dual origin (rumen microbial and mammary gland) of linear odd chain FA.

The *de novo* FA are produced by the activity FA synthetases and Acetil-CoA carboxylase, in the mammary gland, using acetate and beta-hydroxybutyrate, deriving from the rumen, as substrates (Chilliard et al., 2001). Variation of *de novo* FA during lactation (Figure 9) has been largely documented, and it is associated to changes in the energy status of the animals. In early lactation, animals are in negative energy balance and increase body reserves mobilization, including adipose FA (Belyea et al., 1990). As consequence, the increase of FA uptake inhibits the lipogenic activity of mammary gland and the milk concentration of *de novo* FA (Glasser et al., 2008). As lactation proceeds, the animal's energy balance increases and becomes positive; consequently, the

mobilization of body reserves and the milk concentration of FA deriving from the uptake (mainly LCFA; Figure 10) decreases. In this condition, in order to maintain the optimal milk fat fluidity, the synthesis of *de novo* FA increases. This observation is supported by the high negative correlation found between *de novo* and LCFA (r = -0.97, P < 0.0001). In general, the described variations of FA are in accordance with previous works (Van Knegsel et al., 2007; De La Fuente et al., 2009; Martini et al., 2013).In the work of Bauman (1976), the relationship between the synthesis and the mobilization of FA in adipose tissue has been thoroughly investigated. The author stressed that these two functions are not independent but rather under a coordinated control, and defined this relationship as "synthesis/mobilization cycle". The results of the present works, confirmed by previous studies, show that the "cycle" also exist in the mammary gland, where the *de novo* synthesis and the uptake of mobilized FA are under the same coordinated control.

As far as the effect of the diet is concerned, the observed differences in milk fat profile between the two groups were rather unexpected. Diets high in readily digestible carbohydrates (starch diet, S) produce propionate and glucose (Bauman et al., 1971) that should stimulate release of insulin. The increase of circulating insulin concentration decreases the release of FA by adipose tissue (Bauman and Griinari, 2001). This should result in a reduction of NEFA, reduction of uptake of circulating FA by mammary gland, increase of FA synthesis and, consequently, a reduction of LCFA concentration and increase of *de novo* FA in milk. In the present work, the milk of animals fed starch diet showed higher levels of LCFA and lower of *de novo* FA compared to the milk of animals fed fiber diet. These differences and the inconsistence from the expected results should be explained by the different level of FA precursors related to the diets and with a different lipid metabolism in the two groups. The fermentation of digestible fiber diet produces high levels of acetate and, consequently, higher acetate to propionate ratio, compared with the starch diet. Higher level of FA precursors (acetate and beta-hydroxybutyrate) in the F diet compared to S diet may have promoted a higher activity of FA synthesis in mammary gland. As the FA composition is reported in term of g/100 g of total FA, values of *de novo* FA are negatively correlated to that of LCFA. The switch-diet from S to F resulted in a more rapid reduction of the LCFA in F groups compared to the S, perhaps consequent to a decrease of lipid depots mobilization. The higher concentration of MCFA

in the milk of F group can be explained by the inclusion of many FA of *de novo* synthesis, in particular of C16:0. Thus, our results disagree with the work of Philippeau et al. (2017), where the milk of cows fed low or high starch did not show any significant difference in the concentration of *de novo* FA.

3.7. General patterns

Looking at the whole picture and the high similarity of performances in the two different groups a general description of the performance trends can be figured out and possible explanation could be hypothesized. The milk and FPCMY production patterns of the S and F groups, even if not significantly different on average, showed a similar trend to that observed in previous studies (Bovera et al., 2004; Cannas et al., 2004; Lunesu, 2016). Starch group showed numerical higher milk yield and FPCMY from 56 to 110 DIM and then lower persistency(Figure 2 and 4) showing patterns similar to Bovera et al. (2004) at respective DIM stages. DM intake at the end of lactation showed numerical higher values for F than for S group (Figure 1).Body reserves showed more constant trends in F than in S group (Figure 6; Table 7). Looking at the curves of milk component and the curves of body weight and BCS, they consistently indicated a possible different nutrient partitioning in the two groups S and F. The group S showed higher values of milk yield in the first part of lactation (approximately until 110 DIM) and higher body weights in late lactation in respect to F (Figure 2, 4 and 6). Oppositely, the group F in respect to S showed a more persistent pattern of milk production, higher milk yield and milk solids and lower body weight in late lactation. Those observations, only in terms of observed patterns, showed high consistency with the formulated hypotheses of the role of carbohydrate in mid lactation even if experimental evidences did not lead to a significant effect of diet. The main significant effects were observed on milk fatty acid composition in agreement with the dietary content of fat precursors. Several explanations were adduced in order to explain these findings.

As indicated before, the level of NFC in S and F diets is lower than those applied in other studies and might indicate a threshold of NFC that determines a different effect of diets in nutrient partitioning (Chagas et al., 2009; Cannas et al., 2013).

A further factor that might have influenced the experimental results was the choice of the DIM at dietary switch, fixed at 55 DIM. It can be considered quite early in respect to the 110 DIM already pointed above. It has to be noticed that sheep fed high fiber and low NFC diets in previous experiments (Bovera et al., 2004; Cannas et al., 2004; Lunesu, 2016) showed higher persistency approximately after 110 DIM. This choice was driven by the fact that in primiparous Sarda ewes mid lactation period corresponds to the summer time. The risk of the imposition of an early dietary switch (i.e.: feeding of low NFC diets while animals should be still fed with high NFC) might have prejudiced the homeorhetic regulation of the nutrient partitioning in lactation in the opposite direction, with undesired confounding effects in the sequent phases. Previous studies do not give any indication about the criteria for the choice the best moment of dietary switch from glucogenic to lipogenic diets in order to get higher nutrient partitioning to the mammary gland in mid-late lactation.

The DIM of diet switch was considered very important at the time of the experimental planning and relatively to the homeorhetic regulation for the initial assumptions. For this reason, the sheep were divided in S and F groups at 55 DIM, calculated from their lambing date. In fact, when data from animals mated in the same estrus cycle were analyzed separately, the differences in the observed patterns of milk production and FPCMY between the F and S group were more evident compared to when records of all animals were considered (Figure 7 and 8). Significant differences were not observed as well. In fact, beside these dynamics, a large variability on individual performances was observed in each group and during each lactation stage. The experimental groups derived from a balanced mix of animals with different previous nutritional experiences as described in Chapter 2. It might also have induced larger variability in the animal response to the experimental diets in the short term.

The individual diet selection on the offered TMR might have increased the individual variability in the animal response of each group. The nutritional behavior of sheep,which are able to select forage and grain particles showing high individual variability on feed sorting(Kenney and Black, 1984; Cannas et al., 2001) could have hidden the differences in diet composition offered to the S and F groups. It suggests to deeply investigating the composition of the individual eaten diets relatively to the animal response in the lactation curve.

4. CONCLUSIONS

In the present study, the substitution of a part of the dietary starch with soluble fiber after the lactation peak did not induce a clear increase of lactation persistency in sheep. In particular the use of different dietary carbohydrates(glucogenic vs. lipogenic) did not stimulated significant effects on lactation performance from 56 to 165 DIM in primiparous dairy sheepthat were fed a glucogenic diet from parturition to 55 DIM. The patterns of behavior and numerical values of animal performances, observed in this study in response to different dietary carbohydrates, were very similar to those observed in previous studies describing significant increases of sheep lactation persistency with diets based on soluble fiber. Differently, in this study, a large individual variability in response to the diet was observed without significant differences on production performances among groups. It confirms previous literature evidences that the substitution of dietary starch with soluble fiber in mid lactating sheep lead to increases in milk yield and milk solids and reduction of body fattening but these differences are not always significant. These considerations suggest that dietary carbohydrates might have a functional role in determining the homeorhetic regulation of nutrient partitioning to mammary gland from mid to late lactation in dairy sheep, but also that their effects might be subordinated to other physiological conditions and environmental factors. More evidence on the exact stage of lactation for the switching from glucogenic to lipogenic dietary carbohydrates, on the thresholds of NFC of the diet in early and mid lactation, and on the study of previous nutritional experience of the animals should be deeply investigated to better define the effect of different carbohydrates on metabolic regulations of *Homeorhesis*. Moreover, the study of the milk fatty acid profile could provide valuable information for the study of nutrient partitioning in response to the dietary carbohydrates.

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6. TABLES AND FIGURES

Table 1. Diet formulation and chemical composition offered to the experimental groups.

Feed	UM	Milking mix	TMR EL	Offered ELdiet	TMR S	TMR F	Offered S diet	Offered F diet
Rye grass hay chopped	% of DM		27.0		26.7	26.0		
Wheat straw	% of DM		16.2		17.0	15.0		
Soybean meal, 44%	% of DM		19.5		19.1	19.0		
Corn flaked	% of DM		17.2		18.0	5.0		
Corn meal finely ground	% of DM		8.0		10.0	5.0		
Beet pulp	% of DM		6.1		6.0	28.0		
Molasses cane	% of DM		5.0		1.0	1.0		
Vit-min mix	% of DM		1.0		1.0	1.0		
Bicarbonate sodium	% of DM				1.2			
Offered proportion	% of asfed	8.0	92.0		92.0	92.0	100	100
Composition								
Dry matter	% of asfed	88.3	89.3	89.2	89.8	89.9	89.7	89.8
Ashes	% of DM	8.6	8.4	8.4	10.0	10.2	9.9	10.1
Crude protein	% of DM	17.1	17.2	17.2	16.0	16.8	16.1	16.8
NDF	% of DM	23.8	41.3	39.9	40.4	44.9	39.1	43.2
ADF	% of DM	10.9	23.4	22.4	27.3	32.0	26.0	30.3
ADL	% of DM	4.7	3.8	3.9	5.6	4.9	5.5	4.9
Lipids	% of DM	3.9	2.7	2.8	1.6	1.5	1.8	1.7
Starch	% of DM	41.7	25.2	26.5	24.3	12.7	25.7	15.0
Non-starch soluble NDF	% of DM	4.9	5.2	5.2	7.7	13.9	7.5	13.2
NFC	% of DM	46.6	30.4	31.7	32.0	26.6	33.2	28.2
7h IVSD	% of Starch	75.4	73.0	73.2	73.4	73.8	73.6	73.9
NDF- d, 48h	% of NDF	66.0	64.9	65.0	63.4	64.2	63.6	64.3
Digestible NDF	% of DM	15.7	26.8	25.9	24.9	28.9	24.2	27.8
INDF	% of NDF	27.9	15.6	16.6	18.5	22.6	19.3	23.0
INDF	% of DM	6.6	6.5	6.5	7.3	10.2	7.2	9.9
peNDF	% of NDF		21.0		23.0	24.0		
NEL3x, from IV INDF)[1]	Mcal/kg of DM	1.77	1.56	1.575	1.53	1.41	1.55	1.44
NEL3x (SRNS)[2]	Mcal/kg of DM			1.57			1.52	1.43

EL = in early lactation; S = starch diet fed after 55 DIM; F = fiber diet fed after 55 DIM; DM = dry matter; NDF = neutral detergent fiber; ADF = acid detergent fiber; ADL = acid detergent lignin; Non-starch soluble NDF = estimated as difference among NFC and starch; NFC = non fiber carbohydrates; 7h IVSD = 7 hour test in vitro digestibility of starch; NDF-d = degradability of NDF; Digestible NDF = NDF digestible at 48 h; INDF = indigestible NDF at 48 h; peNDF = physically effective NDF.

[1]: dietary energy content determined using the values of INDF and NDF d 48h as described in details by Gallo et al., 2013.[2]: dietary energy estimated with the model Small Ruminant Nutrition System(Tedeschi et al., 2010)

Table 2. Average lactation performances of the experimental groups in the phase of early lactation from lambing to 55 DIM when animals were fed the early lactation diet. S and F groups refer to the animals that will receive S and F diets from 56 to 165 DIM.

Stage		Early lactation	Early lactation groups			P *value*		
Values	UM	All n = 72	S n = 37	F n= 35	SEM	Diet	DIM	Diet×DIM
Phase	DIM	0-55	0-55	0-55				
DIM average	DIM	32	32	32	0.79	-	-	-
Milk yield	kg/d per head	1.66	1.65	1.66	0.02	0.755	0.001	0.947
FPCMY	kg/d per head	1.41	1.42	1.40	0.02	0.619	0.081	0.917
Milk fat	%	5.15	5.25	5.04	0.05	**0.006**	0.000	0.858
Milk protein	%	4.93	4.98	4.88	0.04	0.086	0.078	0.562
Milk lactose	%	5.00	5.02	4.98	0.03	0.422	0.157	0.866
Milk fat	g/d	8.41	8.53	8.28	0.12	0.285	0.461	0.919
Milk protein	g/d	8.09	8.13	8.05	0.12	0.729	0.002	0.748
Milk solids	%	13.4	13.5	13.2	0.12	0.112	0.000	0.878
Milk solids	g/d	29.9	30.2	29.5	0.23	0.103	0.451	0.696
Casein	%	3.73	3.77	3.69	0.03	0.113	0.064	0.552
Milk urea	mg/dl	46.1	46.5	45.8	0.65	0.568	0.000	0.811
Somatic cell count	Log_{10} n/ml	2.61	2.76	2.46	0.04	**0.001**	0.001	0.996
Bacterial count	Log_{10}CFU/ml	2.58	2.64	2.51	0.04	0.026	0.001	0.715
pH	Units	6.55	6.65	6.44	0.07	0.082	0.064	0.750
NaCl	Meq	138.3	140.6	135.9	2.72	0.382	0.026	0.877

DIM = days in milk; FPCMY = fat and protein corrected milk yield.

Table 3. Average lactation performances of the experimental groups in the of mid-late lactation phase and, separately, in the last40 days of lactation. In both phases the animals were divided in two groups fed a starch (S)or fiber(F) diet.

Stage		Mid-late[a]						End[a]			P *value*		
Values	UM	S n =37	F n = 35	SEM	Diet	DIM	Diet×DIM	S n =37	F n = 35	SEM	Diet	DIM	Diet×DIM
Phase	DIM (min-max)	57-170	55-169					133-169	132-170				
DIM average	DIM	112	113	1.14				151	153	0.751			
Milk yield	kg/d per head	1.36	1.32	0.021	0.447	0.001	0.924	1.04	0.92	0.033	0.208	0.101	0.271
FPCMY	kg/d per head	1.28	1.25	0.018	0.650	0.001	0.847	1.04	0.96	0.033	0.417	0.188	0.365
Milk fat	%	6.08	6.17	0.034	0.363	0.001	0.152	6.58	6.91	0.054	**0.026**	0.058	0.922
Milk protein	%	5.50	5.68	0.019	**0.001**	0.001	0.002	5.66	6.03	0.030	**0.001**	0.274	0.651
Milk lactose	%	4.74	4.74	0.019	0.983	0.001	0.666	4.41	4.47	0.032	0.465	0.432	0.329
Milk fat	g/d	8.00	7.88	0.109	0.697	0.001	0.817	6.36	6.82	0.221	0.479	0.267	0.424
Milk protein	g/d	7.34	7.30	0.104	0.888	0.001	0.724	5.55	6.84	0.190	0.598	0.140	0.347
Milk solids	%	16.58	16.49	0.169	0.849	0.001	0.871	18.30	19.42	0.489	0.492	0.238	0.885
Milk solids	g/d	31.92	31.67	0.159	0.610	0.755	0.822	30.96	31.33	0.388	0.771	0.751	0.758
Casein	%	4.31	4.16	0.015	**0.001**	0.001	0.001	4.25	4.57	0.027	**0.001**	0.376	0.838
Milk urea	mg/dl	44.44	41.70	0.379	**0.014**	0.000	0.063	43.82	47.99	0.656	**0.027**	0.560	0.725
Somatic cell count	n/ml x 1000	3.20	3.03	0.026	0.001	0.001	0.850	3.51	3.41	0.038	0.377	0.173	0.785
Bacterial count	CFU/ml x 1000	2.74	2.68	0.021	0.008	0.001	0.002	3.08	3.04	0.030	0.642	0.074	0.987
pH	units	6.77	6.68	0.007	**0.001**	0.001	0.064	6.73	6.53	0.020	0.004	0.054	0.032
NaCl	meq	160.17	157.27	2.520	0.696	0.001	0.626	195.46	182.82	4.78	0.360	0.555	0.431

DIM = days in milk.[a]S = group fed a starch diet from 56 to 165 DIM; F = group fed a fiber diet from 56 to 165 DIM;

Table 4. Body weight (BW) and body condition score (BCS) of experimental groups in the experimental trial.

Stage		Group[a]			P *value*		
Variable	UM	S n =37	F n = 35	SEM	Diet	DIM	Diet×DIM
Pre-partum BW	kg	52.48	54.19	0.203	0.021	1.000	1.000
Post-partum BW	kg	44.30	45.03	0.195	0.301	1.000	1.000
Pre-partum BCS	units	2.90	2.92	0.005	0.725	0.961	0.989
Post-partum BCS	units	2.88	2.91	0.005	0.274	0.990	1.000
Body weight at 55 DIM	kg	46.25	46.63	0.177	0.306	1.000	1.000
BW end of lactation	kg	49.69	50.46	0.204	0.264	0.994	1.000
BCS at 55 DIM	units	2.80	2.83	0.004	0.058	0.999	1.000
BCS at 165 DIM	units	2.94	2.92	0.005	0.091	0.920	0.996
BW variation from partum to 55 DIM	kg	1.94	1.60	0.088	0.891	1.000	0.999
BW variation from partum to 55 DIM	kg/d	0.035	0.029	0.001	0.821	1.000	0.999
BW variation from 56 to 165 DIM	kg	3.445	3.830	0.109	0.676	0.907	1.000
BW variation from 56 to 165 DIM	kg/d	0.038	0.043	0.001	0.615	0.903	1.000
BCS variation from partum to 55 DIM	units	0.078	0.071	0.004	0.480	1.000	1.000
BCS variation from 56 to 165 DIM	units	0.138	0.008	0.005	**0.001**	0.992	**0.005**

[a]S = group fed a starch diet from 56 to 165 DIM; F = group fed a fiber diet from 56 to 165 DIM; DIM = days in milk

Table 5. Ruminal pH, total and individual VFA during pregnancy in rumen of sheep fed high or low starch diets in pregnancy

item	Diet[a] S n = 9	Diet[a] F n = 9	SEM	P-*value* Diet	P-*value* Time	P-*value* Diet×Time
pH	6.50	6.27	0.093	0.233	0.262	0.279
TOT VFA (mmol/L)	73.94	82.23	3.143	0.073	0.105	0.901
acetic (%)	70.18	69.82	0.362	0.640	0.542	0.668
Propionic (%)	18.63	18.39	0.253	0.626	0.157	0.398
butyric (%)	11.19	11.79	0.270	0.367	0.477	0.764

[a]S = starch diet; F = fiber diet.

Table6. Effect of high or low starch diet on ruminal pH and volatile fatty acids (VFA) in lactating sheep

Item	Early lactation	Mid lactation diet[a] S n = 9	Mid lactation diet[a] F n = 9	SEM	P-*value*[b] Diet	P-*value*[b] Time	P-*value*[b] Diet×Time
pH	6.27±0.26	6.42	6.36	0.032	0.380	0.025	0.210
Total VFA(mmol/L)	88.01±16.94	83.45	91.41	3.122	0.220	0.001	0.990
Acetic (%)	63.81±4.13	67.48	67.01	0.301	0.480	0.001	0.690
Propionic (%)	20.86±5.31	17.78	18.50	0.308	0.320	0.267	0.840
Butyric(%)	15.33±2.41	14.74	14.49	0.274	0.670	0.034	0.580

[a]S = starch diet; F = fiber diet; [b]Time = two sampling date at 18 and 69 days from the diet-switch corresponding to 73 and 124 DIM.

Table 7. Effect of diet, sampling date and their interaction on milk fatty acid profile

Trait[1]	Diet[a] S n =6	Diet[a] F n = 6	SEM	P-*values* Diet	P-*values* DIM	P-*values* Diet×DIM
SFA						
C4:0	2.48	2.41	0.033	0.545	<0.001	0.467
C6:0	2.04	1.98	0.022	0.479	<0.001	0.892
C8:0	2.10	2.02	0.025	0.474	<0.001	0.587
C9:0	0.11	0.13	0.005	0.015	<0.001	0.039
C10:0	7.60	8.09	0.089	0.266	0.001	0.811
C11:0	0.35	0.36	0.007	0.691	0.070	0.434
C12:0	0.25	0.27	0.076	0.053	0.094	0.809
C13:0iso	0.02	0.02	0.001	0.106	0.001	0.182
C13:0anteiso	0.06	0.07	0.002	0.425	0.002	0.509
C14:0iso	0.10	0.14	0.003	0.003	0.040	0.053
C14:0	11.77	12.52	0.139	0.241	<0.001	0.962
C15:0iso	0.27	0.22	0.006	0.187	<0.001	0.736
C15:0anteiso	0.45	0.51	0.009	0.110	0.001	0.054
C15:0	0.98	1.23	0.017	<0.001	0.004	0.903
C16:0iso	0.27	0.36	0.009	0.018	<0.001	0.025
C16:0	26.60	30.23	0.364	0.039	<0.001	0.427
C17:0iso	0.45	0.42	0.008	0.553	0.004	0.471
C17:0anteiso	0.50	0.57	0.009	0.037	0.001	0.074
C17:0	0.77	0.90	0.009	<0.001	<0.001	0.598
C18:0iso	0.05	0.07	0.002	0.007	0.027	0.487
C18:0	8.32	6.27	0.182	0.029	0.002	0.164
C20:0	0.28	0.21	0.005	0.002	0.173	0.404
C22:0	0.14	0.14	0.002	0.878	0.386	0.063
C23:0	0.06	0.08	0.002	0.070	0.249	0.002
C24:0	0.05	0.06	0.001	0.784	0.461	0.177
C25:0	0.02	0.02	0.001	0.739	0.148	0.758
C26:0	0.02	0.02	0.001	0.688	0.040	0.060
MUFA						
C10:1	0.04	0.04	0.002	0.228	<0.001	0.082
C14:1c9	0.25	0.27	0.008	0.627	<0.001	0.586
C15:1	0.08	0.13	0.004	0.002	<0.001	0.003
C16:1t4	0.04	0.05	0.002	0.127	<0.001	0.017
C16:1t5	0.03	0.03	0.001	0.231	<0.001	0.301
C16:1t6+t7	0.05	0.07	0.002	0.002	0.001	0.252
C16:1t9	0.09	0.09	0.003	0.730	0.254	0.389
C16:1t10	0.02	0.02	0.001	0.264	0.734	0.343
C16:1t11+t12	0.05	0.04	0.001	0.227	0.001	0.132
C16:1c7	0.24	0.22	0.003	0.250	0.003	0.833
C16:1c9	0.91	1.01	0.025	0.362	<0.001	0.437
C16:1c10	0.03	0.03	0.001	0.050	0.002	0.871
C16:1c11	0.02	0.02	0.001	0.147	0.001	0.708
C17:1 c6-7	0.05	0.06	0.001	0.701	0.015	0.112
C17:1c8	0.02	0.02	0.001	0.019	0.145	0.972
C17:1c9	0.21	0.24	0.003	0.054	0.002	0.797
C18:1t4	0.03	0.02	0.001	0.108	0.096	0.300
C18:1t5	0.03	0.02	0.001	0.382	0.098	0.908
C18:1t6+t8	0.28	0.25	0.007	0.116	<0.001	0.206

Table 7. Continued from the previous page**.** Effect of diet, sampling date and their interaction on milk fatty acid profile.

Trait[1]	Diet[a] S n = 6	Diet[a] F n = 6	SEM	P-*values* Diet	P-*values* DIM	P-*values* Diet×DIM
C18:1t9	0.30	0.25	0.007	0.018	<0.001	0.023
C18:1t10	0.59	0.63	0.024	0.667	<0.001	0.900
C18:1t11	1.40	1.50	0.041	0.564	<0.001	0.984
C18:1t12	0.36	0.29	0.008	0.011	<0.001	0.003
C18:1t13+t14	0.55	0.47	0.013	0.029	<0.001	0.002
C18:1c9	16.18	12.14	0.289	<.0001	0.153	0.933
C18:1c11	0.50	0.47	0.007	0.416	0.028	0.893
C18:1c12	0.38	0.32	0.009	0.051	<0.001	0.199
C18:1c13	0.08	0.07	0.001	0.193	0.002	0.331
C18:1t16+c14	0.31	0.24	0.005	0.000	<0.001	0.000
C18:1c16	0.04	0.03	0.001	0.005	0.022	0.429
C20:1c9	0.02	0.02	0.001	0.970	0.163	0.232
C20:1c11	0.07	0.06	0.001	0.443	0.003	0.996
C20:1c15	0.03	0.03	0.001	0.453	0.022	0.838
C22:1n-9	0.02	0.02	0.001	0.046	0.340	0.713
PUFA						
C18:2t10t14	0.04	0.04	0.001	0.155	<0.001	0.001
C18:2t11t15	0.03	0.02	0.001	0.036	<0.001	0.255
C18:2t9t12	0.03	0.03	0.001	0.992	0.208	0.122
C18:2c9t13	0.25	0.20	0.005	0.005	<0.001	0.042
C18:2t8c13	0.13	0.10	0.003	0.006	<0.001	0.002
C18:2c9t12	0.12	0.10	0.002	0.007	<0.001	0.482
C18:2t9c12	0.04	0.04	0.001	0.142	0.013	0.408
C18:2n-6	2.99	3.23	0.058	0.462	<0.001	0.598
C18:2t12c15	0.04	0.05	0.001	0.036	<0.001	0.064
CLAc9t11	0.84	0.81	0.021	0.711	<0.001	0.708
CLAt9c11	0.09	0.09	0.001	0.368	0.014	0.015
CLAt10c12	0.01	0.01	0.001	0.306	0.206	0.973
CLAt11t13	0.02	0.02	0.001	0.806	0.513	0.867
C20:2n-9	0.02	0.02	0.001	0.006	<0.001	0.010
CLAt9t11	0.02	0.03	0.001	0.008	0.159	0.065
C20:2n-6	0.04	0.05	0.001	0.011	0.037	0.375
C22:2n-6	0.03	0.04	0.001	0.012	0.075	0.294
C18:3n-6	0.07	0.10	0.003	0.082	0.001	0.181
C18:3n-3	0.31	0.36	0.007	0.268	<0.001	0.448
C20:3n-9	0.05	0.04	0.001	0.017	0.001	0.762
C20:3n-6	0.05	0.04	0.001	0.593	0.022	0.781
C20:4n-6	0.24	0.21	0.004	0.374	0.692	0.345
C22:4n-6	0.05	0.04	0.001	0.374	0.267	0.679
EPA	0.03	0.02	0.001	0.217	0.336	0.969
DPA	0.07	0.07	0.001	0.660	0.227	0.441
DHA	0.03	0.03	0.001	0.784	0.709	0.957

[a]S = starch diet; F = fiber diet; [1]*c = cis; t = trans*

Table 8. Effect of diet, sampling date and their interaction on groups of FA and ratio of groups of FA.

Groups and ratios [1]	Diet		SEM	P-Values		
	S n = 6	F n = 6		Diet	DIM	Diet×DIM
SCFA	14.42	14.72	0.135	0.607	<0.001	0.900
MCFA	49.46	55.39	0.511	0.004	<0.001	0.563
LCFA	36.12	29.88	0.502	0.001	<0.001	0.685
SFA	70.63	74.57	0.362	0.002	<0.001	0.966
UFA	29.26	25.32	0.361	0.002	<0.001	0.967
MUFA	23.31	19.19	0.323	<0.001	0.004	0.976
PUFA	5.95	6.13	0.093	0.729	<0.001	0.658
TFA	4.84	4.59	0.101	0.544	<0.001	0.596
CLA	0.98	0.97	0.022	0.880	<0.001	0.739
OBCFA	4.45	5.08	0.054	0.007	0.001	0.460
n-6	3.47	3.74	0.062	0.470	<0.001	0.697
n-3	0.46	0.50	0.008	0.376	0.000	0.676
de novo	41.84	45.52	0.348	0.015	0.001	0.802
n-6/n-3	7.59	7.50	0.047	0.687	<0.001	0.577
TFA-VA	3.44	3.09	0.067	0.164	<0.001	0.149
SCFA/LCFA	0.40	0.50	0.008	0.001	0.148	0.891
SFA/UFA	2.44	3.01	0.052	0.001	0.002	0.953
de novo/LCFA	1.18	1.56	0.031	0.001	0.001	0.667

[a]S = starch diet; F = fiber diet; [1]SCFA = short chain fatty acids, sum of the individual fatty acids from C4:0 to C10:0; MCFA = medium-chain fatty acids, sum of the individual fatty acids from C11:0 to C17:0; LCFA = long-chain fatty acids, sum of the individual fatty acids from C18:0 to DHA; SFA = saturated fatty acids, sum of the individual saturated fatty acids; UFA = unsaturated fatty acids, sum of the individual unsaturated fatty acids; MUFA = monounsaturated fatty acids, sum of the individual monounsaturated fatty acids; PUFA = polyunsaturated fatty acids, sum of the individual polyunsaturated fatty acids; n-6 = sum of individual n-6 PUFA; n-3 = sum of individual n-3 PUFA; TFA = trans fatty acids, sum of the individual trans fatty acids; CLA = sum of individual conjugated of linoleic acids; OBCFA = odd- and branched-chain fatty acids, sum of individual odd- and branched-chain fatty acids; *de novo*, sum of the fatty acids synthetized de novo in the mammary gland (from C6:0 to C16:0); ratios between some groups of FA were also calculated: n-6/n-3; SCFA:LCFA, SFA:UFA and *de novo*:LCFA

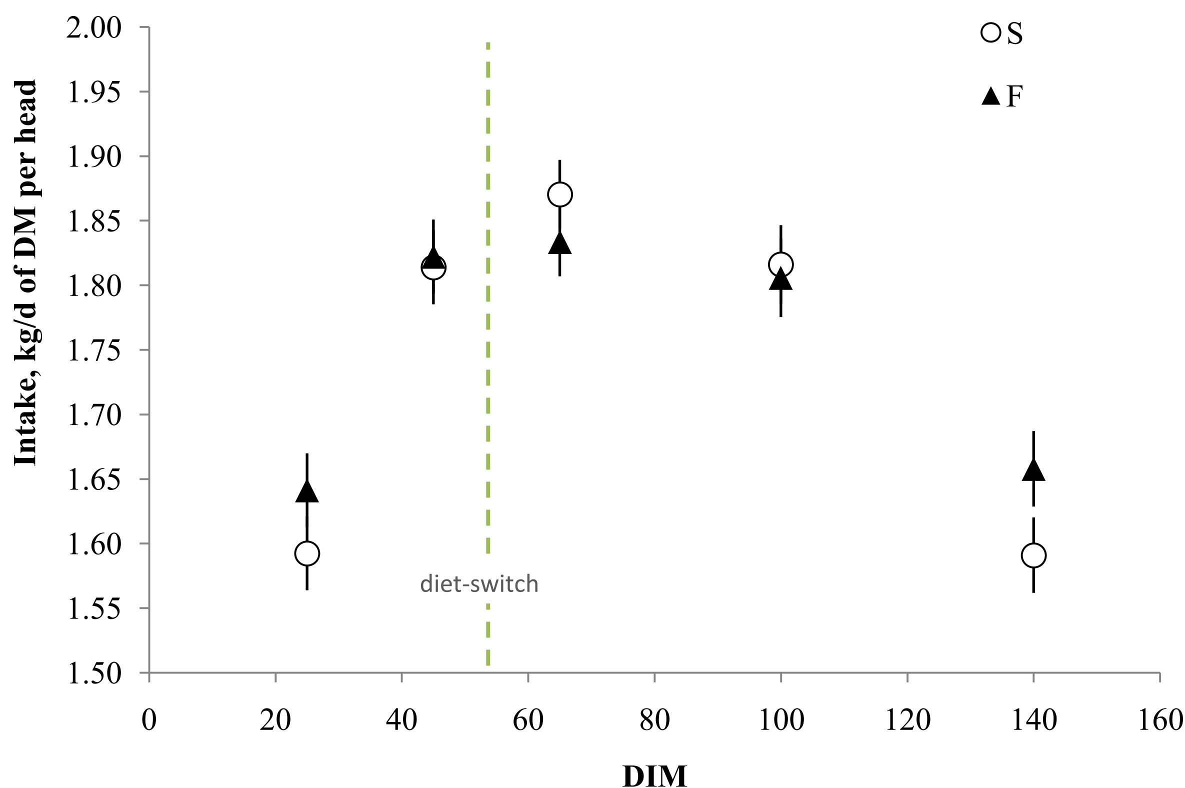

Figure 1. Average daily intake of the experimental groups. The groups S and F from parturition to 55 DIM were both fed a same EL diet (Table 1) then from 56 DIM to end of lactation with starch (S)or fiber (F) diets, respectively.

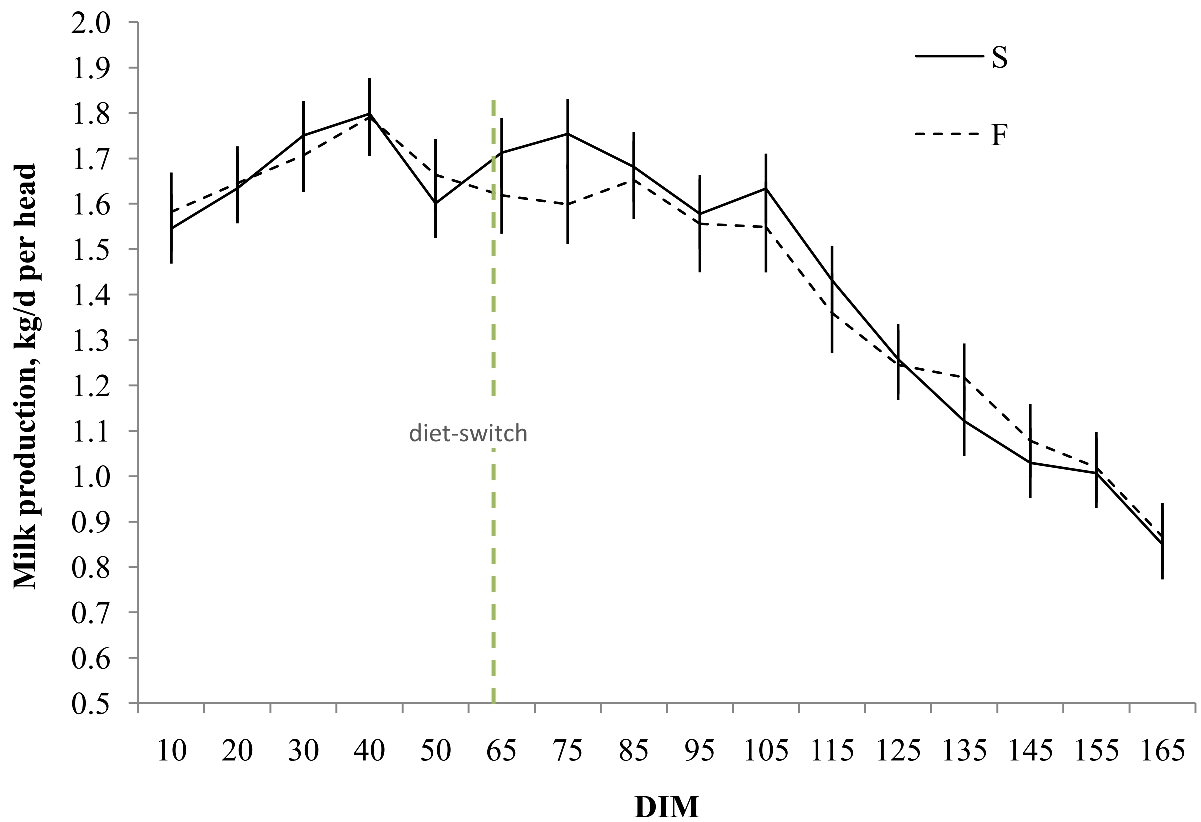

Figure 2. Average milk yield of the experimental groups. The groups S and F from parturition to 55 DIM were both fed a same EL diet (Table 1) then from 56 DIM to end of lactation with starch (S)or fiber (F) diets, respectively.

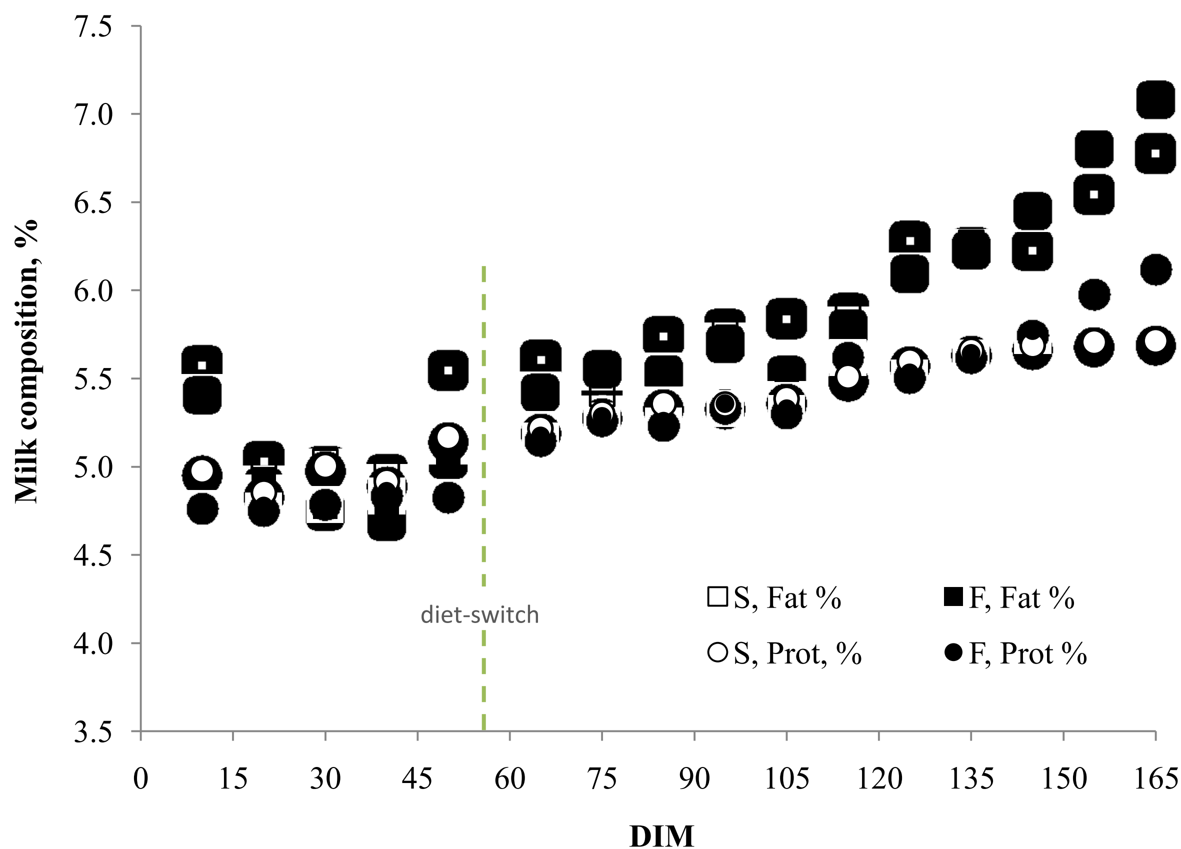

Figure 3. Average milk composition of the experimental groups. The groups S and F from parturition to 55 DIM were both fed a same EL diet (Table 1) then from 56 DIM to end of lactation with starch (S)or fiber (F) diets, respectively.

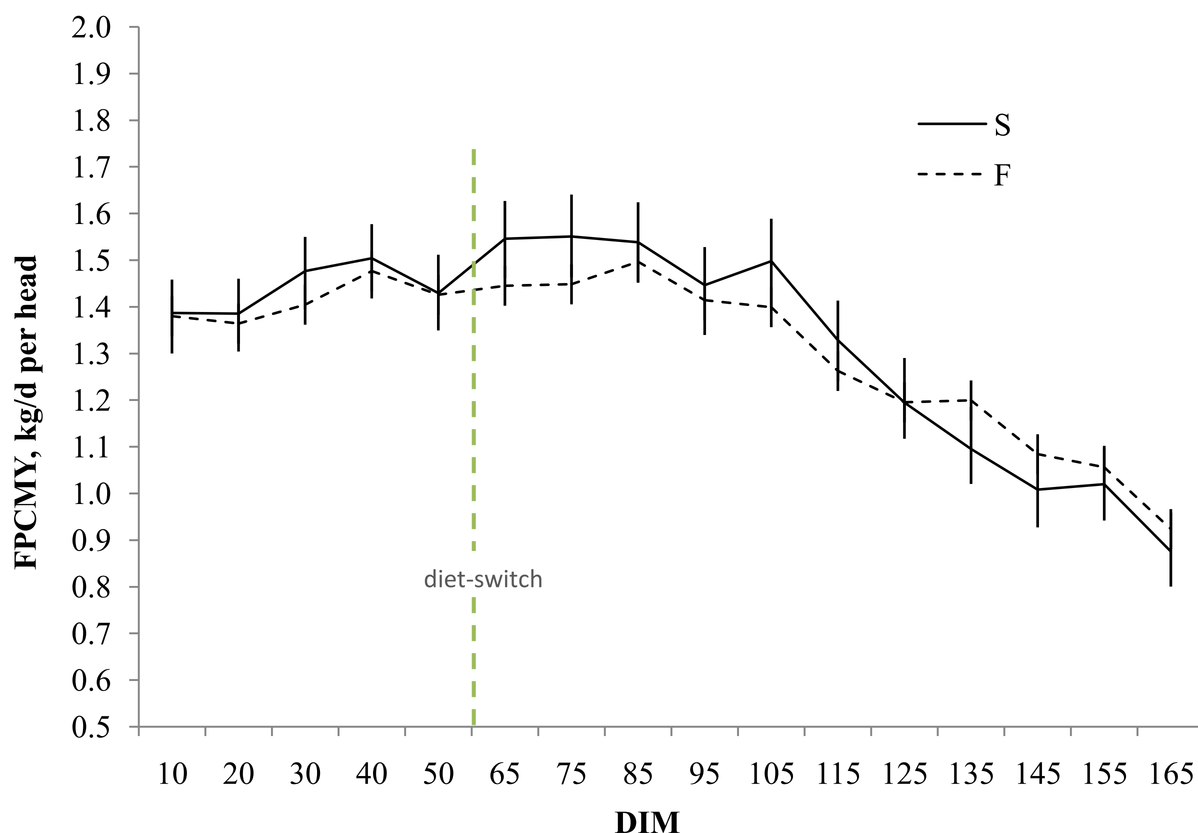

Figure 4. Average fat protein corrected milk yield of the experimental groups. The groups S and F from parturition to 55 DIM were both fed a same EL diet (Table 1) then from 56 DIM to end of lactation with starch (S)or fiber (F) diets, respectively.

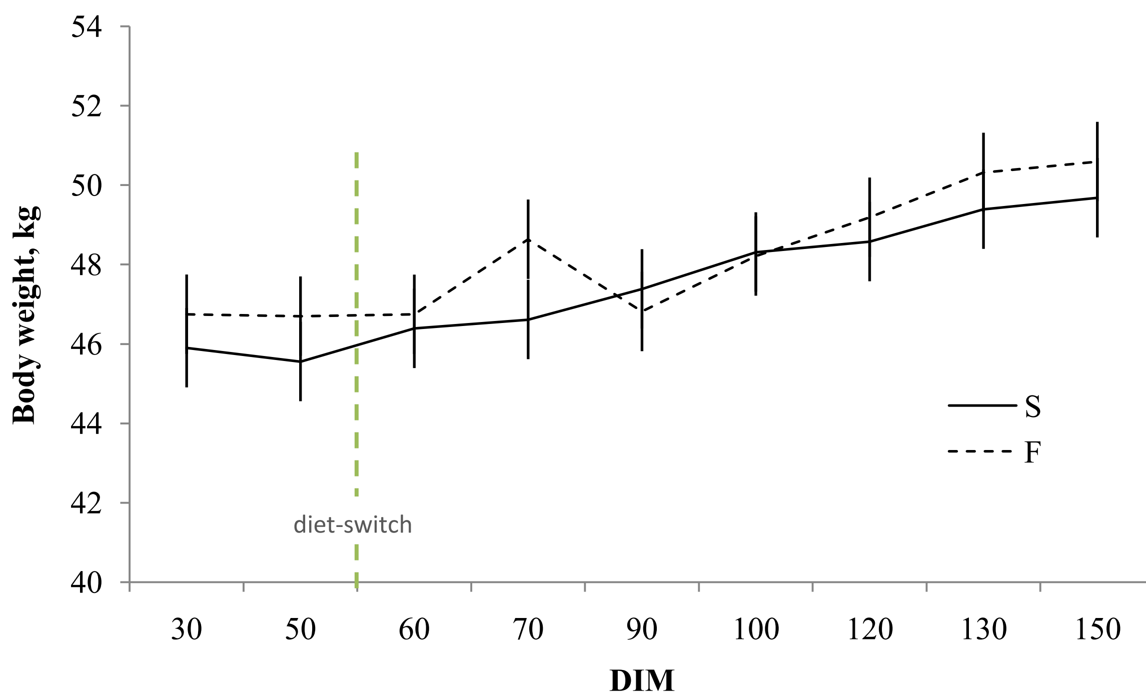

Figure 5. Average body weight trend of the experimental groups. Groups S and F from parturition to 55 DIM were both fed a same EL diet (Table 1) then from 56 DIM to end of lactation with starch (S)or fiber (F) diets, respectively.

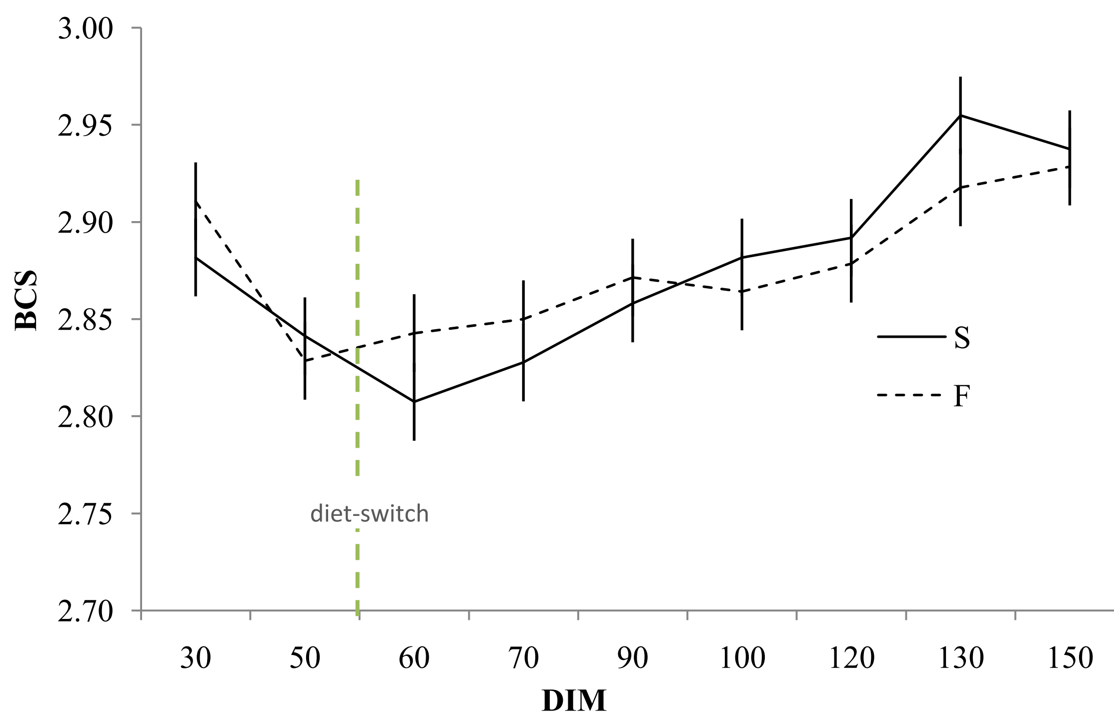

Figure 6. Average body condition score of the experimental groups. The groups S and F from parturition to 55 DIM were both fed a same EL diet (Table 1) then from 56 DIM to end of lactation with starch (S)or fiber (F) diets, respectively.

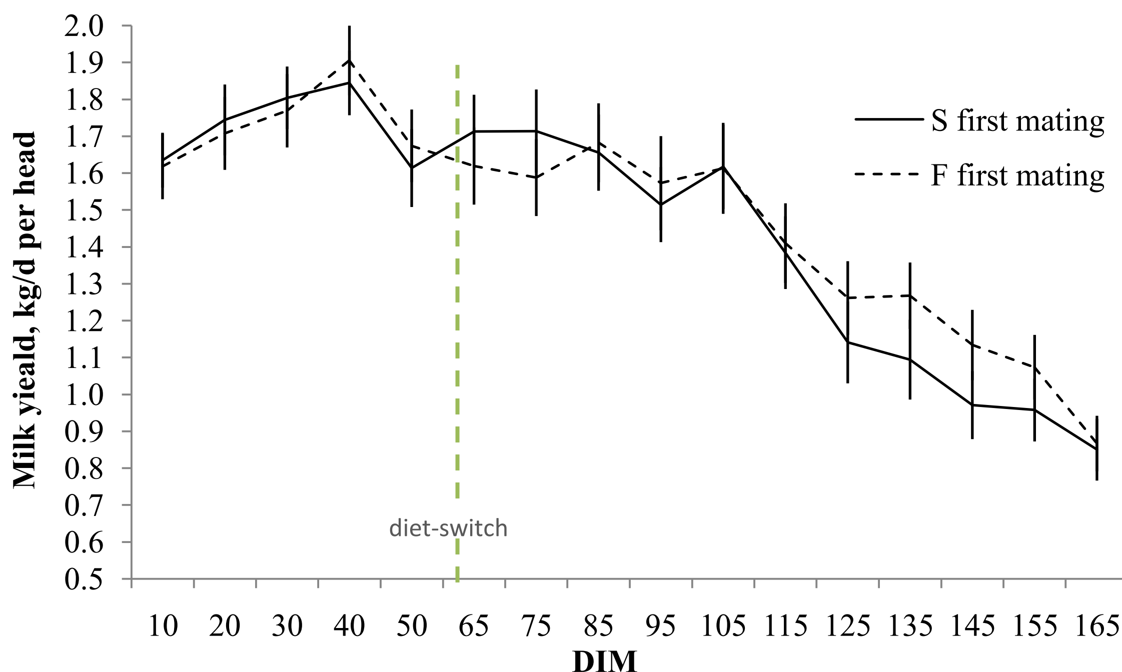

Figure 7. Average milk yield of animals with homogeneous mating period. Groups S and F from lambing to 55 DIM were both fed a same EL diet (table 1) then from 56 DIM to lactation end with starch (S)or fiber (F) diets, respectively.

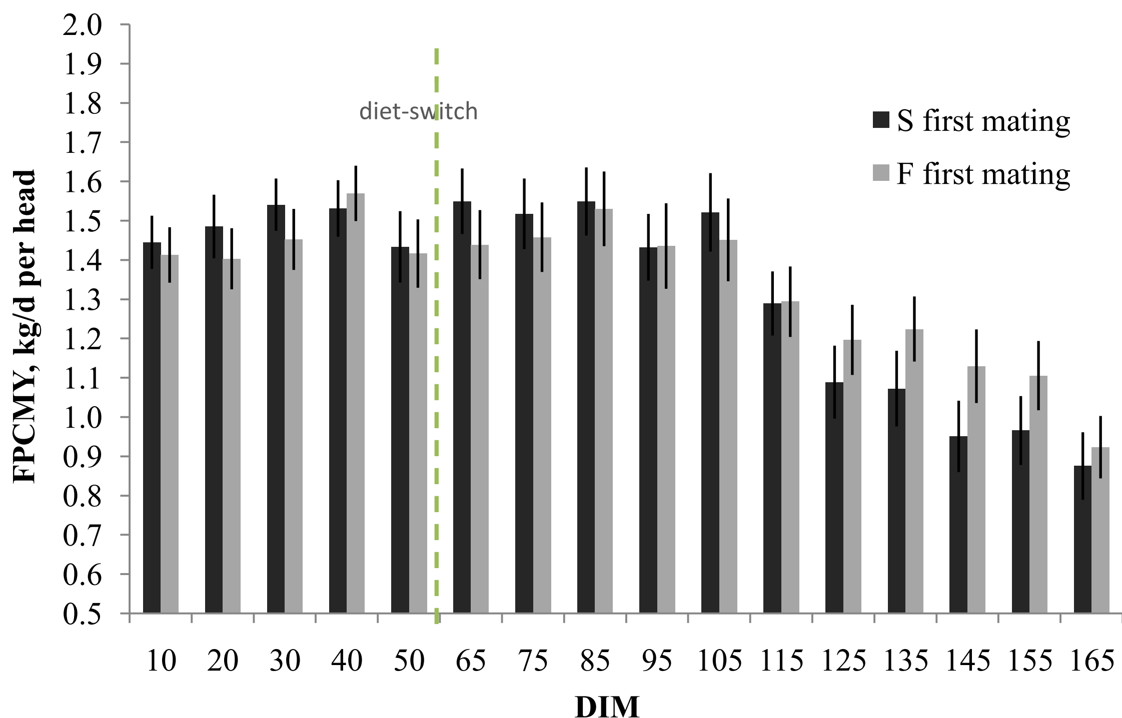

Figure 8. Average fat protein corrected milk yield of the animals with homogeneous mating period. Groups S and F from lambing to 55 DIM were both fed the same EL diet (Table 1) then from 56 DIM to 165 DIMwith starch (S)or fiber (F) diets, respectively.

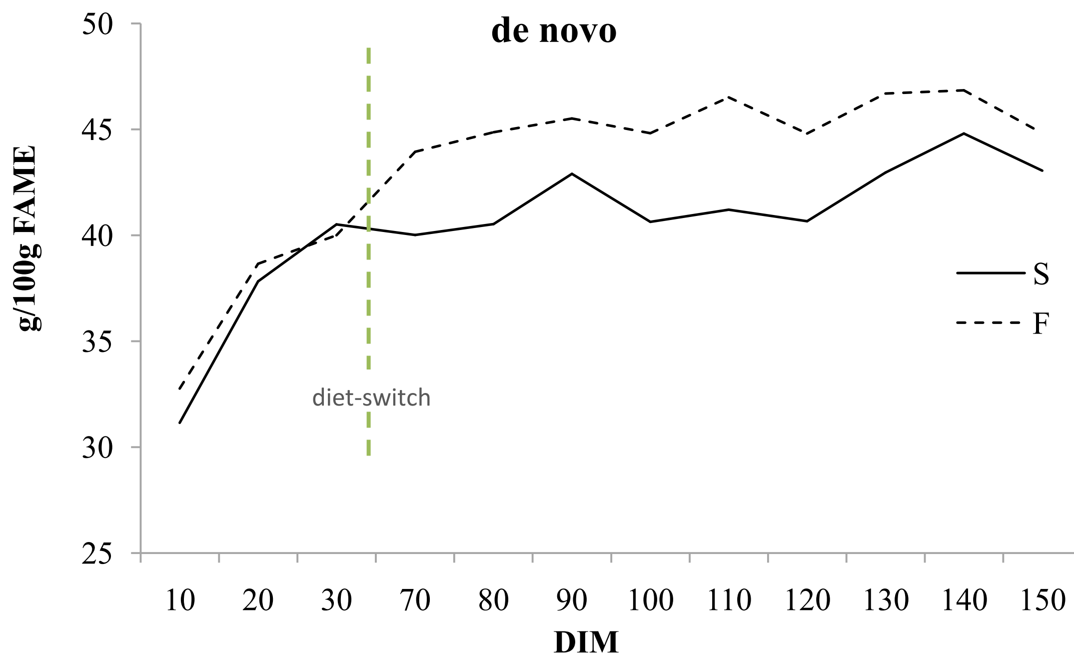

Figure 9. Evolution of de novo FA concentration in milk of ewes included in the experimental groups. Groups S and F from lambing to 55 DIM were both fed a same EL diet (Table 1) then from 56 DIM to lactation end with diets S and F, respectively.

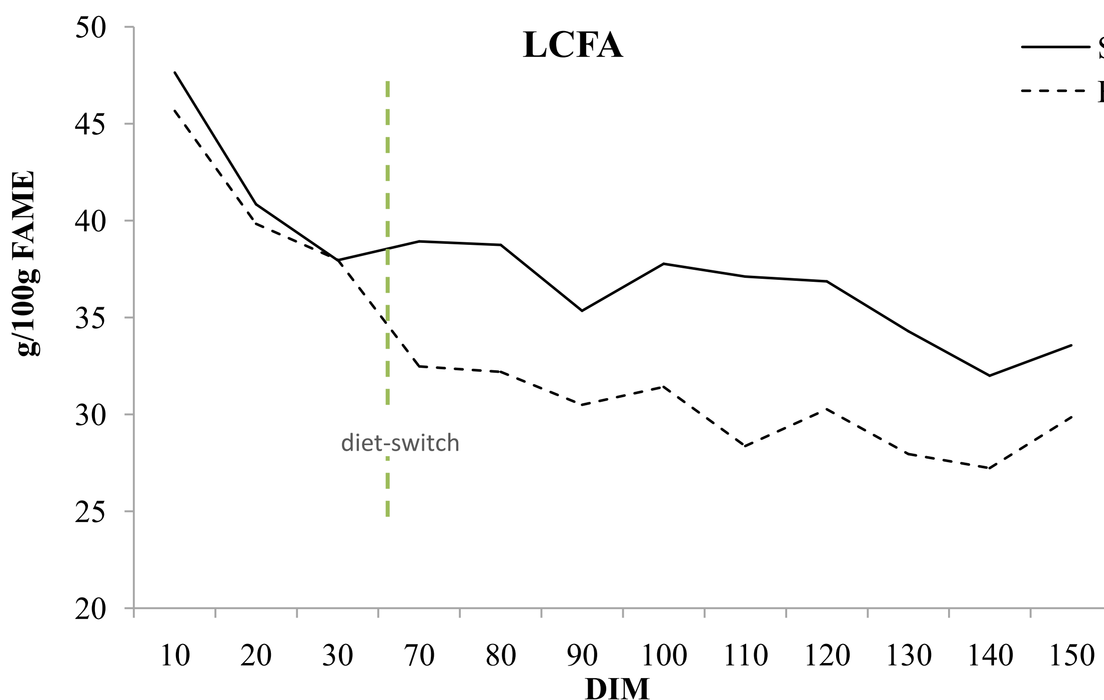

Figure10. Evolution of LCFA concentration in milk of ewes included in the experimental groups. Groups S and F from lambing to 55 DIM were both fed a same EL diet (Table 1) then from 56 DIM to lactation end with diets S and F, respectively.

CHAPTER 4

Effects of dietary carbohydrates, supplied during pre-natal, growing and mid lactation, on lactation performance of primiparous dairy sheep.

ABSTRACT

This research hypothesized that the exposures to diets, either rich in starch or soluble fiber early in life, might affect sheep response to carbohydrates in first lactation. This work specifically aimed to investigate if supplying different sequences of glucogenic and lipogenic diets, from high starch or high soluble fiber respectively, in prenatal life, growing and mid lactation might influence the response to carbohydrate of primiparous dairy sheep in mid lactation stage. Lactation performance of 72 primiparous sheep was analyzed. The sheep were divided in 8 groups identified by a sequence of 3 letters sequences (SSS, SSF, FSS, FSF, SFS, SFF, FFS and FFF) indicating the diets received by each group in the whole experiment. The first two letters (S or F) indicate the diet (starch or fiber) received during the last 75 days of uterine life, and during the growing phase (respectively), whereas the third letter indicates the diet (starch or fiber) received in mid lactation after 56 days in milk (DIM). Data were analyzed with PROC MIXED of SAS to test effects of the dietary treatments and DIM on milk production and composition. A multivariate factor analysis (MFA)was also applied to investigate the latent structure of the animal performance variability. The MFA considered 30 variables including BW, BCS, and milk yield of the mothers of the 72 sheep during pregnancy and lactation, and growing and lactation performances of the 72 sheep included in the experimental trial.

The fat protein corrected milk yield (FPCMY) resulted only numerically different among the 8 groups. The group SSF, and generally the groups fed diet F vs diet S in mid lactation (within same prenatal and growing diets)showed delayed production decline. Only group FFF showed the fastest production decay, whereas the SSF the more persistent pattern. Differences resulted significant only when milk records from a small group of sheep that lambed in the same week was analyzed (n =24): FPCMY at 155 DIM was equal to 1.242 vs. 0.886 kg/d ($P<0.05$) for SSF vs. SSS ewes, and 0.947 vs.

0.770(P>0.80) for FFS and FFF ewes. Prenatal diet also significantly influenced milk conjugated linoleic acid (CLA) until 80 DIM (P = 0.002). Six main factors were extracted by MFA indicating that variability of the dataset was mainly related to milk production and intake in lactation, post-pubertal, prepubertal growth and preweaning growth, and performance of the mothers. Prenatal diet and lactation diet were significantly associated to Factor 4 and Factor 5 (P < 0.05) which both resulted highly correlated to lambing type (single vs. twin). In summary, with a deep analysis it was possible to deduce that effects of dietary treatments on animal response were highlighted by observed patterns. Nevertheless, due to extreme variability observed, they also suggest that carbohydrates might have a mediated or subordinated role, in respect to other environmental or physiological factors, which regulates the animal response in the short term.

1. INTRODUCTION

Metabolic adaptations in pregnancy are driven by the endocrine system with the aim to distribute the nutrients in the maternal body and then, finally, to provide simple elements such as glucose and amino acids to the growing fetus (Gluckman, 1997). Furthermore several authors have observed important effects of maternal nutrition on offspring characteristics, including production performances and metabolism. The maternal environment and in particular the metabolic substrates to which the fetus is exposed during pregnancy, influences epigenetic processes and developmental changes which may have consequences later in life (Poore et al., 2014). These mechanisms aim to have a thrifty metabolic phenotype (Neel, 1962; Parlee and MacDougald, 2014) that is very beneficial in cases of undernutrition experienced in uterine life and/or in postnatal life. Nevertheless it could have detrimental effects later in life even if adequate or excess of nutrients in postnatal or adult life are provided (Duque-Guimaraes et al., 2013). It has been largely demonstrated that prenatal diet can affect body weight, milk yield and milk composition in the subsequent first generation (Blair et al., 2010) or reproductive organs and reproductive performances (Rhind et al., 2001). It has been also observed, either in humans and small ruminants, that overnutrition or undernutrion during prenatal life can impair glucose metabolism in adult life (Paliy et al., 2014; Husted et al., 2008). Effects of dietary factors have also deeply investigated and there are many evidences that exposure to high fat diets in prenatal life are likely to alter the glucose metabolism of the offspring. Furthermore several studies demonstrated that high fat diets induce increases in body lipids, glucose resistance and changes in insulin sensitivity of peripheral tissues (Nielsen et al., 2013; Khanal et al., 2014; 2015).

Effect of lipogenic diet from increases of dietary fat content has been already studied in sheep (Khanal et al., 2015). However, previous studies reported in literature, focusing on the effects of prenatal diets, mainly considered the variation of the dietary fat content, whereas few studies focused on the effect of the type of carbohydrate on offspring metabolism of dairy ruminants (Khanal et al., 2014; Poore et al., 2014). On the other hand experimental evidences focusing on probable effects of different carbohydrate sources supplied in pregnancy and early growing on metabolic programming and, generally, on

sheep performance, are very scarce. Oppositely, Lunesu (2016) reported a list of studies observing significant and numeric increases of milk yield and milk solids, higher or constant DM intake, and general reduction of body reserve accumulation, when feedstuffs rich in high digestible NDF (or soluble fiber) were substituted to corn or barley grains in diets of mid lactating sheep. It evidenced positive short term effects of lipogenic diets, based on fiber, on sheep performances and favored nutrient partitioning toward the mammary gland. Following the evidences on lactation persistency observed by Cannas et al., (2004; 2007) and Lunesu et al., (2016), and considering a fetal programming point of view, this research hypothesized that the exposures to diets, either rich in starch or soluble fiber early in life, might affect the sheep response to carbohydrates in first lactation, possibly in a different manner, in early and mid lactation. This work specifically aimed to investigate if supplying different sequences of glucogenic and lipogenic diets, from high starch or high soluble fiber respectively, in prenatal life, growing and mid lactation might influence the response to carbohydrate in mid lactation of primiparous dairy sheep.

2. MATERIALS AND METHODS

2.1 Experimental design, animals and treatments

The experiment consisted of three main periods, described with more details in Chapter 2:period 1: pre-natal for the last 75 days of uterine life (pregnant mothers);period 2: post-natal growing, from birth to first lambing (growth and pregnancy);period 3: first lactation from parturition to end of lactation (primiparous ewes). A summary of the experimental design was reported in this section. In the whole trial two groups of sheep were feed with different combinations of two main diets, one rich in starch (diet S) and one with low starch but rich in soluble fiber (diet F) from soyhulls or beet pulp from their uterine life (mothers' diet) to the end of their first lactation.

The experiment was conducted at a private farm located in Sassari, in the north of Sardinia, Italy. From the farm flock a group of 202 multiparous sheep on healthy conditions and similar stage of pregnancy was selected. This number was considered

sufficient to get about 10 animals per group in the final phase of the period 3. All animal experiments were performed in accordance with DPR 27/1/1992 (Animal Protection Regulations of Italy) in conformity with European Community regulation 86/609.

2.1.1 Period 1: Prenatal phase

The 202 pregnant sheep were randomly allocated to 2 different groups (101 animals each) homogeneous for body conditions score (BCS), body weight (BW) and gestational age. When groups were created, from a minimum of 75 days from expected day of lambing, all sheep were housed in separated pens, indoor, managed with straw as bedding material. Each group was randomly assigned to one of the experimental dietary treatments either with high content of starch (diet S) or soluble fiber (diet F), as detailed in Chapter 2. In this period all the animals were fed with a diet with mixed hay (clover and ryegrass for the most part) and with a pellet that consisted on the experimental concentrate (Table 1). The diets were supplied twice a day (8:00 a.m. and 7:00 p.m.), the pellets were supplied each time in individual headlocks in order to offer the same average restricted amount of diet to each animal. The mixed hay was supplied in a separate bunk and left available for all day but in a restricted amount in order to respect the forage to concentrate ratio. The offered amount of feeds was estimated proportionally to animal requirements. The lambing period ranged between November 15 and December 10, 2014.

Measurements and samplings in period 1.

Feed samples were collected every 20 days for subsequent chemical analyses. Body weight (BW) and body condition score (BCS) were monitored every 20 days. The BW was measured by using a professional electronic scale (Teobil 6, Electronic Weighing System, Sondrio, Italy). Body condition score was estimated manually by 3 trained operators with manual check of the lumbar fat depots, using a score from 0 to 5 point scale (Russel et al., 1969), approximating 1/8 points.

2.1.2 Period 2: Post-natal growing

The day of lambing, the mothers where moved in the parturition pen and fed a diet that included the same hay and an average mix of the S and F concentrates. Mothers and lambs from the S and F groups remained in the same pen for the first 25 days and lambs

were fed with the mothers' milk. After 25 days mothers and lambs proceeding from S and F prenatal groups were split in two groups and reassigned to the diet, S and F, obtaining 4 groups different for nutritional history plan (SS, n = 28; SF, n = 35; FS, n = 28; and FF, n = 28; Total, n = 119) (Figure 1 of Chapter 2). Only female lambs were kept with the mothers from 25 days until complete weaning at 60 days of age, when they were separated in 4 different pens to continue the experimental trial (Figure 1 in Chapter 2). From weaning to first lambing, the animals were fed twice a day a diet (S or F depending the group) offered in a restricted amount in order to respect the forage to concentrate ratio of the postnatal period (Table 1). The offered amount was calculated on the basis of animal requirements of maintenance and growth. After synchronization the sheep were naturally mated with 8 different rams (ratio 1/15). A group of 23 sheep were not mated and were excluded from the trial. The parturition occurred between mid-March and the end of April 2016.A group of 24 sheep lambed in the first week and were considered separately in statistical analysis.

Measurements and samplings in period 2

Samples of feeds were collected each month for subsequent chemical analysis. Body weight (BW) and body condition score (BCS) were monitored weekly until 4 months of lamb age and then every 21 days. During the last 4 months of pregnancy the sheep were kept indoor and BW and BCS were measured every 15 and 7 days respectively. Body weight and BCS were measured as in period 1.

2.1.3 Period 3: Lactation

After parturition all lambs were separated from their mothers to focus on the mother lactation avoiding the effects of suckling. The ewes were milked twice a day (8:00 a.m. and 6:00 p.m.).

Only 74 sheep completed the period 3 of the trial and were milked for approximately 165 days of lactation until the dry off. Two ewes showed a constant milk production lower than 0.5 kg/d from the first control to the end of lactation and were excluded as outliers.

Lactation diets

Lactation diets were described in detail Chapter 3.

For the first 15 days from lambing: all the sheep were fed with a transition diet with a proportion of 50:50 concentrate to forage ratio based on alfalfa hay and a concentrate with average content of starch and fiber in respect to experimental treatments (Table 1).

From 15 to 55 DIM: the whole group of sheep was fed with a TMR and during milking, a commercial mix was also offered in the dose of 160 g/d per head. Both the TMR and the mix were characterized by high starch content in order to supply a high glucogenic diet to support the lactation peak.

From 56 to 165 DIM: the sheep were assigned to two different groups resulting 36 fed diet F and 38 fed diet S in the mid to late lactation. Almost an equal number of animals, with glucogenic and lipogenic nutritional background, were included in the lactation groups. In fact, taking into account the diet of prenatal life (period 1) and growing phase (period 2), ewes of the groups SS, SF, FS and FF, were randomly assigned either to S, or F,mid lactation diet (Figure 1; Table 2). It resulted in the creation of 8 groups of sheep, (SSS, SSF, SFS, SFF, FSS, FSF, FFS and FFF, as reported in Figure 1) homogeneous per milk yield, BW and BCS, and different for the dietary nutrition plan received from uterine life to first lactation. The letter (S or F) in first position indicates the diet (starch or fiber) fed during last 75 days of uterine life, in second position the diet (starch or fiber) fed during the growing phase and in third position the diet (starch or fiber) fed in mid lactation after 56 DIM. The S and F diets were offered *ad libitum*. Diet S was glucogenic, being rich in starch; diet F was lipogenic with low starch content (Table 1). Both diets S and F offered diets could be considered similar in crude protein (CP) (16.0% and 16.8% of CP) and different for their NFC level and composition, in particular starch content (32.0% and 26.6% of CP, for S and F respectively).

Measurements and samplings of period 3

Samples of experimental unifeed were collected monthly to obtain an average value of composition of the offered TMR. Milk yield was measured every 10 days twice a day and morning milk samples collected for analysis. Body weight (BW) was measured every 15 days and BCS was assessed each 7 with the procedure explained for periods 1 and 2.

2.2 Chemical analysis

Chemical analyses were carried out as described in detail in Chapter 3. The samples of feeds were analyzed for dry matter (DM) after drying at 105 °C for 24 hours. In particular, NDF, ADF, ADL, were analyzed using the filter bag equipment of Ankom (Ankom technology Corp., Fairport, NY; Mertens et al., 2002), for crude protein (CP) Kjeldahl method (A.O.A.C., 1990) for ash (A.O.A.C., 2000) and ether extract (EE) (A.O.A.C., 2005). NFC was calculated according to Weiss (1999) as follows: NFC (g/kg DM) =100-(NDF + CP + ash + EE).
Individual milk samples from the morning milking were analyzed for fat, protein, casein and lactose using a Milkoscan 6000 instrument (Foss Electric, Hillerød, Denmark), for SCC using a Fossmatic 360 instrument (Foss Electric) and for pH.

Fatty acids determination
The milk fat extraction and the fatty acid methylation were carried out as described in detail in Chapter 3 and following the method described by Nudda et al. (2005). The FA concentration of individual FA and that of groups of FA were expressed as g/100 g of total FAME. Groups of FA were calculated as follow and as listed in detail in methods of the Chapter 3.

2.3 Statistical analysis

Data of milk production and milk composition were separated in two datasets. The first included data from early lactation (from parturition to diet switch, 55 DIM; n = 72 sheep). The second one included data from mid to late lactation (from diet switch, 56 DIM to end of lactation, approximately at 165 DIM; n = 72 sheep). A third dataset was created to analyze the FPCMY production, during the entire lactation, of sheep that lambed in the same week (n = 24 sheep).The three datasets were analyzed separately with the PROC MIXED procedure of SAS (SAS version 9.2 SAS Institute Inc., Cary, NC; 2002) by applying the following linear mixed models 1 for the first dataset and 2 for the second and third datasets, respectively.

Model 1: $y_{ijklm} = \mu + dietpregrow_i + DIM_j + diet \times DIM_{(ij)k} + a_l + e_{ijklm}$

where:

- y_{ijkl} is the observed trait (i.e, milk yield, fat corrected milk, milk fat, etc);
- μ is the overall mean;
- $diet\ pre\ grow_i$ is the fixed effect of the ith diet in prenatal life and growing phase (i:4;1 = SS;2 = SF; 3 = FS; 4 = FF);
- dim_j is the fixed effect of the jth class of days in milk (DIM); (j=5);
- $diet\ pre\ grow \times DIM_{(ij)k}$ is the fixed effect of the kth interaction between diet prenatal life and growing phase, and class of DIM;
- a_l is the random effect of the animal (l = 72);
- e_{ijklo} is the random residual term.

Model 2: $y_{ijklmno} = \mu + diet\ pre\ grow_i + diet\ lact_j + diet\ pre\ grow \times diet\ lact_{(ij)k} + DIM_l + diet\ pre\ grow \times DIM_{(il)m} + diet\ pre\ grow \times diet\ lact \times DIM_{(ijl)n} + a_o + e_{ijklmno}$

where:

- $y_{ijklmno}$ is the observed trait (i.e, milk yield, fat corrected milk, milk fat, etc);
- μ is the overall mean;
- $diet\ pre\ grow_i$ is the fixed effect of the ith diet in prenatal life and growing phase (i:4; 1 = SS; 2 = SF; 3 = FS; 4 = FF);
- $diet\ lact_j$ is the fixed effect of the ith diet supplied in mid to late lactation (1: S, 2: F);
- DIM_l is the fixed effect of the lth class of days in milk (DIM); (l= 11 for second dataset and 16 for the third dataset);
- $diet\ pre\ grow \times diet\ lact_{(ij)k}$ is the fixed effect of the kth interaction between diet pre grow and diet lact;
- $diet\ pre\ grow \times DIM_{(il)m}$ is the fixed effect of the mth interaction between diet supplied during prenatal life and growing phase, and class of DIM;
- $diet\ pre\ grow \times diet\ lact \times DIM_{(ijl)n}$ is the fixed effect of the nth interaction between diet supplied during prenatal life and growing phase, diet supplied and class of DIM;
- a_o is the random effect of the animal (o= 72);
- $e_{ijklmno}$ is the random residual term.

The significance of group mean differences was assessed using Tukey Honestly Significant Difference (HSD; P < 0.05) for both models.

Milk fatty acids

Data of milk FA composition (n = 18 sheep) were analyzed by the PROC MIXED procedure of SAS (version 9.2; SAS Institute Inc., Cary, NC). The model was equal to model 1 but testing the effect of the prenatal diet (D; 2 levels: S = starch diet, F = fiber diet), instead of diet pre grow. The significance of group mean differences was assessed using Tukey (P < 0.05).

Multivariate analysis

Data from the experimental design including data from 72 animals that completed their first lactation and also from their mothers, were used to create a dataset summarizing the recorded animal performance. Considered the complex pattern of variables for the period 1,2 and 3, it was decided to carry out a multivariate factor analysis (MFA) in order to highlight the possible existence of latent factors that could be helpful to understand the relationships among variables and explain the observed variability. The MFA was carried out using the FACTOR procedure of SAS. The number of factors to be extracted (n = 6) was based on their readability in terms of biological relationship among variables and on the amount of explained variance. Factor readability was improved through a VARIMAX rotation. A variable was considered to be associated to a specific factor if the absolute value of its loading was ≥ 0.60, in accordance with Macciotta et al. (2015).

Individual factor scores were then calculated and analyzed as new variables with the following mixed linear model:

$$y_{ijklm} = \mu + dietM_i + dietG_j + dietL_k + dietM \times dietG \times dietL_{\,l} + L - type_m + e_{ijklm}$$

where:

-y_{ijkl} is the observed trait (i.e, the factor scores);

-μ is the overall mean;

-$dietM_i$ is the fixed effect of the ith diet of mothers (i: *1=S, starch diet; 2=F, fiber diet*);

-$dietG_j$ is the fixed effect of the jth diet during growing (j: *1=S, starch diet; 2=F, fiber diet*);

-$dietL_k$ is the fixed effect of the kth diet in lactation (*k*: *1=S, starch diet; 2=F, fiber diet*);

-$dietM \times dietG \times dietL_{\,l}$ is the fixed effect of the lth interaction between the three class effects;

-$L - type_m$ is the fixed effect of the mth type of lambing of the mothers (*m: 1= single; 2= twins*);

-e_{ijklo} is the random residual term.

When designs were unbalanced, due to different numbers of animals per treatment, the inclusion of Kenward-Roger correction was tested in the statistical model using the SAS statement “DDFM=KENWARDROGER”. It should be noticed that for the umbalanced designs both the models (with or without the inclusion of the Kenward-Roger correction) were tested, giving no difference of statistical results, in terms of significancy, for each considered variable.

3. RESULTS AND DISCUSSION

Animal performances of the ewes involved in this trial were analyzed mainly focusing the animal response to dietary treatments (S and F supplied from prenatal life to the end of first lactation) in terms of milk production and composition.

3.1 Offered diets

Prenatal and postnatal diets were very similar in terms of chemical composition and nutritive value (Table 1). Small differences in dietary energy content were attributable to the differences in NDF and composition of NFC in the 3 periods (Table 1). The forage concentrate ratio was approximately 45:55 in mother’s diets (prenatal life) and 40:60 in growing postnatal and mid lactation diets (Table 1). Since the differences between S and F were mainly based on the composition of the concentrate, thus a relatively low amount of forage was included in the diet aiming to maintain a constant exposure to the different carbohydrates during the whole experimental period. The diet used in prenatal life consisted of a high and low NDF content (37.0% and 46.5% of DM) in S and F diets

respectively; high and low content of NFC (42.4% and 31.7% of DM) in S and F diets respectively, corresponding to 26.7% and 10.7% of DM of starch, in S and F respectively. The differences were comparable to differences in NFC among dietary treatments reported in previous studies that tested effects of glucogenic and lipogenic diets on performance and metabolism of lactating sheep reported by Lunesu (2016). In particular the difference in NFC between low and high NFC groups in literature studies ranged from a minimum of 4% (from 39.9% and 35.9% of DM, respectively; Bovera et al., 2004), to a maximum of 15% (29.3% and 44.3% of DM, respectively; Zenou and Miron, 2005;Figure 5 in Chapter 1). These differences were maintained almost equal in postnatal growing (39.5% vs. 50.5% of NDF; 40.9% vs. 29.5% of NFC for S and F postnatal diets, respectively; Table 1).In mid lactation, from 56 to 165 DIM, the S and F diets offered to the experimental groups were different for their NDF (40.4% vs. 45.0% of DM, for S and F respectively) and their NFC content (32.0% vs. 26.6% of DM, respectively) in agreement with Bovera et al. (2004; NDF =38.6% vs. 40.8% of DM and NSC = 39.9% and 35.9% of DM for high and low NFC, respectively). The NFC composition was very different among S and F diets, in particular the differences in starch contents between S and F were higher than 10% of DM in each period (Table 1). Offered feed in the period 1 and 2 was proportional to the animal requirements of pregnancy and growth and animal intake was not reported in this work. For the lactation phase, the only one on which animals were fed *ad libitum*, intake was reported in the previous Chapter(Figure 1 of Chapter 3).

3.2 Milk production and composition, prenatal and postnatal effects on lactation performances.

The exposure to different carbohydrate sources did not significantly affect milk production and composition in early lactation (Table 2),nor in mid lactation (Table 3).

In early lactation milk production was, on average, very similar among the treatments SS, SF, FS and FF. From parturition to 55 DIM, SF and FF groups reached the lowest and highest average values of 1.57 and 1.77 kg/d per head, respectively (Table 2; P = 0.4). Similar differences and trends were observed among treatments for FPCMY (Table 2) where the SF and FF groups reached the lowest and highest average values of 1.38 and

1.48 kg/d per head, respectively (Table 2; P = 0.7). A significant effect of DIM was observed both for milk production (P < 0.001) and FPCMY (P < 0.005) for the increasing trend from parturition to the lactation peak (Figure 1 and 2). Milk components were significantly affected by the DIM (P < 0.05; Table 2) showing the typical trends. Oppositely, milk components were not affected by Diet or by the interaction among Diet ×DIM (P > 0.1; Table 2) except for milk urea. Milk urea was affected by diet (P = 0.01) and resulted significantly lower for FF (41.2 mg/dl) than SS (48.2 mg/dl) or SF (48.6 mg/dl) ewes,and numerically lower than FS (46.2 mg/dl) (Table 2). Considering that the dietary protein was similar among treatments (Table 1) and the animals were kept in only 2 pens on the basis of the 2 lactation diets, thus it might indicate that exposure to diet FF might have induced possible effects on the nitrogen use efficiency. It has been largely demonstrated that maternal diets have and strong impact on the microbial communities of the offspring which in turn might have permanent or long term impact on the host metabolism (Spor et al., 2011). Within the same project of this thesis, data on microbial communities that colonized the gastrointestinal tract of the animals of each group were collected but the information was not available at the moment.

From 56 to 165 DIM the milk production and component of SS, SF, FS, FF groups were not statistically different (P > 0.05; Table 3). FPCMY of the primiparous sheep included on the different groups were equal to 1.34, 1.29, 1.33 and 1.19 for SS, SF, FS and FF ewes included in the respective treatment group of prenatal and growing phases. A significant effect of DIM was observed (P < 0.001; Table 3) for all the considered variables of milk production and components whereas the interaction between Diet × DIM did not significantly affected the animal production performances (P = 0.6; Table 3). The milk lactose and NaCl were affected by the interaction between Diet × DIM (P < 0.001; Table 2) in agreement with the involution of the mammary gland in late lactation (Pulina and Nudda 2001). Furthermore, milk lactose tended to be lower in the FF than in other groups (P < 0.06; Table 3) whereas the NaCl tended to be higher in FF than in other groups (P = 0.06; Table 3) indicating a possible worst status of the mammary gland in animals that were exposed to F and FF diet in early life which also showed the fastest decline of milk production from 56 to 165 DIM (Figure 1 and 2). This evidence is consistent with the other observed patterns and, on the best of our knowledge; it has not been previously documented in literature.

Looking at the lactation patterns reported in Figure 1 indicating the first lactation performances in terms of FPCMY of the S and F groups (animals that were fed a different prenatal diet pooled in respect to the diets they were fed in growing and mid lactation), ewes exposed to S evidenced higher numerical yields than ewes exposed to F in early lactation and lower values in mid to late lactation (Figure 1). When lactation performances of the SS, SF, FS and FF groups (animals that were fed a different prenatal and growing diet pooled in respect to their mid lactation diet; Figure 2) it was possible to highlight that SS, SF and FS showed a very similar pattern of FPCMY production during the whole lactation, whereas the FF group showed slightly average values of FPCMY in early lactation and a marked decline of FPCMY from mid to late lactation (Figure 2) was observed. These shapes of lactation curve were different among groups but these differences were not statistically appreciable in the milk records, probably due to the high variability observed within groups (Table 2 and 3). More focus on metabolic patterns and microbial adaptation to the experimental diets could help to understand the individual variability that characterized their first lactation performances. That focus was not included in this research. Possible explanation could be adduced by deeply investigatingthe effects of preweaning growth and postnatal development (Bach et al., 2012; Poore et al., 2014; Rey et al., 2014; Khanal and Nielsen, 2017). Within the growing period, it is difficult to distinguish between dietary effects that affected the postnatal growing and the late part of the first gestation, that usually influence the sequent lactation(Blair et al., 2010; Poore et al., 2014).

3.3 Milk production and composition, prenatal and postnatal effects on carbohydrate response in mid lactation.

The use of different carbohydrates in mid lactation did not significantly affect the animal response in terms of milk production performances (Table 4; $P > 0.1$). From 56 to 165 DIM groups SS, SF, FS, FF were split in two groups each and fed a different mid lactation diet (SSS, SSF; SFS, SFF; FSS, FSF; FFS and FFF) and their lactation performances were not affected by Diet ($P > 0.1$; Table 4). A significant effect of DIM was observed for milk yield and components within each group ($P < 0.001$; Table 4). A already observed in Table 2 and 3 a significant effect of the interaction between Diet

×DIM ($P < 0.001$; Table 2) was observed for milk lactose and milk urea that were lower for the FFS and FFF than other groups and the values of NaCl that was higher for FFS and FFF than in other groups especially in late lactation.

Milk production, FPCMY and milk composition were on average very similar for all the diet histories of the experimental trial, and only numerical differences were observed among these variables (Table 4). In particular, FPCMY ranged from 1.32 to 1.33 kg/d per head in SSS, SFF, FSS, FSF, reached the highest value of 1.37 kg/d per head in the group SSF, and lower values of 1.26, 1.23, and 1.14 kg/d per head in SFS, FFS and FFF, respectively (Table 4). Differently, differences of about 0.1 kg/d of FPCMY per head in average of the mid lactation period resulted significant in previous studies (Bovera et al., 2004; Cannas et al., 2004; Lunesu 2016).

The lactation patterns of the 8 groups with different diet histories were, on average trends, extremely different (Figure 3). The highest persistency was observed in the SSF group and the lowest one in the FFF group (Figure 3).

It has to be highlighted that, all dietary combinations of prenatal and growing phases with exception of the FF (SS, SF, FS), showed high persistent pattern and high production at 165 DIM when associated to a diet F in mid lactation (SSF, SFF, FSF) than when associated to a S mid lactation diet (SSS, SFS, FSS) (Figure 3).

Considering all the 8 dietary histories and groups, the trends observed in this experiment were not clearly defined and quite difficult to explain. To simplify, Figure 4 reported the FPCMY of groups that were exposed to the same diet, both in prenatal and growing periods (SS and FF), but either to S or F diet in mid lactation (SSS, SSF, FFS and FFF). It is possible to see how, from mid to late lactation, the glucogenic feeding sequence SS, seems to induce a high persistent pattern and an apparent better response to diet F (SSF) compared to diet S (SSS) and FFS or FFF combinations (Figure 4).

In addition, both FFS and FFF developed a reduction in FPCMY production yet in early lactation even before the diet switch at 55 DIM (Figure 4). Then, continuing from 56 to 165 DIM, FFF ewes showed a more pronounced decline in FPCMY than FFS ewes (starch) (Figure 4). This variability was not explained by the experimental factors included in the statistical analyses and only effects of DIM within each group were observed ($P < 0.001$) (Table 4). Metabolic information from blood parameters and perhaps information from microbial population at ruminal level might be involved in

physiological mechanisms that created the observed variability. This kind of information was not included in this work and blood and microbial data are only partially available at the moment. Metabolic adaptations to carbohydrate exposure early in life might have negatively influenced the metabolic programming or the mammary gland of the ewes inducing changes in the animal performances. From this point of view, several studies in literature demonstrated that glucose metabolism and microbial imprinting could be strongly affected by diet experienced early in life (Caricilli and Saad, 2013) but without evidences in respect to dietary carbohydrates. Khanal and Nielsen (2017) reviewed several dietary factors related with metabolic programming and observed that exposure to lipogenic diets in prenatal life was associated to impaired glucose metabolism and development of insulin resistance. Furthermore, Abecia et al., (2017)demonstrated that that pre-weaning diet might cause permanent changes in the microbial environment of ruminants without change in animal performances.

However, analyzing the production performance of a smaller group of animals included in the same SSS, SSF, FFS and FFF groups (n = 24; corresponding to the ewes that firstly lambed in the same period of 10 days in order to avoid the effect of DIM from the milk sampling) a significant effect of the interaction among mid lactation diet (S vs. F) and prenatal and growth diets (SS vs. FF) was observed ($P < 0.05$, Table 5; Figures 5 and 6).In particular, from 55 to 160 DIM, the ewes fed SS (thus having a glucogenic background), showed higher FPCMY if fed F (SSF) than fed S (SSS) in mid lactation (1.483 vs. 1.231 kg/d, respectively; $P < 0.05$). In fact, the lactation curve of SSF showed evident higher FPCMY persistency than SSS (Figure 5). Oppositely, the ewes fed FF (thus having a lipogenic background), showed similar performances in terms of FPCMY persistency either if fed with F or S in mid lactation(FFS and FFS; Table 5; Figure 6).

Thus, the use of digestible fiber in mid lactation was significantly associated to maintain FPCMY only in sheep that experienced glucogenic diets early in life (Table 5). In addition the animal response to mid lactation diet (S or F) was evident only after 80 DIM approximately (Figure 4 and 5). FPCMY at 155 DIM was equal to 1.242 vs. 0.886 kg/d ($P<0.05$) for SSF vs. SSS ewes, and 0.947 vs. 0.770($P>0.80$) for FFS and FFF ewes, respectively. These findings are in agreement with the fact that the response to mid lactation diet could be highly dependent from other environmental factors including lactation stage and metabolic status. Considering the delayed lambing of several animals,

possible confounding effects of milk sampling days and DIM could have influenced the experimental records. In particular, the mid to late lactation period of the experimental flock included end of spring and summer time, from May to end of August, with several episodes of heath stress which might have contributed to increase the observed individual variability (Peana et al., 2010). In addition, as well known, heath stress is more detrimental in diets based on fiber than on starch (Stott et al., 1960; Peana et al., 2010).
In terms of lactation shape, results and production patterns observed in this study were in agreement with previous literature indicating high persistent lactation curves (either numerical or statistically significant) in sheep fed diets based on soluble fiber from mid to late lactation (Cannas et al, 2004; Bovera et al., 2004; Lunesu, 2016). However, an adequate modeling approach should be applied in order to test the statistical significance of the graphical indications (Macciotta etal., 2005).
Large variability on milk production was observed within each group and large percentage of the data variability was explained by the individual effect included in the statistical models (unreported data). Considering that the ewes were included in the experimental trial since their prenatal life, these findings might suggest that other factors than dietary carbohydrates might play the fine-tuning of metabolic programming in dairy sheep. In particular, several authors demonstrated that overnutriton and undernutrition during fetal life could impair the metabolic adaptation of the animals in postnatal life and lactation (Husted et al, 2008) and have important consequences on lamb performance in terms of growth, immune system and reproduction and production (Ashwort et al., 2009; Kenyon et al., 2014). Several metabolic factors, stimulated early in life, have been demonstrated to change the metabolic responses with permanent later consequences. Prenatal overnutriton can lead to develop obesity in the offspring, even with changing feeding supply and diet characteristics in adult life (Taylor and Poston, 2007). Overnutriton can also cause overweight in primiparous offspring in respect to underweight (Van Der Linden et al., 2009). Otherwise overfeeding or underfeeding pregnant ewes altered colostrum quality and quantity and in turn reduced offspring birth weight (Swanson, et al., 2008; Kenyon et al., 2014). Colostrum quality and preweaning growth rate have been positively associated to first lactation milk yield (Bach et al., 2012). In this data analysis we did not account of other factors than experimental diets. Literature evidences suggest that dietary effects can be hidden by several metabolic

interferences caused by nutritional, genetic and environmental aspects throughout epigenetic changes (Milagro et al., 2013). The most of the work focused on feed restriction or overfeeding (Kenyon et al., 2014) whereas few evidences were published on dietary effects (Nieelsen et al., 2013). Lipogenic diets rich in fat, fed to pregnant sheep demonstrated ability to induce insulin resistance in offspring and to impair their glucose and insulin metabolism (Nielsen et al., 2103). Prenatal malnutrition differentially programmed glucose –lactate metabolic pathways and cholesterol homeostasis generally predisposing animals to hyperglycemia and hyperlactatemia and to hypercholesterolemia when combined with obesogenic fat diets (Khanal et al., 2014). The same authors highlighted how postnatal nutrition exposure to high fat diets induced hyperlipidemia and reduced glucose clearance and pancreatic insulin response and delayed insulin sensitivity (Khanal et al., 2014; Kongsted et al., 2014). In this experiment a deep investigation of individual animal growth might help to explain the observed variability before to get metabolic and microbial information.

3.4 Carbohydrate exposure early in life and milk fatty acids

Table 6 reported the means of the concentrations of individual FA and groups of FA of animals, allocated to two groups based on the different prenatal diets (S or F diet of the mothers), during the experimental period (mid to late lactation after the dietswitch), and the effects of the diets, sampling dates and their interaction on these traits. In general, the main effect that influenced the concentration of FA was found to be the DIM; this result has been discussed in the Chapter 2. Except for few minor FA the prenatal diet (diet-P) had no effect ($P > 0.05$) on the milk FA composition of ewes considered in the present experiment. Similar results were observed for the effect of the interaction between the diet of the mother and DIM (Diet-P × DIM), where the only important significant effect was observed for the rumenic acid (RA, C18:2 c9,t11), the most abundant conjugated linoleic acid (CLA) isomer. The evolution of this FA, in milk of animals born from mothers fed starch and fiber diets, during the experiment is reported in Figure 7. During early lactation and after the diet-switch, ewes fed F in prenatal period had a higher level of RA than these fed S; from this point the two concentrations seems to become similar as lactation proceeds. Rumenic acid is a very important FA in the milk of ruminants, from a

nutritional and healthy point of view, since it has exhibited important health properties as anticarcinogenic and anti-inflammatory activities (Sofi et al., 2010). It can be found in high concentration only in ruminant milk, as it originates from the biohydrogenation of PUFA operated by ruminal microorganisms. The biohydrogenation process produces RA but also vaccenic acid (VA) that is partially converted in RA by the delta-9 desaturase activity in mammary gland (Griinari et al., 2000; Bernard et al., 2008). The milk concentration of RA depends on the amount of PUFA in the diet of ewes, on the biohydrogenation activity of ruminal microorganisms and on the activity of the delta-9 desaturase in mammary gland (Griinari and Bauman, 2000). Overall, all these variables are usually influenced by the diet. Since in early lactation animals of the two groups were fed the same diet, and were kept in identical conditions, it could be concluded that this effect should be ascribed to the different diets of the mothers. During pregnancy, the diet of mother could have affected the ruminal environment of the new born, the proportions between the different bacterial species and, consequently, the biohydrogenation processes. Otherwise, the effect could be directed on the activity of the desaturase enzyme in mammary gland. Even though this last hypothesis should be excluded, as the other FA produced from the desaturase activity (all *cis*-9 monounsaturated FA) did not differ between the 2 groups ($P > 0.05$; Table 6 and 7), suggesting that the expression and activity of the enzyme was not affected by the mother's diet, on the other hand, a change in microorganism environment between groups should have influenced the concentration of OBCFA, which are of microbial origin FA. The difference in RA concentration between the two groups decrease as lactation proceeds; this result may be ascribed to a possible confounding effect of the two dietary treatments: in fact, at the switch diet, part of the animals born from mothers fed starch diet were fed fiber diet and the other continued to be fed starch diet; the same occurred with the animals born from mothers fed fiber diet. This aspect could be deeply investigated by increasing the analyzed samples in order to consider the interaction among prenatal diet and mid lactation diet.

3.5 Multivariate analysis

Multivariate analysis allowed reducing the information provided by the experimental trial and focusing the main factors related to the animal variability. Six extracted latent factors were able to explain the 70% of the total variance (Table 8) from the correlation matrix of the 30 considered variables. The adequacy of data set used for the factor analysis, was evaluated by calculating the Kaiser Measure of Sampling Adequacy (Kaiser MSA); a value of 0.73 was obtained in the present work, not much different from the value of 0.80 which is assumed to be the empirical threshold that flags a dataset as particularly suitable for multivariate factor analysis (Cerny and Kaiser, 1977). Almost all the investigated variables presented a loading values ≥ 0.60, which represents the threshold to consider a variable to be associated to a specific factor (Macciotta et al., 2015) and to the meaning explained by each factor. Four variables showed values from 0.54 to 0.59 and one presented value < 0.50 (BCS of mothers in mid lactation; -0.48); however, it was possible to associate these variables to a latent factor, considering their biological meanings. Only one variable couldn't be related to a specific factor, due to its very low loading values (≤ 0.31).

The first latent factor (Factor 1) was positively correlated with variables linked to milk production and DM intake. This factor, that was named "Feed efficiency", explained the 26% of the total variance. This factor included variables mainly related with the lactation period (Period 3; Table 8). The strong relationship between milk production and dry matter intake explains high part of variability of livestock performances, as widely recognized.

The second, third and fourth factors were all correlated with variables of BCS and body weight during the lifetime and at different phases of growing (Table 8). In particular the Factor2 was associated to the post pubertal and pubertal growth, from 10 to 15 months; the Factor3 was correlated with measures of BCS and BW of animals at pre pubertal stages (from 3 to 6 months); whereas the Factor4 was related to pre-weaning period, that was 60 days long in the experimental trial, with measures of BW at birth day, at 1 and 2 months (Table 8). These phases have strong importance for mammary development of the mammary gland.

The allocation of these groups of measures into three different latent factors seems to suggest that three important phases can differently influence the lamb performance. These phases are deeply discussed as important for lamb performances and metabolic programming in the reviews of Ashwort et al. (2009) and Kenyon et al. (2014).

The fifth factor was correlated to some measures related to the characteristics of the mothers. In particular, weight and BCS during pregnancy and the total weight of lambs were positively associated to this factor.

The sixth factor was correlated with the average milk production (positive loading) and with the BCS (negative loading) of the mothers, during pregnancy. It could indicate possible effects of nutrient partitioning of their mothers on fetal programming.

Considering the large individual variability observed in lactation performances of the experimental animals, several key experiences in young life (including undernutrition or overnutriton in uterine life and growing when forages were offered per group) might have impaired the metabolic response to carbohydrates in first lactation and induced large part of lactation variability.

Overall the extracted factors used as new variables were slightly affected by the considered effect (Table 2). Only the 4th and 5th factors were influenced by the diet of the mothers and by the mother's type of lambing. In particular, higher scores for Factor4 (Figure 8) and Factor 5 (Figure 9) were detected for animals whose mothers were fed starch diet, compared to those fed fiber diet. Regarding the type of lambing, animal born as single birth had higher scores for Factor4 and lower for Factor5, compared to animals born from twin birth. The interaction between the dietary treatments during different stages of the trial was not effective ($P > 0.05$) in influencing the new variables. It has to be noticed that gender and type of lambing (single vs. twins) explains high variability in animal performances and it is often related to differential response to dietary and other environmental factors (Kenyon et al., 2014; Nielsen et al., 2014).

4. CONCLUSIONS

Dietary carbohydrates, used in different sequences, seem to not significantly affect lactation performances of Sarda primiparous dairy sheep. With a deep analysis, it was possible to deduce that dietary treatments might have induced changes in the animal response which were partially hidden by confounding effects that increased the variability of the animals within groups. A significant effect was observed with ewes that were homogenous for lambing date. It suggests that dietary carbohydrates used in mid lactation might have a mediated or subordinated role, in respect to other environmental or physiological factors acting on the animal response in the short or long term. Large variability of the dataset was associated to animal weight and BCS in well defined growing phases (birth, pre weaning, pre pubertal and post pubertal). Further investigations should consider a deep focus on animal performances in these phases in order to explain the differential response of the animals to dietary treatments.

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6. TABLES AND FIGURES

Table 1. Chemical composition of the offered experimental diets in the last two months of prenatal life (last 75 days of gestation), growing phases (from weaning to first lambing) and lactation (from lambing until 160 days).

Offered diets	Prenatal diet		Growing diet		Mid to late lactation diet[1]	
Item	Diet S	Diet F	Diet S	Diet F	Diet S	Diet F
Dry matter, % of as fed	88.6	88.0	88.5	88.3	89.8	89.9
Crude protein, % of DM	14.6	14.8	16.0	15.5	16.0	16.8
NDF, % of DM	37.0	46.5	39.5	50.5	40.4	44.9
ADF, % of DM	25.9	37.8	27.2	37.3	27.3	32
ADL, % of DM	2.42	2.69	2.25	2.62	5.6	4.9
Ether extract, % of DM	2.09	2.26	2.04	2.15	1.6	1.5
Ash, % of DM	9.76	10.75	7.505	8.33	10.0	10.2
NFC, % of DM	42.4	31.7	40.9	29.5	32.0	26.6
Starch, % of DM	26.7	10.7	25.5	9.8	24.3	12.7
Forage: concentrate ratio	45/55	45/55	40/60	40/60	38/62	38/62
[2]NEL (SRNS)	1.47	1.40	1.55	1.42	1.52	1.43

[1]TMR was fed ad libitum; during milking each ewe received 160 gr/d of DM of pelleted mix (17% of CP, 24% of NDF, 4% of EE and 9% of ashes).[2]NEL = Net energy content fo the diet estimated with the model Small Ruminant Nutrition System (Tedeschi et al., 2010).

Table 2. Effect of prenatal-growing diet, class of DIM and their interaction on daily individual milk production and composition, during early lactation (from lambing to 55 DIM).

		Prenatal and growing diets				SEM	P value		
item	UM	SS n = 18	SF n = 19	FS n = 18	FF n = 17		Diet	DIM	Diet×DIM
Milk	kg/d	1.64	1.57	1.66	1.77	0.02	0.412	<0.001	0.306
FPCMY	kg/d	1.39	1.38	1.41	1.48	0.02	0.739	0.005	0.355
Milk fat	%	5.16	5.34	5.05	5.05	0.04	0.190	<0.001	0.377
Milk protein	%	4.83	5.03	4.97	4.86	0.03	0.373	0.013	0.811
Lactose	%	5.05	5.05	4.96	4.96	0.02	0.462	0.025	0.127
Milk solids	%	13.37	13.90	13.24	12.86	0.10	0.122	<0.001	0.541
Casein	%	3.66	3.82	3.75	3.65	0.02	0.275	0.005	0.826
Urea	mg/dl	48.16^{a}	48.61^{a}	46.19^{ab}	41.20^{b}	0.62	0.013	<0.001	0.271
NaCl		131.34	130.94	138.01	151.72	2.43	0.157	0.000	0.044

a-b different superscript letters within the same row indicate significant differences ($P < 0.05$).
The sequence of letters indicates the nutritional plan received by each group in the whole experiment. Letters sequences (SS, SF, FS, SS) referred to animal groups: the first letter (S or F) indicates the diet (starch or fiber) received during last 75 days of uterine life, the second letter the diet (S or F) received during the growing phase. In this phase all the animals received the same diet rich in starch (Table 1).DIM = Days in milk; FPCMY = fat and protein corrected milk

Table 3. Effect of prenatal-growing diet, class of DIM and their interaction on daily individual milk production and composition, during mid to late lactation (from dietary switch at 56 DIM to end of lactation art approximately 165 DIM).

		Prenatal and growing diets				SEM	P value		
item	UM	SS n = 18	SF n = 19	FS n = 18	FF n = 17		Diet	DIM	Diet×DIM
Milk	kg/d	1.42	1.37	1.42	1.26	0.02	0.698	<0.001	0.531
FPCMY	kg/d	1.34	1.29	1.33	1.19	0.02	0.640	<0.001	0.573
Milk fat	%	6.16	6.09	6.07	5.98	0.03	0.825	<0.001	0.424
Milk protein	%	5.46	5.50	5.57	5.61	0.02	0.598	<0.001	0.987
Lactose	%	4.85	4.82	4.79	4.60	0.02	0.058	<0.001	0.000
Milk solids	%	16.41	15.86	16.20	15.88	0.16	0.940	<0.001	0.889
Casein	%	4.14	4.17	4.21	4.22	0.02	0.816	<0.001	0.878
Urea	mg/dl	43.99	44.29	42.89	40.77	0.37	0.431	<0.001	<0.001
NaCl		143.97	148.07	150.03	177.57	2.16	0.060	<0.001	<0.001

The sequence of letters indicates the nutritional plan received by each group in the whole experiment. Letters sequences (SS, SF, FS, SS) referred to animal groups: the first letter (S or F) indicates the diet (starch or fiber) received during last 75 days of uterine life, the second letter the diet (starch or fiber) received during the growing phase. In this phase, within each group, data from animal receiving (starch or fiber) in mid lactation have been pooled. DIM = Days in milk; FPCMY = fat and protein corrected milk

Table 4. Effect of diet history (in terms of exposure to the type of carbohydrates from prenatal life to mid lactation), class of DIM and their interaction, on daily individual milk production and composition, during mid to late lactation (from dietary switch at 56 DIM to end of lactation art approximatively 165 DIM).

		Diet history								SEM	P value		
item	UM	SSS n = 10	SSF n = 8	SFS n = 10	SFF n = 9	FSS n = 9	FSF n = 9	FFS n = 8	FFF n = 9		Diet	DIM	Diet×DIM
Milk	kg/d	1.42	1.43	1.35	1.40	1.41	1.43	1.31	1.22	0.02	0.980	<0.001	0.166
FPCMY	kg/d	1.32	1.37	1.26	1.33	1.32	1.33	1.23	1.14	0.02	0.962	<0.001	0.163
Milk fat	%	6.01	6.33	6.06	6.12	6.14	6.00	6.01	5.95	0.03	0.912	<0.001	0.404
Milk protein	%	5.31	5.63	5.50	5.49	5.61	5.51	5.55	5.66	0.02	0.467	<0.001	0.097
Lactose	%	4.81	4.90	4.76	4.88	4.78	4.80	4.61	4.59	0.02	0.302	<0.001	0.002
Milk solids	%	16.91	15.93	16.23	15.45	16.08	16.25	15.70	16.05	0.16	0.990	<0.001	0.565
Casein	%	4.01	4.29	4.16	4.18	4.25	4.16	4.18	4.26	0.02	0.495	<0.001	0.019
Urea	mg/dl	43.37	44.85	43.63	44.98	41.20	44.52	40.75	40.80	0.37	0.780	<0.001	0.001
NaCl		153.81	132.45	156.21	139.35	149.37	151.42	176.65	178.49	2.16	0.241	<0.001	0.001

The sequence of letters indicates the nutritional plan received by each group in the whole experiment. Letters sequences (SSS, SSF, FSS, FSF, SFS, SFF, FFS and FFF) referred to animals groups: the first letter (S or F) indicates the diet (starch or fiber) received during last 75 days of uterine life, the second letter the diet (starch or fiber) received during the growing phase and the third letter the diet (starch or fiber) received in mid lactation after 56 DIM. DIM = Days in milk; FPCMY = fat and protein corrected milk

Table 5. Effect of diet history (in terms of exposure to the type of carbohydrates from prenatal life to mid lactation), class of DIM and their interaction, on daily individual milk production and composition, during mid to late lactation (from dietary switch at 56 DIM to end of lactation at approximately 165 DIM). In the table are reported the lactation performances of the animals which had lambing on the same week (n=24).

Prenatal and growing diet		Glucogenic (SS)			Lipogenic (FF)				*P value*		
Mid lactation diet		Starch n = 6	Fiber n = 6	All n= 12	Starch n = 6	Fiber n = 6	All n=12				
Whole diet history	UM	SSS	SSF	Mean	FFS	FFF	Mean	SEM	Prenatal and growth	Mid lactation	Diet history ×DIM
Sheep	n.	6	6	12	6	6	12	24	12	12	24
Milk	kg/d	1225	1.61	1434	1.37	1.25	1.31	0.06	0.560	0.08	0.01
Fat	%	6.10	5.96	6,02	5.91	6.04	6.0	0.08	0.689	NS	0.321
Protein	%	5.59	5.45	5.51	5.43	5.69	5.56	0.05	0.456	NS	0.047
Lactose	%	4.75	4.95	4,86	4.78	4.51	4,65	0.04	0.001	NS	0.01
FPCMY	kg/d	1.23	1.48	1.38	1.27	1.20	1.23	0.04	0.05	0.07	0.01

Letters sequences (SS; SF; FS; FF; SSS, SSF, FSS, FSF, SFS, SFF, FFS and FFF) referred to animals groups: the first letter (S or F) indicates the diet (starch or fiber) received during last 75 days of uterine life, the second letter the diet (starch or fiber) received during the growing phase and the third letter the diet (starch or fiber) received in mid lactation after 56 DIM. DIM = Days in milk; FPCMY = fat and protein corrected milk

Table 6. Effect of prenatal diet (starch, S; fiber, F), class of DIM and their interaction on milk fatty acids, during the first lactation of primiparous dairy sheep. In this analysis, within each group, data from animal receiving (starch or fiber) in lactation were pooled.

Trait[1]	Prenatal diet		SEM	P-Values		
	S n = 9	F n = 9		Diet-P	S	Diet-P×S
SFA						
C4:0	2.52	2.37	0.033	0.223	<0.001	0.635
C6:0	2.04	1.98	0.022	0.484	<0.001	0.659
C8:0	2.08	2.05	0.025	0.771	<0.001	0.104
C9:0	0.12	0.12	0.005	0.839	<0.001	0.557
C10:0	7.82	7.88	0.089	0.897	0.000	0.019
C11:0	0.33	0.38	0.007	0.106	0.070	0.535
C12:0	0.23	0.29	0.076	0.541	0.094	0.010
C13:0 iso	0.02	0.02	0.001	0.835	0.000	0.876
C13:0 anteiso	0.06	0.07	0.002	0.045	0.002	0.836
C14:0 iso	0.12	0.11	0.003	0.510	0.040	0.695
C14:0	12.15	12.13	0.139	0.979	<0.001	0.595
C15:0 iso	0.25	0.24	0.006	0.774	<0.001	0.724
C15:0 anteiso	0.50	0.46	0.009	0.313	0.001	0.733
C15:0	1.11	1.10	0.017	0.741	0.004	0.598
C16:0 iso	0.32	0.30	0.009	0.606	<0.001	0.370
C16:0	28.33	28.51	0.364	0.921	<0.001	0.621
C17:0 iso	0.44	0.42	0.008	0.617	0.004	0.655
C17:0 anteiso	0.54	0.53	0.009	0.814	0.001	0.631
C17:0	0.83	0.84	0.009	0.799	<0.001	0.939
C18:0 iso	0.06	0.06	0.002	0.543	0.027	0.934
C18:0	7.91	6.68	0.182	0.192	0.002	0.215
C20:0	0.25	0.24	0.005	0.507	0.173	0.933
C22:0	0.14	0.14	0.002	0.625	0.386	0.839
C23:0	0.07	0.07	0.002	0.624	0.249	0.966
C24:0	0.06	0.05	0.001	0.452	0.461	0.646
C25:0	0.02	0.02	0.000	0.992	0.148	0.500
C26:0	0.02	0.02	0.001	0.628	0.040	0.881
MUFA						
C10:1	0.03	0.05	0.002	0.001	<0.001	0.003
C14:1 c9	0.22	0.29	0.008	0.058	<0.001	0.853
C15:1	0.10	0.11	0.004	0.925	<0.001	0.304
C16:1 t4	0.04	0.05	0.002	0.764	<0.001	0.105
C16:1 t5	0.03	0.03	0.001	0.891	<0.001	0.222
C16:1 t6+t7	0.06	0.06	0.002	0.878	0.001	0.225
C16:1 t9	0.08	0.10	0.003	0.236	0.254	0.075
C16:1 t10	0.02	0.02	0.001	0.744	0.734	0.429
C16:1 t11+t12	0.05	0.05	0.001	0.825	0.000	0.273
C16:1 c7	0.24	0.23	0.003	0.640	0.003	0.302
C16:1 c9	0.85	1.07	0.025	0.079	<0.001	0.566
C16:1 c10	0.03	0.03	0.001	0.338	0.002	0.439
C16:1 c11	0.02	0.02	0.001	0.006	0.001	0.647
C17:1 c6-7	0.06	0.05	0.001	0.783	0.015	0.195
C17:1 c8	0.02	0.02	0.001	0.685	0.145	0.863
C17:1 c9	0.22	0.23	0.003	0.180	0.002	0.225
C18:1 t4	0.02	0.03	0.001	0.112	0.096	0.406
C18:1 t5	0.02	0.03	0.001	0.005	0.098	0.334
C18:1 t6+t8	0.25	0.28	0.007	0.385	<0.001	0.718
C18:1 t9	0.26	0.28	0.007	0.222	<0.001	0.330
C18:1 t10	0.57	0.64	0.024	0.385	<0.001	0.792
C18:1 t11	1.39	1.51	0.041	0.514	<0.001	0.117

Table 6. Continue from the previous page. Effect of prenatal diet (starch, S; fiber, F), class of DIM and their interaction on milk fatty acids, during the first lactation of primiparous dairy sheep. In this analysis, within each group, data from animal receiving (starch or fiber) in lactation were pooled.

Trait[1]	Prenatal diet		SEM	P-Values		
	S n = 9	F n = 9		Diet-P	S	Diet-P×S
C18:1 t12	0.32	0.33	0.008	0.800	<0.001	0.406
C18:1 t13+t14	0.52	0.50	0.013	0.651	<0.001	0.223
C18:1 c9	14.10	14.22	0.289	0.861	0.153	0.983
C18:1 c11	0.48	0.49	0.007	0.755	0.028	0.662
C18:1 c12	0.35	0.36	0.009	0.765	<0.001	0.268
C18:1 c13	0.07	0.08	0.001	0.226	0.002	0.509
C18:1 t16+c14	0.28	0.27	0.005	0.493	<0.001	0.010
C18:1 c16	0.04	0.04	0.001	0.355	0.022	0.668
C20:1 c9	0.02	0.02	0.001	0.851	0.163	0.688
C20:1 c11	0.06	0.06	0.001	0.729	0.003	0.779
C20:1 c15	0.02	0.03	0.001	0.059	0.022	0.252
C22:1 n-9	0.02	0.02	0.001	0.787	0.340	0.749
C24:1 c15	0.02	0.02	0.000	0.641	0.273	0.965
PUFA						
C18:2 t10t14	0.04	0.04	0.001	0.665	<0.001	0.299
C18:2 t11t15	0.02	0.03	0.001	0.056	<0.001	0.271
C18:2 t9t12	0.03	0.03	0.001	0.278	0.208	0.710
C18:2 c9t13	0.21	0.24	0.005	0.169	<0.001	0.481
C18:2 t8c13	0.11	0.12	0.003	0.321	<0.001	0.714
C18:2 c9t12	0.11	0.11	0.002	0.256	<0.001	0.144
C18:2 t9c12	0.04	0.04	0.001	0.413	0.013	0.944
C18:2 n-6	3.04	3.18	0.058	0.682	<0.001	0.393
C18:2 t12c15	0.04	0.05	0.001	0.011	<0.001	0.938
C18:2 c12c15	0.01	0.01	0.000	0.014	0.251	0.031
Δ7,9 17:2	0.02	0.02	0.000	0.153	0.019	0.198
CLA c9t11	0.75	0.90	0.021	0.059	<0.001	0.001
CLA t9c11	0.09	0.09	0.001	0.733	0.014	0.363
CLA t10c12	0.01	0.01	0.000	0.652	0.206	0.953
CLA t12t14	0.01	0.01	0.000	0.624	0.419	0.337
CLA t11t13	0.02	0.02	0.001	0.982	0.513	0.934
C20:2 n-9	0.02	0.02	0.001	0.478	<0.001	0.718
CLA t9t11	0.02	0.02	0.001	0.587	0.159	0.017
C20:2 n-6	0.04	0.04	0.001	0.463	0.037	0.813
C22:2 n-6	0.04	0.04	0.001	0.690	0.075	0.387
C18:3 n-6	0.09	0.09	0.003	0.789	0.000	0.173
C18:3 n-3	0.33	0.33	0.007	0.924	<0.001	0.678
C20:3 n-9	0.05	0.04	0.001	0.144	0.001	0.268
C20:3 n-6	0.05	0.04	0.001	0.785	0.022	0.230
C20:3*	0.01	0.01	0.000	0.852	0.246	0.199
C20:3 n-3	0.01	0.01	0.000	0.981	0.021	0.711
C22:3 n-6	0.01	0.01	0.000	0.694	0.575	0.822
C18:4 n-3	0.01	0.01	0.000	0.574	0.000	0.749
C20:4 n-6	0.21	0.24	0.004	0.392	0.692	0.307
C20:4 n-3	0.01	0.01	0.000	0.290	0.097	0.630
C22:4 n-6	0.04	0.05	0.001	0.197	0.267	0.312
EPA	0.02	0.03	0.000	0.604	0.336	0.869
DPA	0.06	0.07	0.001	0.279	0.227	0.223
DHA	0.02	0.03	0.001	0.701	0.709	0.472

[1]*c* = *cis*; *t* = *trans*.

Table 7. Effect of prenatal diet (starch, S; fiber, F), class of DIM and their interaction on the groups and ratios of milk fatty acids, during the first lactation of primiparous dairy sheep. In this analysis, within each group, data from animal receiving (starch or fiber) in lactation were pooled.

Trait[1]	Prenatal diet		SEM	P-Values		
	S n = 9	F n = 9		Diet-P	S	Diet-P×S
Groups and ratios						
SCFA	14.66	14.48	0.135	0.765	<0.001	0.134
MCFA	52.10	52.75	0.511	0.745	<0.001	0.779
LCFA	33.24	32.76	0.502	0.800	<0.001	0.840
SFA	73.15	72.05	0.362	0.380	<0.001	0.819
UFA	26.74	27.84	0.361	0.379	<0.001	0.825
MUFA	20.90	21.60	0.323	0.456	0.004	0.946
PUFA	5.84	6.24	0.093	0.456	<0.001	0.269
TFA	4.56	4.87	0.101	0.460	<0.001	0.541
CLA	0.90	1.05	0.022	0.092	<0.001	0.002
OBCFA	4.80	4.74	0.054	0.804	0.000	0.518
n-6	3.52	3.69	0.062	0.655	<0.001	0.426
n-3	0.47	0.48	0.008	0.858	0.000	0.744
de novo	43.52	43.84	0.348	0.829	0.001	0.382
n6/n3	7.45	7.65	0.047	0.402	<0.001	0.147
TFA-VA	3.17	3.36	0.067	0.453	<0.001	0.829
SCFA/LCFA	0.45	0.46	0.008	0.764	0.148	0.818
SFA/UFA	2.78	2.67	0.052	0.462	0.002	0.973
de novo/LCFA	1.34	1.40	0.031	0.578	0.000	0.866

[1]SCFA = short chain fatty acids, sum of the individual fatty acids from C4:0 to C10:0; MCFA = medium-chain fatty acids, sum of the individual fatty acids from C11:0 to C17:0; LCFA = long-chain fatty acids, sum of the individual fatty acids from C18:0 to DHA; SFA = saturated fatty acids, sum of the individual saturated fatty acids; UFA = unsaturated fatty acids, sum of the individual unsaturated fatty acids; MUFA = monounsaturated fatty acids, sum of the individual monounsaturated fatty acids; PUFA = polyunsaturated fatty acids, sum of the individual polyunsaturated fatty acids; n-6 = sum of individual n-6 PUFA; n-3 = sum of individual n-3 PUFA; TFA = trans fatty acids, sum of the individual trans fatty acids; CLA = sum of individual conjugated of linoleic acids; OBCFA = odd- and branched-chain fatty acids, sum of individual odd- and branched-chain fatty acids; *de novo*, sum of the fatty acids synthetized de novo in the mammary gland (from C6:0 to C16:0); ratios between some groups of FA were also calculated: n-6/n-3; SCFA:LCFA, SFA:UFA and *de novo*:LCFA

Table 8. Rotated factor pattern and variable communality (Commun) in the dataset of 72 ewes including information from their prenatal life to end of first lactation.

	Extracted Factors						Commun
Item	Factor1	Factor2	Factor3	Factor4	Factor5	Factor6	
Average FPCMY	**0.88**	0.01	0.11	-0.01	-0.11	0.37	0.93
Milk yield in early lactation	**0.86**	0.03	0.08	-0.05	-0.08	0.42	0.93
Milk yield mid-late lactation	**0.83**	-0.06	0.14	0.14	-0.18	-0.23	0.82
FPCMY in mid-late lactation	**0.81**	-0.11	0.09	0.15	-0.15	-0.28	0.80
DM Intake early lactation	**0.77**	-0.07	0.12	0.19	0.12	-0.13	0.74
DM intake mid-late lactation	**0.74**	0.28	-0.14	-0.02	0.18	-0.26	0.69
Milk yield at130-170 DIM	**0.71**	0.05	0.07	-0.07	-0.03	0.55	0.82
Average DMI	**0.59**	0.38	-0.21	0.24	0.14	0.19	0.65
BW postpartum	0.06	**0.84**	0.08	0.31	0.13	0.13	0.85
BW mating	0.09	**0.79**	0.40	0.26	0.10	-0.12	0.89
BCS pre partum	-0.09	**0.74**	0.18	-0.08	-0.26	0.14	0.69
BW pre partum	0.12	**0.74**	0.32	0.36	0.21	-0.01	0.83
BCS post partum	-0.02	**0.68**	0.17	-0.05	-0.41	0.32	0.77
BW puberty	0.04	**0.66**	0.54	0.28	0.09	-0.07	0.82
BCS mating	0.05	**0.65**	0.11	-0.45	0.07	-0.07	0.65
BCS 6 months	-0.07	0.28	**0.84**	0.07	0.03	-0.12	0.81
BCS 4 months	0.10	0.11	**0.82**	0.07	-0.16	0.02	0.72
BCS 3 months	0.15	0.16	**0.72**	-0.06	-0.14	0.19	0.63
BW 6 months	0.09	0.45	**0.68**	0.39	0.06	-0.05	0.83
BW 4 months	0.22	0.34	**0.56**	0.53	-0.10	-0.06	0.77
BCS puberty	-0.10	0.53	**0.54**	-0.31	-0.02	-0.11	0.68
BW 1 month	0.05	0.00	0.14	**0.86**	-0.04	0.00	0.77
BW birth	0.15	0.16	-0.15	**0.74**	-0.03	-0.12	0.64
BW 2 months	0.11	0.08	0.41	**0.73**	-0.05	0.24	0.78
Weight gained in pregnancy	0.18	0.10	-0.19	-0.02	**0.71**	-0.06	0.59
BCSgained in pregnancy	-0.09	0.03	0.24	-0.15	**0.70**	0.19	0.61
Litter weight of the mother	-0.20	-0.01	-0.17	-0.03	**0.58**	-0.10	0.41
Litter weight	-0.01	-0.22	-0.03	0.15	0.31	0.25	0.23
Milk yield of the mother	0.01	0.19	-0.09	0.06	-0.03	**0.65**	0.47
BCS mother in mid lactation	0.03	0.04	-0.04	0.03	-0.04	**-0.48**	0.24
EIGENVALUE	7.84	4.81	2.66	2.29	1.86	1.57	
Var. explained (%)	26.13	16.04	8.88	7.63	6.22	5.23	

DMI : dry matter intake; DIM = Days in milk; FPCMY = fat and protein corrected milk

Table 9. Effect of diet received in prenatal life (diet of the mother), during postnatal growing, and lactation, their interaction and type of lambing of the mother on the 6 extracted factors

Item	P-*Values*				
	Diet-M	Diet-G	Diet-L	M×G×L	L-Type
Factor1	0.481	0.206	0.719	0.571	0.126
Factor2	0.298	0.783	0.207	0.368	0.576
Factor3	0.178	0.514	0.158	0.794	0.367
Factor4	**0.027**	0.069	0.407	0.117	**0.044**
Factor5	**0.040**	0.441	0.511	0.525	**<0.001**
Factor6	0.262	0.262	0.310	0.962	0.488

1: p-value of the fixed effect of Diet-M = dietary treatment of the mothers, Diet-G = dietary treatment of the growing lambs, Diet-L = dietary treatment of the lactating ewes, M×G×L = interaction among the 3 diet effects, L-Type = effect of the mother's type of lambing.

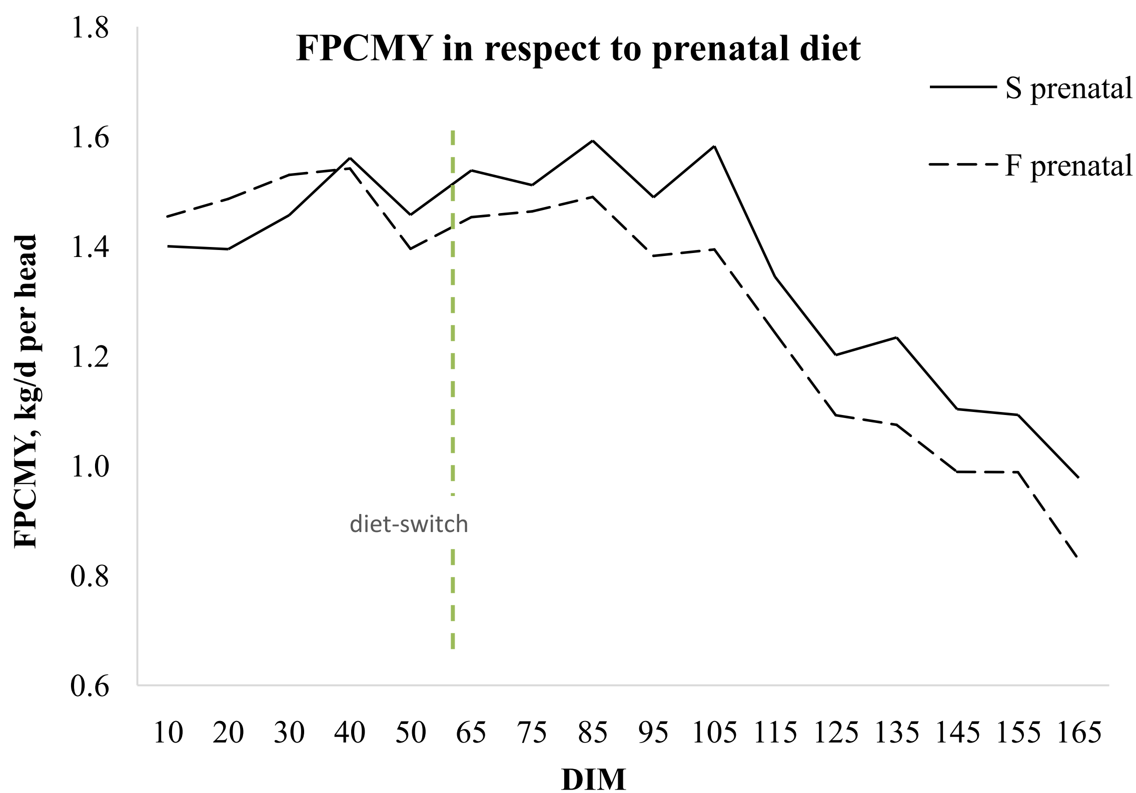

Figure 1. Evolution of fat and protein corrected milk (FPCMY) during lactation in the animal groups that were exposed to a diet rich in starch (S) or fiber (F) in last 75 days of uterine life. Data of animals (n =72) were plotted in respect to the prenatal diet (period 1) without considering dietary treatments of Period 2 and 3. DIM: days in milk.

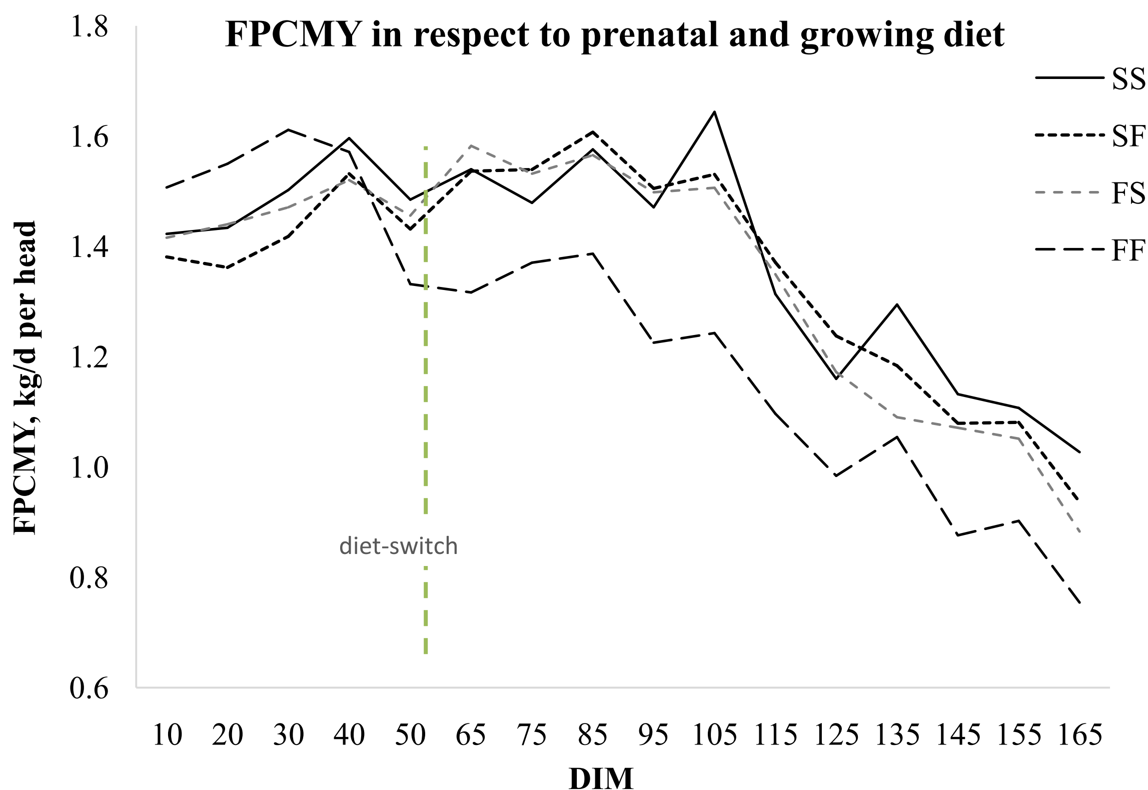

Figure 2. Evolution offat and protein corrected milk (FPCMY) during lactation in the prenatal-growing groups. Letters sequences (SS; SF; FS; FF) referred to animals groups: the first letter (S or F) indicates the diet (starch or fiber) received during last 75 days of uterine life, the second letter the diet (starch or fiber) received during the growing phase. Data of animals (n =72) were plotted in respect to the prenatal and growing diets (period 1+2) without considering dietary treatments of Period 3.DIM = Days in milk.

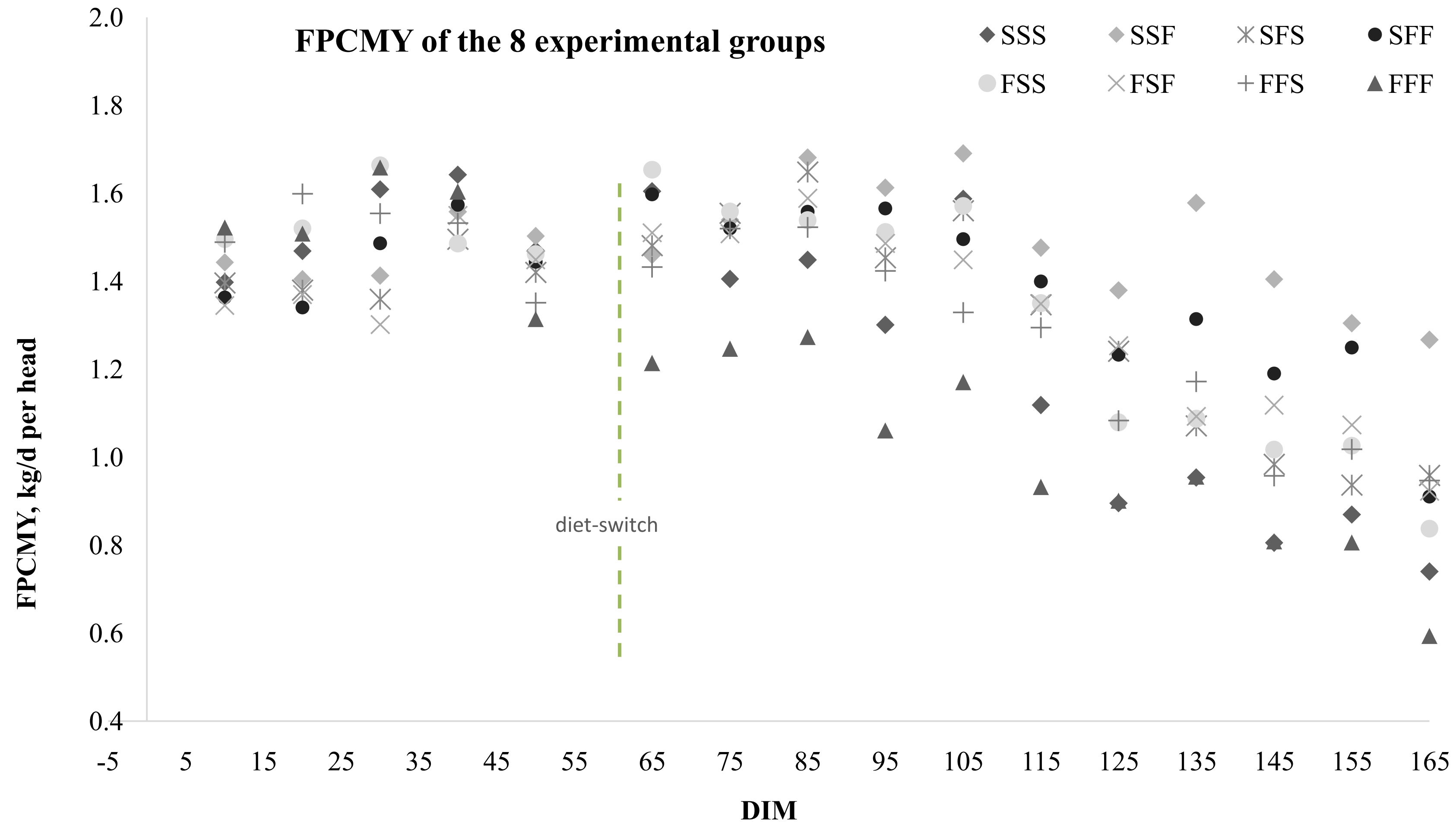

Figure 3. Evolution of fat and protein corrected milk (FPCMY) during lactation in the 8 groups of period 3 (n =72). Letters sequences (SSS, SSF, FSS, FSF, SFS, SFF, FFS and FFF) referred to animals groups: the first letter (S or F) indicates the diet (starch or fiber) received during last 75 days of uterine life, the second letter the diet (starch or fiber) received during the growing phase and the third letter the diet (starch or fiber) received in mid lactation after 56 DIM. DIM = Days in milk.

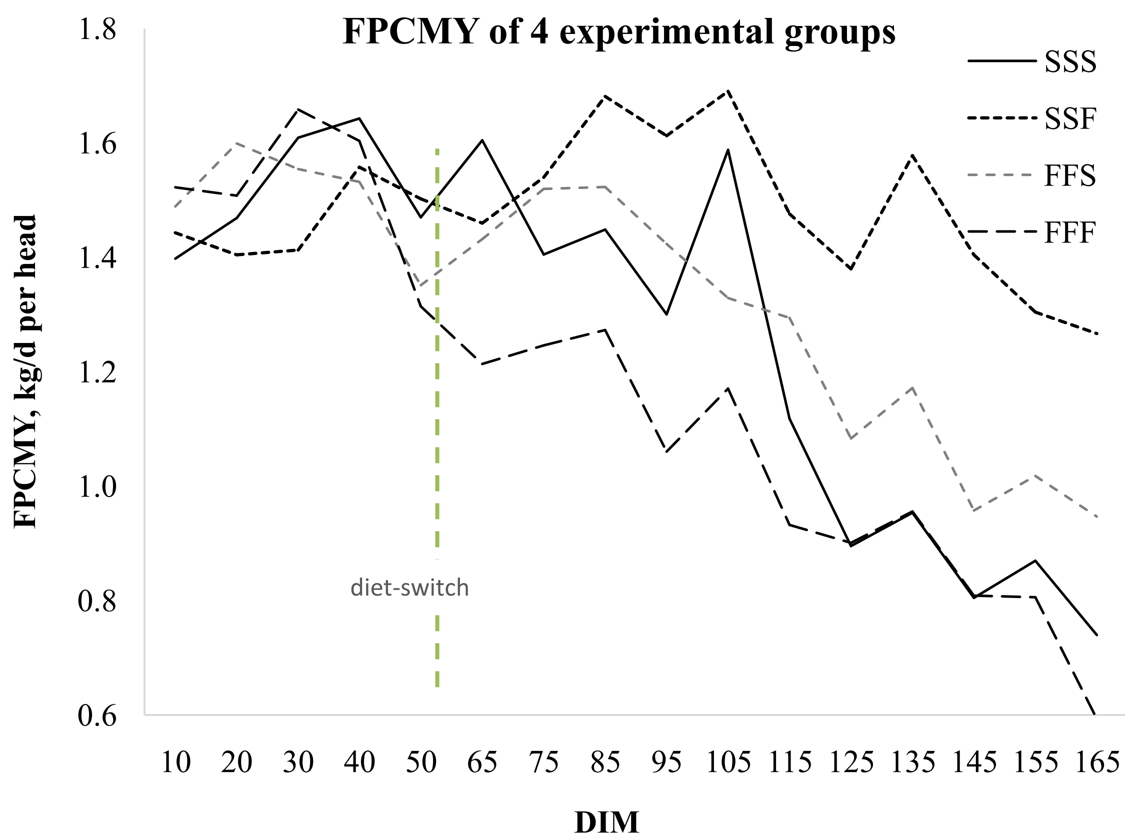

Figure 4. Evolution of fat and protein corrected milk (FPCMY) during lactation in only 4 groups of period 3(n= 35). Letters sequences (SSS, SSF, FSS, FSF, SFS, SFF, FFS and FFF) referred to animals groups: the first letter (S or F) indicates the diet (starch or fiber) received during last 75 days of uterine life, the second letter the diet (starch or fiber) received during the growing phase and the third letter the diet (starch or fiber) received in mid lactation after 56 DIM. DIM = Days in milk.

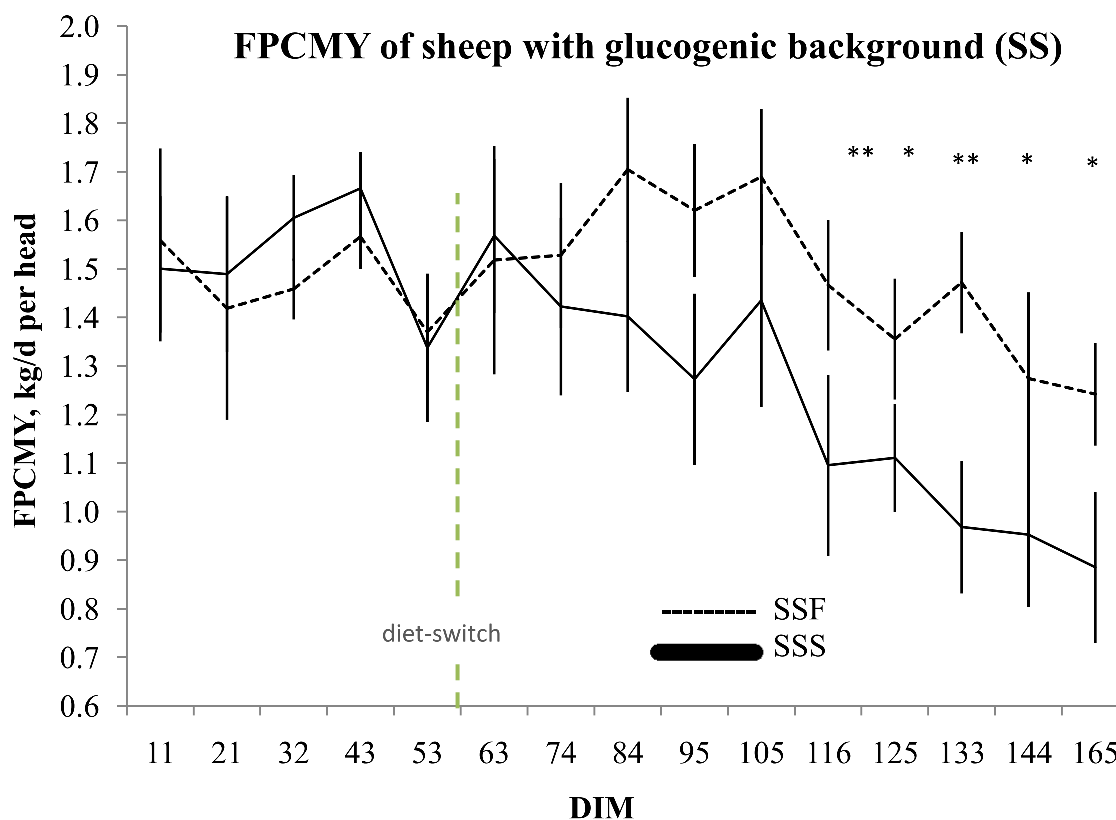

Figure 5. Production of fat and protein corrected milk (FPCMY) during the first lactation of the experimental ewes SS (n= 12; ewes with glucogenic background; the letter (SS) indicates the diet (starch) received during last 75 days of uterine life, and during the growing phase. In the first 55 days of lactation all the groups were fed with the glucogenic (S) diet then from 56 to 165 DIM with starch (SSS) or fiber (SSF) DIM. * and ** indicates that observed FPCMY of the two groups were significantly different for P<0.1 and P<0.05.DIM: days in milk.

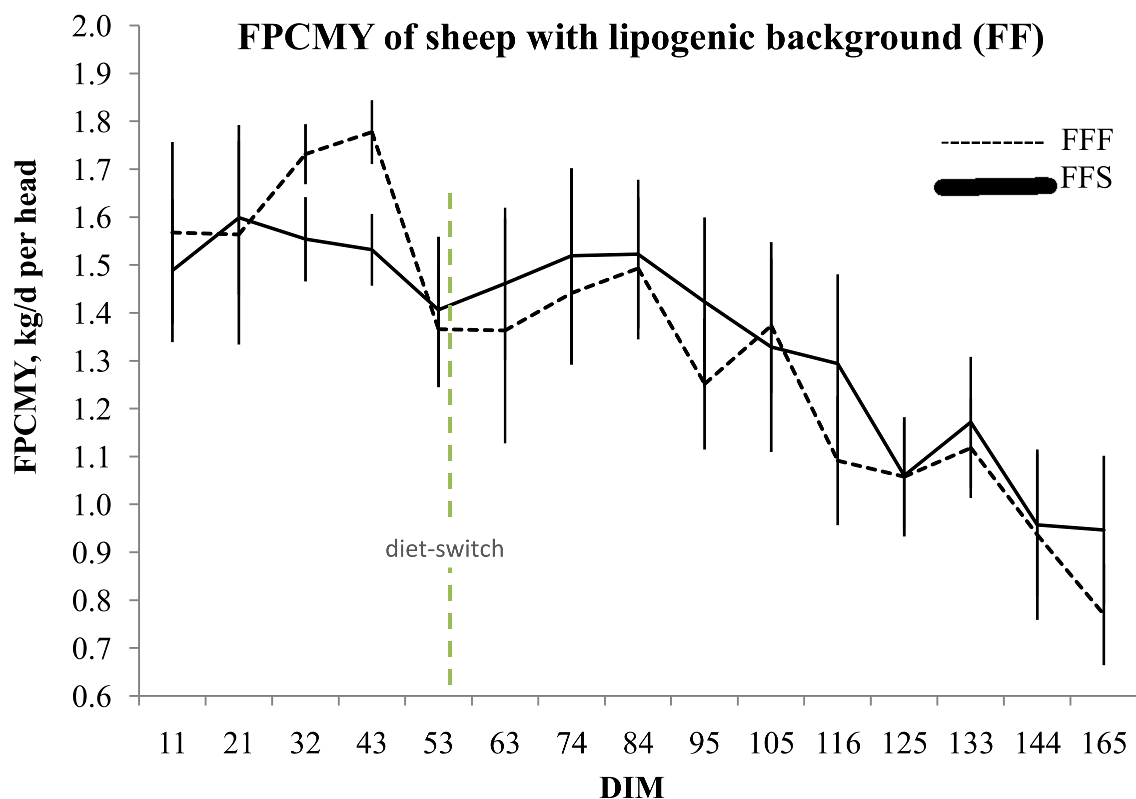

Figure 6. Production of fat and protein corrected milk yield (FPCMY) during the first lactation of the experimental ewes FF (n = 12; ewes with lipogenic background; the letter (FF) indicates the diet (fiber) received during last 75 days of uterine life, and during the growing phase). In the first 55 DIM all the groups were fed with the glucogenic (S) diet then from 56 to 165 DIM with starch (FFS) or fiber (FFF).DIM: days in milk.

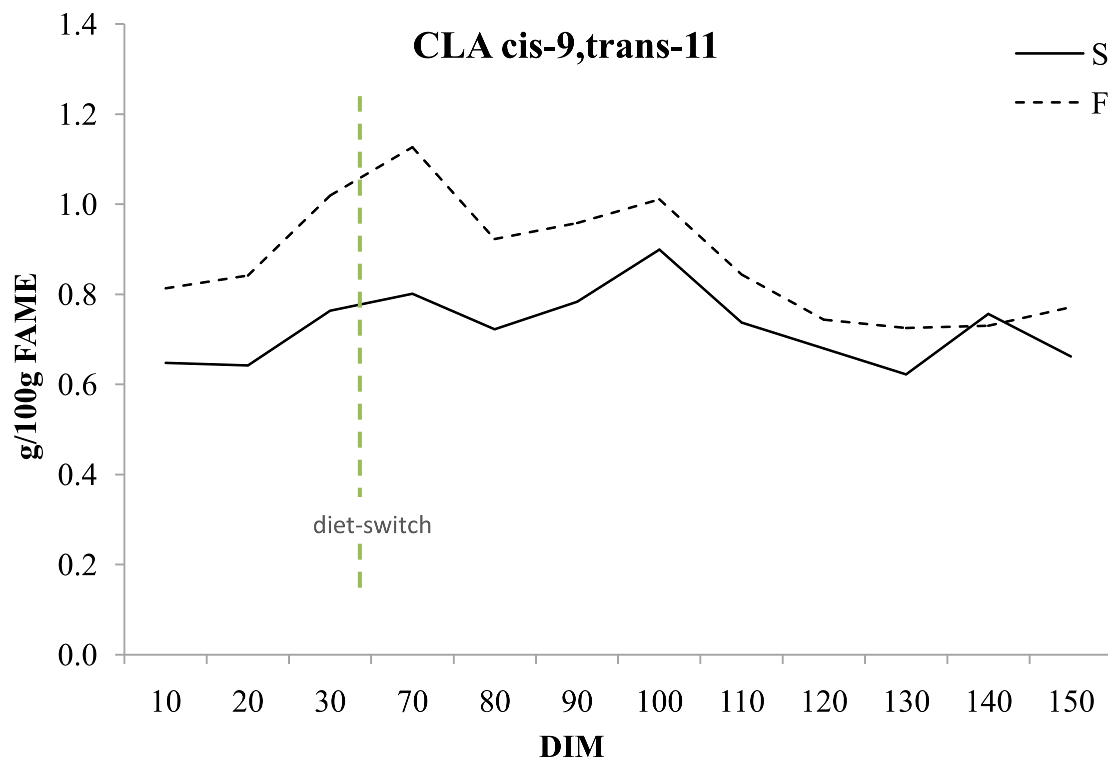

Figure. 7. Evolution of rumenic acid (CLA cis-9, trans-11) concentration in milk of ewes exposed to starch (S) or fiber (F) diets during their uterine life.

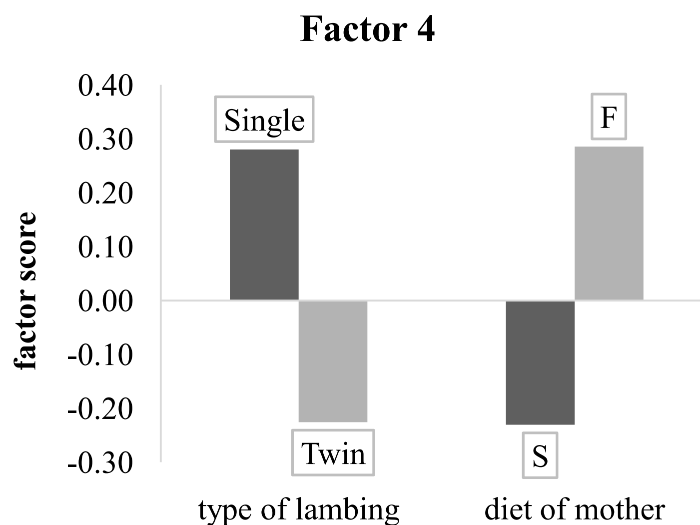

Figure 8. Effect of type of lambing (single or twin) and diet of mothers (starch or fiber, S or F) on the 4th latent factor.

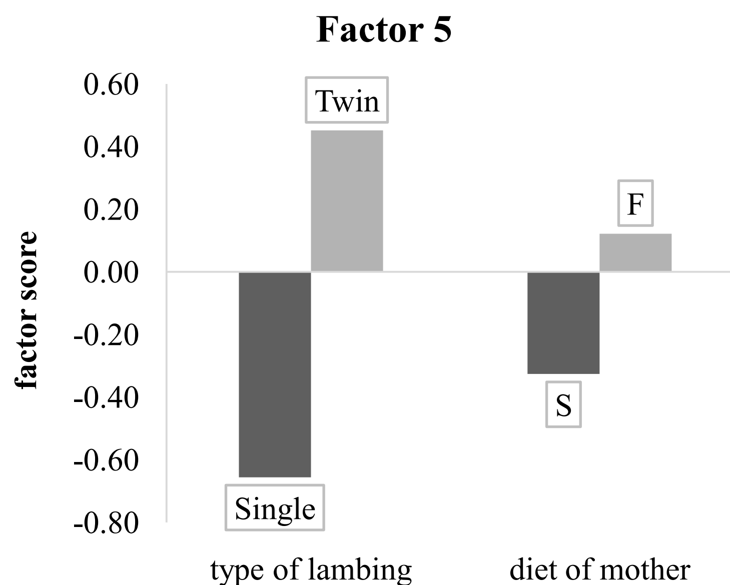

Figure 9. Effect of type of lambing (single or twin) and diet of mothers (starch or fiber, S or F) on the 5th latent factor.

CHAPTER 5

Effect of glucogenic and lipogenic carbohydrates, supplied during uterine life, post-natal growing and first lactation, on glucose and insulin tolerance tests in Primiparous Sarda ewes

ABSTRACT

The aim of this work was to evaluate if the exposure to different carbohydrates in early stage of life (prenatal life and post-natal growing) (PGdiet) and then in mid lactation (Ldiet) could affect glucose and insulin metabolism in dairy ewes, both during pregnancy and first lactation. A flock of dairy sheep was divided in two groups and fed with two diets, being one rich in starch (S) and one rich in soluble fiber (F). Their offsprings were in turn split in two groups and randomly assigned either to S or F until first lambing. Only lambs that followed the same diet of the mother were considered: i) FF (fiber-fiber); and ii) SS (starch-starch) ewes fed both during pre-natal life and during growing with high fiber or starch diets, respectively; (PGdiet). The two groups were further split, from 56 to 165 days of the first lactation (DIM), and randomly assigned either to S or F, mid lactation diet. From the split were obtained 4 groups (SSS, SSF, FFS and FFF) indicating with the first two letters the diet received from prenatal life until first lambing, and with the third letter the mid lactation diet.

During pregnancy a glucose tolerance test (GTT) was performed in 10 pregnant primiparous Sarda ewes (PREG; 23 days before parturition (DBP)). Ewes belonged to SS (n = 5) and FF diets (n = 5).

During lactation, glucose and insulin metabolism were evaluated through a GTT and an insulin tolerance test (ITT). The GTT and ITT were performed in 12 mid lactating primiparous Sarda ewes (LACT; 96 and 121 days in milk (DIM); respectively). The ewes beloged to 4 respective with different nutritional history for carbohydrate exposure (FFF, n= 3; SSS, n = 3, FFS, n = 3; SSF; n= 3).

For GTT a glucose solution (50%) was infused at a dose of 1 ml/kg of BW into the jugular vein of each animal before the morning meal. Blood samples were taken at following time points: -15, + 5, + 10, + 15, + 30, + 45, + 90 and + 180 min after glucose infusion. For ITT, insulin was infused at a dose of 0.15 IU/kg of BW into the jugular vein, 1 hour after the morning meal. Blood samples were collected 10 min before the morning

meal, - 15 and - 5 min before insulin injection and + 2.5, + 5, + 10, + 20, + 30, + 60 min after insulin injection. Area under the curve (AUC), fractional turnover rate (k) and half time (T ½) were calculated. Insulin sensitivity and insulin resistance were estimated through a quantitative insulin check index (QUICKI) and a homeostasis model assessment (HOMA).Data were analyzed by using a PROC MIXED procedure of SAS with repeated measures. Experimental results showed that SS based-PGdiet increased basal plasma insulin in PREG ewes during GTT (mean ± standard error of the mean (SEM)) 1.09 vs. 0.53 ± 0.18 µg/L; SS vs. FF diets; respectively; P=0.019) and plasma insulin in LACT ewes during ITT (12.47, 12.08, 8.94, 11.40µg/L; SSS, SSF, FFS and FFF diets; respectively; P=0.042). The F based-Ldiet positively affected (P=0.027) plasma glucose in LACT ewes during GTT (212.8 vs. 207.5 mg/dl; FFF and FFS diets; respectively; 219.11 vs. 139.33 mg/dl; SSF vs. SSS diets; respectively).

For both tests, AUC, k, T ½, HOMA and QUICKI did not differ between the SS and the FF diets in PREG ewes and among the SSS, SSF, FFS and the FFF diets in LACT ewes. It could be concluded that, the type of dietary carbohydrates offered during pre and postnatal life seems to influence glucose and insulin metabolism in the offspring. Starch rich-diets offered during pre-natal and early post-natal life (SS) increased basal blood insulin and seem to induce insulin resistance in ewes both during pregnancy and during lactation. Whereas high fiber rich diet offered during pre-natal and early post-natal life (FF) seems to induce more insulin sensitivity during lactation. Fiber rich-diets offered in mid lactation positively affected glucose concentration. However, glucose and insulin evolution in GTT and ITT could have been influenced by pregnancy and lactation requirements. Additional studies are needed to *i)* better understand the complex mechanisms involved in the transmission of information from the mother to the offspring *ii)* improve GTT and ITT both in pregnant and in lactating animals.

Key words: pre-natal diet, post-natal diet, fiber, starch, ewes, insulin sensitivity, insulin resistance.

1. INTRODUCTION

The effect of nutritional status, during pre and post-natal life, on the influence of metabolic status of adult offspring has been documented in humans (Elahi et al, 2009; Hanley et al., 2010; Godfrey et al., 2011; Milagro et al., 2013; Smith and Ryckman, 2015), rats (Samuelsson et al., 2008; Zeisel, 2009) and ruminants (Poore et al., 2013; Khanal et al., 2015).

Exposure in fetal life or in the first phases of postnatal life to excesses of dietary fat, undernutrition and overnutriton are commonly related to higher risk of development of insulin resistance status and obesity (Duque-Guimarães and Ozanne, 2013), lower glucose tolerance, altered lipid and glucose metabolism both in monogastric and in ruminant species (Parlee et al., 2014). As suggested by Paily et al. (2013), women who are overweight before conception period, during and after pregnancy, are associated to weight gain in their offspring.

Intense activity research is focusing on these mechanisms in humans to overcome and prevent obesity problems and encompassing multidisciplinary approaches including social aspects related to income and dietary habits (Duque-Guimaraes and Ozanne, 2013), microbiological environment (Clemente et al., 2012; Shen et al, 2013), genetics (Milagro et al., 2013), physiological and health features (Lawrence et al., 2008; Catalano et al., 2009).

As pointed by Arabin and Stupin (2014), "*the time before, during and after pregnancy should be viewed as a window of opportunities to minimize the short- and long-term health risks for women and their children at an early stage in their life span*".

In ewes, overnutrition of the mother during pregnancy seems to be associated to lower weight gain and glucose tolerance in their adult offspring (Ford et al. 2007; Tygesen et al., 2007); in addition, it impairs pancreatic insulin secretion capacity (Gardner et al., 2005; Husted et al., 2007; 2008). The prenatal nutrition also appears to influence reproductive (Rhind et al., 2001) and productive performances (Van Der Linden et al., 2009). In fact, maternal overnutrition status favored milk yield in their offspring (1st lactation), in contrast to that observed for offspring born from underweight mothers (Van Der Linden et al., 2009).

If the pre-natal and post natal diet can influence animal performances in adult life, it will be very interesting to investigate if the type of dietary carbohydrates (fiber or starch) supplied in prenatal life, growing and lactation can influence glucose and insulin metabolism in ewes during the first pregnancy and lactation. Regarding the influence of diet on glucose and insulin metabolism, it seems to be clearerin non-ruminant species compared to ruminants. It is well known that in monogastric species high carbohydrates diets (cereals-based diet) increase glycemia values and insulin concentration. In lactating animals high sensitivity to insulin of the adipose tissue and high basal glucose levels have been correlated with decreases of milk production due to the competition for nutrients between mammary gland and adipose uptake for reserves (Baumgard et al., 2017). In this sense the use of glucose tolerance test (GTT) and insulin tolerance test (ITT) might be useful to get information on glucose metabolism and insulin sensitivity of the animals that were exposed to nutritional and environmental factors that could have modified their glucose metabolism (De Koster and Opsomer, 2013; Khanal et al., 2016) or the priorities of their homeorhetic regulation of nutrient partitioning (Baumgard et al., 2017). From this point of view the aim of this work was to test if the carbohydrates-based diet (starch or fiber) offered during pre-natal life, growing and lactation could affect glucose and insulin metabolism in pregnant and primiparous Sarda ewes and indirectly affect their lactation persistency.

2. MATERIALS AND METHODS

The experiment was conducted at the private farm located in the north of Sardinia (Porto Torres, Sassari, Italy).

2.1.Experimental procedure

Glucose and insulin metabolism were evaluated in pregnant (PREG, n=10) and mid lactating (LACT, n=12) primiparous Sarda ewes. In particular, glucose tolerance test (GTT) was performed in PREG and LACT animals, whereas insulin tolerance test (ITT) was performed only in LACT animals.

During pregnancy, 10 animals at 23 days before parturition (DBP) were selected from two larger flocks fed a starch-starch (SS; fed a starch diet both during pre-natal life and during growing (PGdiet); BW: 49.83 kg; BCS: 3.00) and fiber-fiber (FF; ewes fed a fiber diet both during pre-natal life and during growing (PGdiet): BW: 49.0 kg; BCS: 2.88) diets according to the description reported in the Chapter 2.

During lactation, 12 mid lactating ewes at 96 and at 121 days in milk (DIM) were involved in a GTT and ITT, respectively. They were selected from 2 larger flocks fed a starch or fiber diet during mid lactation (Ldiet) and divided in 2 subgroups in relation to the pre-natal and growing diet (PGdiet) as follow: 1) starch-starch-starch group (SSS): 3 ewes fed a starch diet during pre-natal life + growing (PGdiet) and in mid lactation (Ldiet) (GTT, milk yield: 2.07 kg/d per head; BW: 48.2 kg; BCS: 2.8; ITT, milk yield:1.87 kg/d per head; BW: 50.13 kg; BCS: 2.81; respectively); 2) starch-starch-fiber group (SSF): 3 ewes fed a starch diet during pre-natal life + growing (PGdiet) and a fiber diet during mid lactation (Ldiet) (GTT, milk yield:1.91 kg/d per head; BW: 46.0 kg; BCS: 2.8; ITT, milk yield:1.62 kg/d per head; BW:47.7 kg; BCS:2.75; respectively); 3) fiber-fiber-starch group (FFS): 3 ewes fed a fiber diet during pre-natal life + growing (PGdiet) and a starch diet during mid lactation (Ldiet) (GTT, milk yield:1.86 kg/d per head; BW: 47.2 kg; BCS: 2.8; ITT, milk yield:1.55 kg/d per head; BW:47.47 kg; BCS: 2.79; respectively) and 4) fiber-fiber-fiber group (FFF): 3 ewes fed a fiber diet during pre-natal life + growing (PGdiet) and in mid lactation (Ldiet) (GTT, milk yield:1.91 kg/d per head; BW: 44.1 kg; BCS: 2.9; ITT, milk yield:1.34 kg/d per head; BW:45.93 kg; BCS: 2.88; respectively) diet according to the description reported in the Chapter 2.

Within each group, three animals with low, medium and high milk yield were included in order to have a range of milk production similar in each group. The ITT was performed after few weeks after GTT on the same animals, for that reason animal performances appear slightly different.

Basal insulin and growth hormone concentration in lactation

Insulin and GH levels were evaluated during lactation in 16 Sarda ewes (for insulin at 45, 65, 85, 125, 145 and 165 DIM; for GH at 85, 125 and 145 DIM). Ewes were selected from 4 groups considering their prenatal, growing diet and lactation diet: 1) starch-starch-starch or SSS: 4 ewes (BW: 48.50 kg; BCS: 3.00; milk yield:1.56 kg/d at diet switch); 2)

starch-starch-fiber group or SSF: 4 ewes (BW: 47.58 kg; BCS: 2.94; milk yield:1.64 kg/d at diet switch); 3) fiber-fiber-starch group or FFS: 4 ewes (BW: 48.73 kg; BCS: 2.84; milk yield:1.51 kg/d at diet switch) and 4) fiber-fiber-fiber group or FFF: 4 ewes (BW: 45.40 kg; BCS: 2.88; milk yield:1.78 kg/d at diet switch).

GH concentration at 160 DIM

Growth hormone levels were evaluated during late lactation at 160DIM in16 Sarda ewes divided as follow: 1)SSS group: 4 ewes(BW: 50.35 kg; BCS: 2.90; milk yield: 1.27 kg/d); 2)SSF group: 4 ewes(BW: 51.37 kg; BCS: 2.87; milk yield:1.23kg/d); 3) FFS group: 4 ewes(BW: 51.75 kg; BCS: 2.93; milk yield: 1.22kg/d); and 4) FFF group: 4 ewes (BW: 47.45 kg; BCS: 2.94; milk yield: 1.27kg/d)

2.2.Measurements and samplings

For GTT, a 50% of glucose solution was infused at a dose of 1 ml/kg of BW into the jugular vein of each animal before the morning meal (fasting animals). In addition, in LACT animals it was carried out after milking (empty mammary gland).

Blood samples were taken at following time points: -15, 5, 10, 15, 30, 45, 90 and 180 min after glucose infusion modifying partially the procedure described by Morgante et al. (2012). For LACT animals, blood samples were taken also at -20 min.

In addition, for PREG animals, 1 animal per group (1 SS, 1 FF) did not receive glucose infusion and were considered as control animals.

For ITT, an insulin bolus (1000 IU/ml, Recombinant human insulin; Eli Lilly, Lyngby, Denmark) was infused at a dose of 0.15 IU/kg of BW into the jugular vein 1 hour after the morning meal and after milking. Thus, the test was conducted in fed animals and with empty mammary gland. In particular, the animals received 600 g of the experimental diet related to their feeding group (SSS, SSF, FFS and FFF); the diet was available to each animal for 45 minutes. Blood samples were collected 10 min before morning meal, - 15 and - 5 minutes before insulin injection and + 2.5, + 5, + 10, + 20, + 30, + 60 min after insulin injection according to Khanal et al. (2015).

For both tests, blood samples were collected in evacuated tubes inclusive for anticoagulants, *i.e* lithium heparin (Venosafe VF-109SHL Lithium Heparin, Terumo

Europe NV, Leuven, Belgium; 9 ml) for plasma insulin determination, and glucose-NaF/NH (Vacucheck, Nuova Aptaca s.r.l, Italy; 4 ml) for plasma glucose determination. All blood samples were immediately centrifuged for 10 min (3500 rpm, 4°C) and plasma was stored at -80°C until analysis.

For insulin and GH levels, before the morning meal and after milking, blood samples were collected from the jugular vein in vacuum tubes with lithium heparin (Venosafe VF-109SHL Lithium Heparin, Terumo Europe NV, Leuven, Belgium; 9 ml)for subsequent determination of hormones.

To determine pulsatile activity of GH, blood samples were collected three times every 5 minutes: T1 (0 min), T2 (+5 min) and T3 (+10 min). All blood samples were immediately centrifuged for 10 min (3500 rpm, 4°C) and plasma was stored at -80°C until analysis.

2.3.Chemical analyses

Plasma glucose concentration was determined using a glucose oxidase-peroxidase method (GOD-POD) (Shenzen Mindray Bio-Medical Electronics Co., Shenzen, China). Plasma insulin concentration was determined using an ovine-specific commercial ELISA kit (Ovine Insulin ELISA, Mercodia AB, Uppsala, Sweden) based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule.

Plasma concentrations of GH were evaluated by radio-immuno assay (RIA) technique in the laboratory of Veterinary Physiology. Circulating bovine growth hormone was measured by a heterologous double antibody RIA, using a purified oGH preparation (LER 1774) both as the standard and tracer. The tracer was prepared following the methods described by Salacinski et al. (1981) and 10 000 cpm of the 125I-oGH solution (specific activity: 7.7 uCi/ug) obtained were added to each assay tube. The antiserum was raised in rabbit against oGH (LER 1774) and used at the final dilution of 1/7,000.Separation of free hormone from hormone–antibody complexes was achieved using an anti-rabbit gamma globulin serum raised in goat at the final dilution of 1/500. The sensibility of the analyses, in terms of the interpolated dose as a response to zero concentration, minus the statistical error (Programme Riastar, Canberra-Packard), was 0.28 ng/ml.

2.4 Calculations

For glucose, area under the curve (AUC) was calculated using a trapezoidal summation method (linear and transformed logarithmically). The fractional turnover rate (k) was calculated as *k (%/min)= 100 x {[ln(glucose T15)-ln (glucose T45)]/(T45-T15)}* whereas the half time (T ½) was calculated as *T ½ (min) =100* (0.693/k)* in accordance to Zachut et al. (2013). The same equations were used to calculate AUC, k and T ½ for insulin during ITT.

For GTT, insulin resistance was estimated through a homeostasis model assessment (HOMA), calculated as *HOMA=[fasting insulin (µIU/ml) x fasting glucose (mmol/l)]/22.5* in accordance to Matthews et al. (1985), whereas insulin sensitivity was estimated through a quantitative insulin check index (QUICKI), calculated as *QUICKI=1/[log (fasting glucose, mg/dl)+ log(fasting insulin, µIU/ml)]* in accordance to Katz et al. (2000).

2.4.Statistical analysis

- For PREG animas, data on glucose and insulin concentration, k and T ½ were analyzed by the PROC MIXED procedure of SAS (Version 9.0, SAS Institute Inc., Cary, NC, USA) with repeated measurements, according to the following model:

$$Y_{ijl} = \mu + diet_i + Time_j + diet \times Time_{ij} + \gamma_l + \varepsilon_{ijl}$$

where:

- Y_{ij} is the dependent variable (I =SS diet; FF diet; j =15 min before and 180 min after glucose infusion; from 5 to 90 min after glucose infusion);
- μ is the general mean;
- $diet_i$ is the effect of diet (I = SS diet; FF diet);
- $Time_j$ is the effect of time (j=15 min before and 180 min after glucose infusion; from 5 to 90 min after glucose infusion);
- $diet \times Time_{ij}$ is the diet × time interaction (I = SS diet; FF diet; j=15 min before and 180 min after glucose infusion; from 5 to 90 min after glucose infusion);
- γ_l is the random effect of animal;
- ε_{ijl} is the residual error.

Data on QUICKI, HOMA and AUC concerning the same period (pregnancy) were analyzed considering the only fixed effect of diet (SS, FF).

- For GTT performed in LACT animals, data on glucose and insulin concentration, k and T ½ were analyzed by the PROC MIXED procedure of SAS (Version 9.0, SAS Institute Inc., Cary, NC, USA) with repeated measurements, according to the following model:

$$Y_{ijk} = \mu + PGdiet_i + Ldiet_j + \gamma_k + PGdiet \times Ldiet \times Time_{ijk} + \delta + \varepsilon_{ij}$$

where:

- Y_{ijk} is the dependent variable (I =SS diet; FF diet; j =S diet, F diet; k = -20, -15 min before and 180 min after glucose infusion; from 5 to 90 min after glucose infusion);
- μ is the general mean;
- $PGdiet_i$ is the effect of pre-natal and growing diet (PGdiet; i= SS diet; FF diet);
- $Ldiet_j$ is the effect of lactation diet (Ldiet; j=S diet, F diet);
- $Time_k$ is the effect of time (k= -20, -15 min before and 180 min after glucose infusion; from 5 to 90 min after glucose infusion);
- $PGdiet \times Ldiet \times Time_{ijk}$ is the pre-natal and growing diet × lactation diet × time interaction (i =SS diet; FF diet; j=S diet, F diet; k= -20, -15 min before and 180 min after glucose infusion; from 5 to 90 min after glucose infusion);
- δ is the random effect of animal;
- ε_{ij} is the residual error.

Data on QUICKI, HOMA and AUC concerning the same period (mid lactation) were analyzed considering the fixed effect of prenatal and growing diet (PGdiet: SS and FF diet), the fixed effect of lactation diet (Ldiet: S and F diet), and their interaction (PGdiet × Ldiet).

- For ITT performed in LACT animals, data were analyzed by the PROC MIXED procedure of SAS (Version 9.0, SAS Institute Inc., Cary, NC, USA) with repeated measurements, according to the following model:

$$Y_{ijk} = \mu + PGdiet_i + Ldiet_j + Time_k + PGdiet \times Ldiet \times Time_{ijk} + \delta + \varepsilon_{ij}$$

where:

- Y_{ijk} is the dependent variable (I =SS diet; FF diet; j =S diet, F diet; k = -10 min before the morning meal and -15, -5 min before the insulin infusion, from 2.5 to 60 min after insulin infusion);
- μ is the general mean;
- $PGdiet_i$ is the effect of pre-natal and growing diet (PGdiet; i = SS diet; FF diet);
- $Ldiet_j$ is the effect of lactation diet (Ldiet; j = S diet, F diet);
- $Time_k$ is the effect of time (k= -10 min before the morning meal and -15, -5 min before the insulin infusion, from 2.5 to 60 min after insulin infusion);
- $PGdiet \times Ldiet \times Time_{ijk}$ is the pre-natal and growing diet × lactation diet × time interaction (i = SS diet; FF diet; j = S diet, F diet; k = -10 min before the morning meal and -15, -5 min before the insulin infusion, from 2.5 to 60 min after insulin infusion);
- δ is the random effect of animal;
- ε_{ij} is the residual error.

- For blood basal concentration of GH and Insulin performed in LACT animals,data were analyzed by the PROC MIXED procedure of SAS (Version 9.0, SAS Institute Inc., Cary, NC, USA) with repeated measurements, according to the following model:

$$Y_{ijk} = \mu + PGdiet_i + Ldiet_j + PGdiet \times Ldiet \times Time_{ijk} + \delta + \varepsilon_{ij}$$

where:

- Y_{ijk} is the dependent variable (I =SS diet; FF diet; j =S diet, F diet;
- μ is the general mean;
- $PGdiet_i$ is the effect of pre-natal and growing diet (PGdiet; i= SS diet; FF diet);
- $Ldiet_j$ is the effect of lactation diet (Ldiet; j=S diet, F diet);

- $Time_k$ is the effect of time (k= from 45 to 165 DIM for insulin; from 85 to 145 DIM for GH; and Time 0, +5 minutes and + 10 minutesfor the GH sampling at 160 DIM);
- $PGdiet \times Ldiet \times Time_{ijk}$is the pre-natal and growing diet × lactation diet × time interaction (i =SS diet; FF diet; j=S diet, F diet; k= from 45 to 165 DIM for insulin; from 85 to 145 DIM for GH; and Time 0, + 5 minutes and + 10 minutesfor the GH sampling at 160 DIM);
- δ is the random effect of animal;
- ε_{ij} is the residual error.

Pearson correlations have been tested to study relationship among milk yield, body weight and glucose and insulin concentrations

3. RESULTS AND DISCUSSION

3.1. GTT during pregnancy: effect of prenatal and growing diet (PGdiet)

Basal glucose concentration was not influenced by the PGdiet in fact its concentration before and 180 minutes after glucose infusion did not differ between the FF and the SS diets (70.12 vs. 76.75 ± 9.82 mg/dl; P>0.05; Table 2) in contrast to what observed for basal insulin concentration, which was statistically higher in the SS than in the FF groups (1.09 vs. 0.53 ± 0.18 µg/L; P=0.019; Table 3). Following the glucose infusion (from + 5 to + 90 min), glucose (Table 4) and insulin (Table 5) concentration were not affected by the PGdiet (P>0.05); however, glucose concentration was numerically higher in the FF than in the SS groups (248.25 vs. 223.71 ± 36.88 mg/dl; table 4) in contrast to what observed for insulin which was numerically highest in the SS group (2.57 vs. 1.54 ± 0.60 µg/L; SS vs. FF group; respectively) (Table 5).

Following their trend, blood glucose increased, reaching the peak 5 min after the infusion and decreased until 180 min reaching the basal value (Figure 1; effect of time P=0.0009; Table 4) in accordance to what observed previously in pregnant ewes (Morgante et al., 2012; Schmitt et al., 2012). In addition, in the FF group, glucose concentration decreased after 5 min until 45 min, then increased again until 90 min and hence decreased until 180 min (Figure 1).

As suggested by Baumgard et al. (2017), glucose metabolism can be insulin dependent or insulin independent. In insulin dependent metabolism, insulin dependent-organs and tissues such as skeletal muscle and adipose tissue are involved whereas insulin independent metabolism includes insulin independent-organs and tissues such as the mammary gland and the uterus (De Koster et al., 2017). Probably, in PREG animal, glucose bolus could be redirected toward the pregnant uterus (De Koster et al., 2017) and, thus, the decrease in glucose concentration observed 5 min after glucose infusion could be independent by the insulin activity of the animal. Thereafter, is difficult to discriminate glucose uptake from non-insulin dependent rather than insulin dependent organs as previously observed in pregnant ewes (Duehlmeier et al., 2013). In addition, insulin depend-organs are easily affected by the intravenous glucose infusion in contrast to what observed for the insulin independent-organs and tissues (De Koster et al., 2017). Thus,

data derived from GTT should be considered and interpreted differently in relation to the physiological status of the animals (De Koster et al., 2017).

The trend observed for insulin differed compared to that observed for glucose. In fact, after glucose infusion, insulin concentration reached the peak 10 min after glucose infusion in the FF group and at 30 min in the SS group (Figure 2; effect of time P=0.001; Table 5) in contrast to what observed previously by Morgante et al. (2012). The FF than the SS group also had a lower rise in plasma insulin concentration (Figure 2). In both groups (SS and FF), insulin concentration reached the peak after that of glucose; its release seems to be biphasic in analogy to what occurs in man. In fact, as suggested by Caumo and Luzi (2004), the response of insulin to glucose infusion shows a biphasic profile: 1) in the first phase (called acute response), insulin is released in the first 5 min, after glucose infusion 2) after the acute response and thus in a second moment, insulin decreases until 10 min and thus rises gradually until 30 min after glucose infusion. Generally, this phenomenon (biphasic release of insulin) occurs when β-cells are exposed to a fast change in glucose concentration whereas do not occur under physiological conditions (Caumo and Luzi, 2004).

It is well known that in monogastric species high carbohydrates diets (cereals-based diet) increase glycaemia values and insulin concentration. The influence of diet on glucose and insulin metabolism seems to be less strong in ruminant than non-ruminant species since the amount of glucose absorbed in the intestine is lower compare to non-ruminant species and concentration of blood glucose depends mostly on the hepatic gluconeogenesis (Van Soest, 1994). However, in our experiment the lack of differences in glucose concentration between the SS and the FF groups was not expected. Since propionic acid, derived mostly from starch rich-diets, is considered the most important glucose precursor (De Koster and Opsomer, 2013), a highest glucose concentration in the SS diet was expected. It could be adduced to the fact that in this phase of pregnancy uterus requirements are high and in turn the placental glucose uptake.

The highest insulin concentration observed in the SS than in the FF groups is in accordance with Cannas et al. (2004) and in discordance with Lunesu et al. (2017a, b) and Lunesu (2016). However, in these cases the authors performed their experiments with lactating ewes rather than pregnant ewes. Other studies observed that concentrates-based diet did not influence insulin concentration in heifers (Sternbauer and Luthman, 2002).

The positive relation between insulin and dietary carbohydrates in ruminant species is more documented than the relation between glucose and dietary carbohydrates. The relation between volatile fatty acids (VFA) and endocrine secretion in ruminants have been well documented by Harmon (1992). The author reviewed the literature observing that the infusion of VFA as propionate, butyrate, valeriate, isovaleriate can increase insulin production in cows, goats and sheep in contrast to what observed for acetate infusion (Harmon, 1992). For example, in sheep and cows, the infusion of propionate and butyrate increased insulin production, without affecting plasma glucose, whereas the infusion of acetate did not affect plasma insulin (Horino et al., 1968). Harmon (1992) pointed that only high levels of acetate infusion can stimulate insulin production.

Not only the type of diet but also the amount of feed offered can influence tissues insulin sensitivity. In fact, as suggested by Sternbauer and Luthman (2002), the lack of differences found in insulin concentration between heifers fed a high or a low energy diet probably was due to the low amount of concentrate offered.

In our experiment, considering that the diet offered was high starch or high fiber-based diets both during pre-natal life and during growing (until pregnancy), it is important to understand if one of them (pre-natal diet or growing diet) or both had an influence on insulin metabolism. In fact, in some studies,it has been shown that the offspring performances could be linked to prenatal and early post-natal life. For example, in sheep, maternal nutrition during the last period of gestation seems to influence glucose metabolism in their offspring, increasing insulin sensitivity in female offspring (Owens et al., 2007). Todd et al. (2009) found that moderate maternal undernutrition from 2 months before, until 1 month after conception impairs glucose tolerance in the adult offspring. This effect seemed to be stronger in female than male offspring at 10 months (Todd et al., 2009).

Table 6 reported the mean values of glucose and insulin observed in pregnant ewes that did not receive glucose infusion. During all period, glucose and insulin values were numerically greater in the FF group than in the SS group in contrast to what observed for basal insulin and glucose of animals that received glucose infusion. However, considering the limited number of animals, statistical analysis was not carried out and this should be taken into account when data are discussed.

The type of diet did not influence $AUC_{glucose}$ (Table 7), k (Table 8), T ½ (Table 8); T ½ was numerically higher in the FF than in the SS diet both at 45 min (60.93 vs. 12.65 min) that at 90 min (318.71 vs. 0.70 min) after glucose infusion (Table 8). The same trend was observed for linear and log AUC (Table 7). These results are in contrast with previous studies that analyzed the effect of maternal nutrition (before conception and during pregnancy) on offspring metabolism. For example, glucose concentration and AUC were higher (P=0.006) in ewes (10 months old) born from undernourished than nourished mothers during peri-conceptional period (Todd et al., 2009).
The indicators of insulin sensitivity (QUICKI; Table 7) and insulin resistance (HOMA; Table 7) were not affected by the diet even though HOMA index was numerically higher in the SS group (10.19 vs. 6.85; SS vs. FF group; respectively; Table 7).
The effect of time was statistically significant for basal insulin concentration (P=0.0024; Table 3) whereas the effect of diet ×time interaction was statistically significant only for basal insulin concentration at - 15 min before glucose infusion (P=0.028; Table 3) and for mean basal insulin concentration (P=0.032; Table 3).

3.2. GTT during lactation: effect of prenatal, growing and lactation diet

In LACT animals, the PGdiet and the Ldiet did not affect basal glucose and insulin concentration (-20 min, -15 min before and +180 min after glucose infusion) (Tables 9, 10). In fact, mean of basal glucose (-20, -15 min and +180 min) and that of insulin concentration did not differ among the SSS (76.78 mg/dl; 0.35 µg/L), SSF (71.22 mg/dl; 0.28 µg/L), FFS (72.62 mg/dl; 0.56 µg/L) and the FFF (68.33 mg/dl; 0.26 µg/L) groups (P> 0.05) (Table 9, 10).
The means of basal insulin observed in this stage (0.35, 0.28, 0.56, 0.26 µg/L; SSS, SSF, FFS and FFF groups; respectively; Table 10), were numerically lower than the values of basal insulin observed in PREG animals (0.53 vs. 1.09 µg/L; FF and SS groups; respectively; Table 3) mostly for animals fed starch diets, whereas basal glucose concentration was similar.
Previous studies observed no differences in the basal glucose and insulin concentration between pregnant nonlactating heifers (12 to 7 before calving; 64.5 mg/dl and 5.91

μIU/ml) and lactating primiparous cows (11 to 14 d after calving; 59.5 mg/dl and 3.39 μIU/ml) (De Koster et al., 2017). The same authors observed that nonpregnat-nonlactating heifers had greater basal glucose (P < 0.01) and insulin (P = 0.001) concentration and peak glucose (P = 0.005) than pregnant and lactating cows, probably due to their lowest requirements (De Koster et al., 2017). In fact, the greater animal requirements the lower glucose and insulin concentration and this is in agreement with the milk production status in our experiment.

Probably, the numerically lower values of basal insulin observed in the LACT than in the PREG animals could be due to their requirements, which, generally, are higher in lactating than pregnant animals. It seems that the nutrients are partitioned more to mammary gland in LACT group than to uterus in PREG group (De Koster et al., 2017). However, this result is in contrast to that what reported in the literature for insulin resistance status that occurs from end of pregnancy to early lactation. In particular, insulin resistance can be defined as the ability of cells to modify their response to insulin (Soeters and Soeters, 2012) reducing their sensibility to keep the glucose available for insulin non-dependent tissues. In fact, as suggested by several authors, during pre and post gestation, the low tissue insulin sensitivity is a metabolic adaptation to reduce nutrient uptake from insulin dependent tissues and to drive blood glucose towards fetus or mammary gland (insulin independent sites) (Bell and Bauman, 1997; De Koster and Opsomer, 2013; De Koster et al., 2017). Insulin level begin to rise during lactation, when the requirements of mammary gland are lower and thus, nutrients can redirect to insulin dependent tissues (Bell and Bauman, 1997). Thus, it was expected to observe lower insulin level in PREG than in the LACT animals. However, data were performed in a limited number of animals; in addition, the statistical comparison between the PREG and the LACT groups involved in GTT was not performed.Following glucose infusion (from + 5 to + 90 min), glucose concentration was little affected by the PGdiet (P = 0.087; Table 11) and positively affected by the F diet offered in mid lactation (Ldiet) (Table 11). In fact, glucose concentration was higher in the SSF than in the SSS groups (219.11 vs. 139.33 mg/dl) and in the FFF than in the FFS groups (212.78 vs. 207.50 mg/dl) (effect of Ldiet: P = 0.027; Table 11). This result was not expected since previous studies observed no differences or highest glucose concentration in animals fed starch diets. In fact, glucose concentration was higher in mid lactating Sarda ewes fed a high starch than low starch

diet (Cannas et al., 2004; Cannas et al., 2013) whereas Lunesu et al. (2017a, b) and Lunesu (2016) did not observe differences between ewes fed a high or low starch diets in mid lactation.

In others studies, high energy diet (concentrates-based diet) increased glucose concentration in female calves (4-5 months old) (Sternbauer and Luthman, 2002).

Considering that blood glucose concentration is high when the requirements of animals are lower, the highest values of blood glucose observed in our experiment in mid lactating ewes fed a fiber diet probably means that this type of diet is negatively affecting milk production, in contrast to what observed in the literature. In fact, previous studies observed a positive effect of diets rich in highly digestible fiber on milk yield of primiparous (Bovera et al., 2004) and pluriparous (Cannas et al., 2004; Cannas et al., 2013; Lunesu, 2016; Lunesu et al., 2017a) ewes in mid lactation.

Concerning glucose evolution during the test, blood glucose reached the peak around 5 min after the infusion, then decreased until 180 min reaching the basal value (Figure 3; effect of time $P < 0.0001$; Table 11) in accordance to that observed in previous studies (Morgante et al., 2012; Schmitt et al., 2012) and slightly different than the trend observed in the PREG group. In addition, the SSS group had a lesser rise in plasma glucose concentration (glucose concentration at the peak= 175.67 mg/dl Table 11) than the others groups (Figure 3).

The trend observed for insulin differed than the trend observed for glucose. In fact, after glucose infusion, insulin concentration reached the peak 5 min after glucose infusion only in the FFS group and at 10 min in the SSS, SSF and FFF groups (Figure 4; effect of time $P < 0.001$; Table 12) in contrast to what previously observed in pregnant sheep by Morgante et al. (2012). The SSS group also had a lower rise in plasma insulin concentration (Figure 4). In all groups, insulin release seems to be biphasic according to what suggested previously (Caumo and Luzi, 2004).

Considering that in LACT group, GTT was performed after morning milking and thus with empty mammary gland, it could be hypothesized that the reduction of glucose during the test may be due to the uptake from the mammary gland (Marett et al., 2015) rather than insulin activity; in other words, the decrease in glucose concentration observed 5 min after glucose infusion could be independent by the insulin activity of the animal and highly correlated to the mammary glucose uptake proportional to milk yield. Therefore,

considering that is impossible to discriminate the two pathways (Duehlmeier et al., 2013), mentioned before, data should be interpreted more carefully (De Koster et al., 2017). In addition, as suggested by De Koster et al. (2017) is necessary to improve GTT techniques both in pregnant and lactating animals.

In opposition to that observed for glucose, after glucose infusion (from + 5 to + 90 min), insulin concentration was not affected by the PGdiet and/or Ldiet (P > 0.05; Table 12).

Linear and log $AUC_{glucose}$ were numerically greater in animals fed a F diet during lactation (SSF and FFF) (effect of Ldiet: P=0.074 andP=0.08; respectively; Table13) in accordance to that observed during pregnancy (Table 7). Generally, higher AUC for glucose means a lower response which could be interpreted as the highest peripheral tissue insulin resistance as suggested by De Koster et al. (2017). This is in line to what observed in pregnancy that SS group showed higher numerical HOMA as reported in the previous paragraph. However, no differences were observed in the indicators of insulin resistance and insulin sensitivity (HOMA and QUICKI, respectively; Table 13).

K was numerically greater in animals fed a F diet during pre-natal and growing diet (effect of PGdiet: P=0.06; Table 14) and was not affected by Ldiet. In addition, the type of diet (PGdiet and Ldiet) did not influence T ½ (Table 14).

The effect of time was statistically significant for basal glucose (P = 0.013; Table 9), glucose and insulin concentration after glucose infusion (P < 0.001; Tables 11, 12).

The effect of PGdiet × Ldiet interaction was never significant whereas the effect of PGdiet × Ldiet × time interaction was statistically significant only for plasma glucose concentration after glucose infusion (from + 5 to + 90 min) (P < 0.001; Table 11).

3.3. ITT during lactation: effect of prenatal, growing and lactation diet

The PGdiet and the Ldiet did not affect basal glucose and insulin concentration (Tables 15, 16) and plasma glucose after insulin infusion (Tables 17). In contrast blood insulin concentration after the infusion was significantly higher (effect of PGdiet, P = 0.042; Table 18) in the SSS (12.47 µg/L; Table 18) and SSF (12.08 µg/L; Table 18) groups than in the FFS (8.94 µg/L; Table 18) and FFF (11.40 µg/L; Table 18) in accordance to what observed for basal insulin concentration in PREG animals (Table 3).

Figure 5 reported the evolution of blood glucose during the ITT. After insulin infusion, plasma glucose markedly decreased (Figure 5; effect of time: $P < 0.001$; Table 18) reaching the lowest values (around 30 mg/dl) at 30 min except for the FFS group where the fall of glucose was lower drastically reaching lowest value of 50 mg/dl.
Regarding the trend of blood insulin, its values reached the peak 2.5 min after insulin infusion for the SSS, SSF and FFS in contrast to what observed for the FFF group who reached the peak 5 min after insulin infusion (Figure 6; effect of time $P < 0.001$; Table 18). In addition, the highest values at peak were only reached by SSS and SSF groups (Figure 6). The FFS group, in contrast to what observed for the others groups, showed a decrease in insulin concentration from 2.5 min until 5 min, thus increased until 10 min and decreased again reaching basal values 60 min after insulin infusion (Figure 6).The effect of PGdiet × Ldiet × Time interaction was statistically significant only for plasma insulin concentration after insulin infusion (from + 2.5 to 60 min) ($P < 0.001$; Table 18). It might partially indicate that FFS had an impaired insulin regulation and glucose uptake.

3.4. Correlations of glucose and insulin with milk yield in GTT and ITT

Considering the findings from GTT and ITT a correlation analysis was performed to investigate possible relationships among basal glucose, insulin concentrations and animal lactation performances. In particular, data of all animals involved in GTT and ITT tests were pooled and only basal values and milk production were considered. In the whole dataset high negative correlation among milk yield and DIM ($r = - 0.48$; $P < 0.05$) and among milk yield and glucose concentration ($r = -0.57$; $P < 0.01$) was observed; similar values were observed among FPCMY and the glucose and FPCMY and insulin (Table 19). As expected BW and BCS resulted positively correlated ($r = 0.48$; $P < 0.05$). In addition a positive correlation was found between insulin and DIM ($r= 0.68$; $P = 0.001$; Table 19). It agrees with all literature on endocrine regulation of energy partitioning, also confirming that basal insulin levels increase form early to end of lactation (Bauman and Currie,1980; Baumgard et al., 2017). When correlations were tested within treatment, considering 4 nutrition plans of the experimental groups (SSS, SSF, FFS and FFF) correlation pattern, resulted different (Table 20).

In the SSS group high negative correlation among glucose and basal insulin (r = -0.84) even if with poor significant difference P < 0.10; Table 20) was observed.
Oppositely, in SSF group a strong correlation among glucose and basal insulin (r =0.96; P <0.05), and a positive correlation among DIM and BW were observed (r =0.90; P = 0.01). In both, SSS and SSF groups milk yield seems not related to basal levels of insulin and glucose perhaps confirming the effects of homeorhetic insulin resistances, already highlighted in pregnancy and in ITT. In addition, the relation between glucose and insulin was positive in SSS and negative in SSF (Table 20). It might be related to the fact that in SSS group propionate, more than glucose, stimulates the insulin secretion in ruminants (Rhoads et al., 2004) and that the nutrient partitioning is already directed to the adipose tissue. It also agrees with higher uptake of nutrients from the mammary gland and patterns of high and low milk persistency already reported in the Figure 4 of Chapter 4 for SSF and SSS groups, respectively.
In FFS group BW was positively correlated to BCS (r = 0.86; P < 0.05), and insulin with DIM (r = 0.90; P < 0.01) in agreement to what reported by De Koster and Opsomer (2013) and Bell and Bauman (1997) and Baumgard, et al. (2017) which explained the usual patterns of nutrient partitioning and glucose and insulin in lactation.
In FFF group milk yield resulted negatively and highly correlated with insulin and basal glucose concentrations (r = - 0.90; P < 0.1; -0.89; P < 0.05, respectively). Glucose also tended to positively correlate with BW (r = 0.81; P < 0.10). In FFF group milk yield was negatively correlated to insulin (r = -0.90; P < 0.10) and glucose (r = - 0.89; P < 0.05). In the same group, glucose tended to be correlated to BW (r = 0.81; P < 0.1). It might be related to impaired regulation of insulin already highlighted in the ITT. It also agrees with low uptake of nutrients from the mammary gland and patterns of low milk persistency already reported in the Figure 4 of Chapter 4.
Considering that groups of ewes included in GTT and ITT were initially balanced for milk yield, the high correlations observed among variables within groups, might help to explain some effect of exposure to carbohydrates on milk production and lactation persistency.

3.5. Basal levels of insulin and growth hormonein mid to late lactation

A further check of growth hormone and insulin concentration was performed on basal concentrations of insulin and growth hormone from mid to late lactation in the groups SSS, SSF, FFS and FFF.

Differently from GTT and ITT, basal insulin concentration from mid to late lactation was not influenced by the PGdiet and the Ldiet and their interaction resulting equal to 0.70, 0.60, 0.98 and 0.80µg/L for SSS, SSF, FFS and FFF groups from 45 to 165 DIM without significant differences among them (Table21). Insulin concentration was influenced by the stage of lactation showing increasing significant trends in mid to late lactation (P = 0.05; Table 21). In terms of patterns, the group FFF showed a rapid increase of basal insulin concentration after 60 DIM, whereas in the other groups SSS, SSF and FFS the insulin remains quite stable until 120 DIM then increased with lactation progress, particularly in the FFS group (Figure 7). A significant interaction among PG diet, Ldiet and DIM was not observed but the observed pattern in FFF is in line to what observed in the previous paragraphs about the impaired insulin regulation in the FFF group and its milk production decline. These patters are in agreement with increasing insulin trends with the lactation progress (Cannas et al., 2004; Lunesu, 2016: Baumgard et al., 2017). The earlier increase of insulin basal concentration in the FFF group is in line with the observed lower milk production of this group, thus resulting in higher nutrient partitioning to the adipose tissue (De Koster et Opsomer, 2013)

GH concentration from mid to late lactation was not influenced by the PGdiet and the Ldiet and their interaction resulted equal to 1.11, 1.09, 1.10 and 1.41 ng/ml for SSS, SSF, FFS and FFF groups from 85 to 145 DIM, respectively (Table 22). Differently, GH concentration was influenced by the stage of lactation showing increasing and significant trends in mid to late lactation (P = 0.002; Table 22). In terms of patterns, the GH showed an increasing trend from 85 to 125 and then a decline until 145 DIM. These patterns are partially in contrast to what reported in literature about increases of GH in early lactation and then decreases from mid to late lactation (Bauman, 2000; Baumgard et al., 2017). Similar pattern were observed in multiparous ewes by Lunesu (unpublished data) with

increased levels of GH from parturition to 40 DIM and then declining trends until late lactation.

Unexpectedly, the group FFF showed higher numerical GH concentration than other groups in mid to late lactation (Figure 8) which is in disagreement with the lactation pattern of the same group observed in Chapter 4 which declined very fast (Figure 4 of Chapter 4). It has been observed in literature that GH concentration is affected by many factors, in addition increases of GH were related to the blood glucose decline after insulin action (Trenkle, 1977 and are higher in growing animals (Laurentie et al., 1987). Nevertheless these factors were considered not sufficient to explain the patters observed in this study.

The GH shows a marked pulsatile activity that is influenced by many factors (Plouzek, 1998) including photoperiod (Kasuya et al., 2016), feeding time (Driven and Forbes, 1981) blood sampling (Trenkle, 1977). For this reason the concentration of GH of the SSS, SSF, FFS and FFF groups was assessed with 3 sequent samplings at 160 DIM (Table 23 and Figure 9). Not significant differences were observed for the effects of PG diet, Ldiet, Time of sampling and their interactions. The final level of GH resulted, on average 1.38, 1.20, 1.44 and 1.53 ng/ml for SSS, SSF, FFS and FFF (Table 23). The average values were numerically slightly higher than those observed at 125 and 145 DIM and keeping the proportions among groups confirming the consideration already adduced for the whole mid to late lactation phase.

4. CONCLUSIONS

This research explored, with the aid of GTT and ITT,glucose and insulin metabolism in ewes fed a different amount of starch or fiber diet in different physiological conditions (pregnancy or mid lactation). The evolution of insulin during the GTT followed a similar pattern to that observed in human (biphasic pathway). The evolution of glucose and insulin in GTT and ITT followed expected patterns. Furthermore, the type of dietary carbohydrates offered during pre and postnatal life seemed to influence glucose and insulin metabolism in the offspring.

Significant effects of prenatal and growing diet and lactation diet were observed for basal insulin concentration of animals tested with GTT in pregnancy (P = 0.02) indicating that animals fed SS have higher basal insulin concentration. During lactation, Ldiet tended to influence the area under curve of GTT with higher values for diet F than S (P = 0.07) and PGdiet tended to influence the fractional turnover rate of glucose clearanceinducing higher K from 0 to +45 min for FF animals (P = 0.06). In addition, plasma insulin concentration during ITT was higher for the group SS than for the group FF (P = 0.04), confirming the results obtained in pregnancy. Significant increases of insulin were observed moreover after 125 DIM in all groups except for the FFF group which basal insulin started increasing at 60 DIM. Higher numerical values of HOMA index were observed for the SS group.

When GTT and ITT basal blood values of insulin concentration were correlated with BW, BCS and FPCMY the correlations resulted different depending on the nutritional plan experienced by the group. In particular, insulin high and significantly correlation were observed: negatively with blood glucose in SSS, positively with blood glucose in SSF, with BW and BCS in FFS. In FFF insulin was not significantly correlated with other variableswhereas blood basal glucose was negatively correlated to FPCMY and tended to be positively correlated with BW.

These results, although deduced from poor significanteffects, were in agreement with the lactation pattern observed for the experimental treatments. A possible explanation of the these evidences is that exposure to high starch diets in prenatal and growing life (SS) was related with reduced insulin sensitivity favoring the nutrient partitioning to mammary gland in late lactation. Oppositely, exposure to low starch and high fiber diets (FF) in

prenatal and growing life might induce to develop an impaired regulation of insulin secretion and/or higher insulin sensitivity of adipose tissue and possibly low lactation persistency. With higher nutrient partitioning to body reserved in late lactation. Additional studies are needed to *i)* better understand the complex mechanisms involved in the transmission of information from the mother to the offspring *ii)* improve GTT and ITT both in pregnant and in lactating animals.

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6. TABLES AND FIGURES

Table 1. Diet formulation and chemical composition offered to the experimental groups. In the day of performing glucose tolerance test (GTT) and insulin tolerance test (ITT) lactating animals did not received the morning dose of milking concentrate.

Offered feeds	Prenatal diet		Growing diet		Mid to late lactation, diet*	
Item	Diet S	Diet F	Diet S	Diet F	Diet S	Diet F
Dry matter, % of as fed	88.6	88.0	88.0	88.0	89.8	89.9
Crude protein, % of DM	14.6	14.8	16.0	15.5	16.0	16.8
NDF, % of DM	37.0	46.5	39.5	50.5	40.4	44.9
ADF, % of DM	25.9	37.8	27.2	37.3	27.3	32
ADL, % of DM	2.42	2.69	2.25	2.62	5.6	4.9
Ether extract, % of DM	2.09	2.26	2.04	2.15	1.6	1.5
Ashes, % of DM	9.76	10.75	7.505	8.33	10.0	10.2
NFC, % of DM	42.4	31.7	40.9	29.5	32.0	26.6
Starch, % of DM	26.7	10.7	25.5	9.8	24.3	12.7
Forage:concentrate ratio	45/55	45/55	40/60	40/60	38/62	38/62

EL = in early lactation; S = starch; F = fiber; Non-starch soluble NDF = estimated as difference among NFC and starch; 7h IVSD = 7 hour test in vitro digestibility of starchNDF-d = degradability of NDF; Digestible NDF = NDF digestible at 48 h; INDF = indigestible NDF at 288 h.

Table 2. Basal plasma glucose concentration 15 min before and 180 min after glucose infusion in pregnant primiparous Sarda ewes fed a fiber-fiber (FF) and a starch-starch (SS) diet.

		Group*			*P level*		
		FF	SS	SEM[a]	Diet	Time	Diet×Time[b]
Glucose (mg/dl)	-15 min	68.00	74.75	4.13			N.S.[c]
	+180 min	72.25	78.75	17.95			N.S.
	Mean	70.12	76.75	9.82	N.S.	N.S.	N.S.

[a] Standard error of the mean; [b] effect of diet x time interaction; [c] not significant; * FF= fiber-fiber group (4 ewes fed a fiber diet during pre and post-natal life or growing); SS=starch-starch group (4 ewes fed a starch diet during pre and post-natal life or growing)

Table 3. Basal plasma insulin concentration 15 min before and 180 min after glucose infusion in pregnant primiparous Sarda ewes fed a fiber-fiber (FF) and a starch-starch (SS) diet.

		Group*			*P level*		
		FF	SS	SEM[a]	Diet	Time	Diet×Time[b]
Insulin (µg/L)	-15 min	0.61	1.37	0.19			0.028
	+180 min	0.45	0.81	0.19			N.S.[c]
	Mean	0.53	1.09	0.18	0.019	0.0024	0.032

[a] Standard error of the mean; [b] effect of diet x time interaction; [c] not significant; * FF= fiber-fiber group (4 ewes fed a fiber diet during pre and post-natal life or growing); SS=starch-starch group (4 ewes fed a starch diet during pre and post-natal life or growing)

Table 4. Plasma glucose concentration during glucose tolerance test in pregnant primiparous Sarda ewes fed a fiber-fiber (FF) and a starch-starch (SS) diet**.**

		Group*			*P level*		
		FF	SS	SEM[a]	Diet	Time	Diet×Time[b]
Glucose (mg/dl)	+5 min	341.00	305.25	49.18			N.S.[c]
	+10 min	293.75	270.00	49.18			N.S.
	+15 min	272.75	244.00	49.18			N.S.
	+30 min	230.25	198.75	49.18			N.S.
	+45 min	177.25	178.00	49.18			N.S.
	+90 min	174.50	146.25	49.18			N.S.
	Mean	248.25	223.71	36.88	N.S.	0.0009	N.S.

[a] Standard error of the mean; [b] effect of diet x time interaction; [c] not significant; * FF= fiber-fiber group (4 ewes fed a fiber diet during pre and post-natal life or growing); SS=starch-starch group (4 ewes fed a starch diet during pre and post-natal life or growing)

Table 5. Plasma insulin concentration during glucose tolerance test pregnant primiparous Sarda ewes fed a fiber-fiber (FF) and a starch-starch (SS) diet**.**

		Group*			*P level*		
		FF	SS	SEM[a]	Diet	Time	Diet×Time[b]
Insulin (µg/L)	+5 min	1.17	1.89	0.68			N.S.[c]
	+10 min	1.80	2.83	0.68			N.S.
	+15 min	1.76	2.95	0.68			N.S.
	+30 min	1.55	3.10	0.68			N.S.
	+45 min	1.50	2.73	0.68			N.S.
	+90 min	1.43	1.89	0.68			N.S.
	Mean	1.54	2.57	0.60	0.13	0.001	N.S.

[a] Standard error of the mean; [b] effect of diet x time interaction; [c] not significant; * FF= fiber-fiber group (4 ewes fed a fiber diet during pre and post-natal life or growing); SS=starch-starch group (4 ewes fed a starch diet during pre and post-natal life or growing)

Table 6. Descriptive statistical analysis (mean, standard deviation, minimum, maximum) of plasma glucose and insulin concentration in pregnant primiparous Sarda ewes fed a fiber-fiber (FF) and a starch-starch (SS) dietthat did not receive glucose infusion.

		FF*				SS*			
		Mean	Sd.	Min	Max	Mean	Sd.	Min	Max
-15 min before and +180 min after	Basal glucose	87.50	10.61	80.00	95.00	71.00	5.66	67.00	75.00
	Basal insulin	1.21	0.61	0.78	1.64	0.28	0.11	0.20	0.36
From +5 to +90 min after	Glucose	93.67	5.46	88.00	100.00	64.60	0.89	63.00	65.00
	Insulin	1.30	0.13	1.13	1.53	0.28	0.05	0.23	0.36

* FF= fiber-fiber group (1 ewes fed a fiber diet during pre and post-natal life or growing); SS=starch-starch group (1 ewes fed a starch diet during pre and post-natal life or growing)

Table 7. Quantitative insulin check index (QUICKI), homeostasis model assessment (HOMA) and area under the curve ($AUC_{glucose}$) in pregnant primiparous Sarda ewes fed a fiber-fiber (FF) and a starch-starch (SS) diet**.**

	Group*			*P level*
	FF	SS	SEM[a]	Diet
QUICKI**	0.29	0.28	0.006	N.S.[b]
HOMA***	6.85	10.19	1.60	N.S.
Linear $AUC_{glucose}$	29911	27154	1575	N.S.
Log $AUC_{glucose}$	29079	26528	1433	N.S.

[a] Standard error of the mean; [b] not significant
*FF=fiber-fiber group (4 ewes fed a fiber diet during pre and post-natal life or growing); SS=starch-starch group (4 ewes fed a starch diet during pre and post-natal life or growing)
** calculated as*QUICKI=1/[log (fasting glucose, mg/dl)+ log(fasting insulin, µU/ml)]* (from Katz et al., 2000)
*** calculated as HOMA=[fasting insulin (µIU/ml) x fasting glucose (mmol/l)]/22.5 (from Matthews et al., 1985)

Table 8. Fractional turnover rate (k) and half time (T ½) in pregnant primiparous Sarda ewes fed a fiber-fiber (FF) and a starch-starch (SS) diet**.**

	Group*			*P level*		
	FF	SS	SEM[a]	Diet	Time	Diet×Time[b]
K +45 (%/min)	1.51	0.93	0.46	N.S.[c]	N.S.	N.S.
K +90 (%/min)	0.64	0.72				
T ½ +45 (min)	60.93	12.65	155.07	N.S.	N.S.	N.S.
T ½ +90 (min)	318.71	0.70				

[a] Standard error of the mean; [b] effect of diet x time interaction; [c] not significant
*FF=fiber-fiber group (4 ewes fed a fiber diet during pre and post-natal life or growing); SS=starch-starch group (4 ewes fed a starch diet during pre and post-natal life or growing); SS=starch-starch group (4 ewes fed a starch diet during pre and post-natal life or growing).

Table 9. Basal plasma glucose concentration (mg/dl) 20 and 15 min before and 180 min after glucose infusion in mid-lactating primiparous Sarda ewes.

	Time					*P level*			
Group*	-20 min	-15 min	+180 min	Mean	SEM[a]	PGdiet[b]	Ldiet[c]	Time	PGdiet ×Ldiet ×Time[d]
SSS	75.33	77.00	78.00	76.78					
SSF	74.67	75.67	63.33	71.22	1.31	N.S.[e]	N.S.	0.013	N.S.
FFS	70.67	81.18	68.33	72.62					
FFF	67.67	70.67	66.67	68.33					

[a] Standard error of the mean; [b] effect of pre-natal and growing diet; [c] effect of lactation diet; [d] effect of pre-natal and growing diet × lactation diet × time interaction; [e] not significant;

* SSS= starch-starch-starch group (3 ewes fed a starch diet during pre-natal life, growing and in mid lactation), SSF=starch-starch-fiber group (3 ewes fed a starch diet during pre-natal life, growing and a fiber diet during mid lactation), FFS=fiber-fiber-starch group (3 ewes fed a fiber diet during pre-natal life, growing and a starch diet during mid lactation) and FFF=fiber-fiber-fiber group (3 ewes fed a fiber diet during pre-natal life, growing and in mid lactation)

Table 10. Basal plasma insulin concentration (µg/L) 20 and 15 min before and 180 min after glucose infusion in mid-lactating primiparous Sarda ewes.

	Time					*P level*			
Group*	-20 min	-15 min	+180 min	Mean	SEM[a]	PGdiet[b]	Ldiet[c]	Time	PGdiet×Ldiet×Time[d]
SSS	0.27	0.35	0.42	0.35					
SSF	0.26	0.41	0.16	0.28	0.05	N.S.[e]	N.S.	N.S.	N.S.
FFS	0.27	0.72	0.65	0.56					
FFF	0.26	0.34	0.17	0.26					

[a] Standard error of the mean; [b] effect of pre-natal and growing diet; [c] effect of lactation diet; [d] effect of pre-natal and growing diet × lactation diet × time interaction; [e] not significant

* SSS= starch-starch-starch group (3 ewes fed a starch diet during pre-natal life, growing and in mid lactation), SSF=starch-starch-fiber group (3 ewes fed a starch diet during pre-natal life, growing and a fiber diet during mid lactation), FFS=fiber-fiber-starch group (3 ewes fed a fiber diet during pre-natal life, growing and a starch diet during mid lactation) and FFF=fiber-fiber-fiber group (3 ewes fed a fiber diet during pre-natal life, growing and in mid lactation)

Table 11. Plasma glucose concentration (mg/dl) in mid-lactating primiparous Sarda ewes during glucose tolerance test (from 5 to 90 min after glucose infusion).

	Time								*P level*			
Group*	+5 min	+10 min	+15 min	+30 min	+45 min	+90 min	Mean	SEM[a]	PGdiet[b]	Ldiet[c]	Time	PGdiet×Ldiet×Time[d]
SSS	175.67	164.00	153.33	135.00	120.33	87.67	139.33	10.3	0.087	0.027	<0.001	<0.001
SSF	336.00	284.67	259.00	198.67	156.33	80.00	219.11					
FFS	326.00	281.33	254.33	185.67	136.00	61.67	207.50					
FFF	322.67	284.33	251.67	188.00	151.67	78.33	212.78					

[a] Standard error of the mean; [b] effect of pre-natal and growing diet; [c] effect of lactation diet; [d] effect of pre-natal and growing diet × lactation diet × time interaction
* SSS= starch-starch-starch group (3 ewes fed a starch diet during pre-natal life, growing and in mid lactation), SSF=starch-starch-fiber group (3 ewes fed a starch diet during pre-natal life, growing and a fiber diet during mid lactation), FFS=fiber-fiber-starch group (3 ewes fed a fiber diet during pre-natal life, growing and a starch diet during mid lactation) and FFF=fiber-fiber-fiber group (3 ewes fed a fiber diet during pre-natal life, growing and in mid lactation)

Table 12. Plasma insulin concentration (µg/L) in mid-lactating primiparous Sarda ewes during glucose tolerance test (from 5 to 90 min after glucose infusion).

	Time								*P level*			
Group*	+5 min	+10 min	+15 min	+30 min	+45 min	+90 min	Mean	SEM[a]	PGdiet[b]	Ldiet[c]	Time	PGdiet×Ldiet×Time[d]
SSS	1.13	1.49	1.00	0.77	1.10	0.39	0.98	0.14	N.S.[e]	N.S.	<0.0001	N.S.
SSF	1.57	2.84	2.12	2.30	1.99	0.49	1.88					
FFS	3.13	2.96	2.64	2.61	2.69	0.20	2.37					
FFF	1.09	2.28	1.33	1.40	0.92	0.40	1.24					

[a] Standard error of the mean; [b] effect of pre-natal and growing diet; [c] effect of lactation diet; [d] effect of pre-natal and growing diet × lactation diet × time interaction; [e] not significant

* SSS= starch-starch-starch group (3 ewes fed a starch diet during pre-natal life, growing and in mid lactation), SSF=starch-starch-fiber group (3 ewes fed a starch diet during pre-natal life, growing and a fiber diet during mid lactation), FFS=fiber-fiber-starch group (3 ewes fed a fiber diet during pre-natal life, growing and a starch diet during mid lactation) and FFF=fiber-fiber-fiber group (3 ewes fed a fiber diet during pre-natal life, growing and in mid lactation)

Table 13. Quantitative insulin check index (QUICKI), homeostasis model assessment (HOMA) and area under the curve ($AUC_{glucose}$) in mid-lactating primiparous Sarda ewes before and after glucose infusion.

	Group*					*P level*		
	SSS	SSF	FFS	FFF	SEM[a]	PGdiet[b]	Ldiet[c]	PGdiet×Ldiet[d]
QUICKI**	0.33	0.32	0.33	0.35	0.009	N.S.[e]	N.S.	N.S.
HOMA***	2.22	3.20	2.15	2.45	0.36	N.S.	N.S.	N.S.
Linear $AUC_{glucose}$	18533	21927	19878	21472	659	N.S.	0.074	N.S.
Log $AUC_{glucose}$	18441	21665	19594	21216	645	N.S.	0.08	N.S.

[a] Standard error of the mean; [b] effect of pre-natal and growing diet; [c] effect of lactation diet; [d] effect of pre-natal and growing diet × lactation diet interaction; [e] not significant

* SSS= starch-starch-starch group (3 ewes fed a starch diet during pre-natal life, growing and in mid lactation), SSF=starch-starch-fiber group (3 ewes fed a starch diet during pre-natal life, growing and a fiber diet during mid lactation), FFS=fiber-fiber-starch group (3 ewes fed a fiber diet during pre-natal life, growing and a starch diet during mid lactation) and FFF=fiber-fiber-fiber group (3 ewes fed a fiber diet during pre-natal life, growing and in mid lactation)

**calculated as*QUICKI=1/[log (fasting glucose, mg/dl)+ log(fasting insulin, µU/ml)]* (from Katz et al., 2000)

***calculated as HOMA=[fasting insulin (µIU/ml) x fasting glucose (mmol/l)]/22.5 (from Matthews et al., 1985)

Table 14. Fractional turnover rate (k) and half time (T ½) in mid-lactating primiparous Sarda ewes during glucose tolerance test.

	Group*					*P level*			
	SSS	SSF	FFS	FFF	SEM[a]	PGdiet[b]	Ldiet[c]	Time	PGdiet×Ldiet×Time[d]
K +45 (%/min)	0.71	1.69	2.10	1.70	0.14	0.06	N.S.[e]	N.S.	N.S.
K +90 (%/min)	0.72	1.58	1.90	1.57					
T ½ +45 (min)	-4.79	41.93	33.45	41.23	33.7	N.S.	N.S.	N.S.	N.S.
T ½ +90 (min)	-204.8	44.74	36.77	44.90					

[a] Standard error of the mean; [b] effect of pre-natal and growing diet; [c] effect of lactation diet; [d] effect of pre-natal and growing diet × lactation diet × time interaction; [e] not significant

* SSS= starch-starch-starch group (3 ewes fed a starch diet during pre-natal life, growing and in mid lactation), SSF=starch-starch-fiber group (3 ewes fed a starch diet during pre-natal life, growing and a fiber diet during mid lactation), FFS=fiber-fiber-starch group (3 ewes fed a fiber diet during pre-natal life, growing and a starch diet during mid lactation) and FFF=fiber-fiber-fiber group (3 ewes fed a fiber diet during pre-natal life, growing and in mid lactation).

Table 15. Basal plasma glucose concentration (mg/dl) during insulin tolerance test in mid-lactating primiparous Sarda ewes (insulin at - 15 min before insulin infusion was not analyzed).

	Time*				*P level*			
Group**	-10 min	-5 min	Mean	SEM[a]	PGdiet[b]	Ldiet[c]	Time	PGdiet×Ldiet×Time[d]
SSS	68.50	74.33	72.00	2.44	N.S.[e]	N.S.	N.S.	N.S.
SSF	78.00	81.00	79.80					
FFS	66.00	72.33	69.80					
FFF	76.00	70.00	72.40					

[a] Standard error of the mean; [b] effect of pre-natal life and growing diet; [c] effect of lactation diet; [d] effect of pre-natal life and growing diet x lactation diet x time interaction; [e] not significant

* -10 min before the morning meal; -5 min before the insulin infusion

* SSS= starch-starch-starch group (3 ewes fed a starch diet during pre-natal life, growing and in mid lactation), SSF=starch-starch-fiber group (3 ewes fed a starch diet during pre-natal life, growing and a fiber diet during mid lactation), FFS=fiber-fiber-starch group (3 ewes fed a fiber diet during pre-natal life, growing and a starch diet during mid lactation) and FFF=fiber-fiber-fiber group (3 ewes fed a fiber diet during pre-natal life, growing and in mid lactation)

Table 16. Basal plasma insulin concentration (µg/L) during insulin tolerance test in mid-lactating primiparous Sarda ewes (insulin at - 15 min before insulin infusion was not analyzed).

	Time*				*P level*			
Group**	-10 min	-5 min	Mean	SEM[a]	PGdiet[b]	Ldiet[c]	Time	PGdiet ×Ldiet ×Time[d]
SSS	0.57	0.71	0.64	0.06	N.S.[e]	N.S.	N.S.	N.S.
SSF	0.62	0.68	0.65					
FFS	0.66	0.90	0.78					
FFF	0.46	0.35	0.40					

[a] Standard error of the mean; [b] effect of pre-natal life and growing diet; [c] effect of lactation diet; [d] effect of pre-natal life and growing diet × lactation diet × time interaction; [f] not significant

* -10 min before the morning meal; -5 min before the insulin infusion

* SSS= starch-starch-starch group (3 ewes fed a starch diet during pre-natal life, growing and in mid lactation), SSF=starch-starch-fiber group (3 ewes fed a starch diet during pre-natal life, growing and a fiber diet during mid lactation), FFS=fiber-fiber-starch group (3 ewes fed a fiber diet during pre-natal life, growing and a starch diet during mid lactation) and FFF=fiber-fiber-fiber group (3 ewes fed a fiber diet during pre-natal life, growing and in mid lactation)

Table 17. Plasma glucose concentration (mg/dl) in mid lactating primiparous Sarda ewes during insulin tolerance test(from 2.5 to 60 min after glucose infusion).

	Time								*P level*			
Group*	+2.5 min	+5 min	+10 min	+20 min	+30 min	+60 min	Mean	SEM[a]	PGdiet[b]	Ldiet[c]	Time	PGdiet × Ldiet × Time[d]
SSS	65.73	68.67	61.67	39.67	34.67	51.33	53.35	1.91	N.S.[e]	N.S.	<0.0001	N.S.
SSF	72.52	67.67	60.00	41.33	33.67	50.67	53.18					
FFS	71.33	69.67	59.67	51.00	52.00	50.67	59.05					
FFF	70.33	64.67	52.06	39.33	36.67	52.91	52.81					

[a] Standard error of the mean; [b] effect of pre-natal life and growing diet; [c] effect of lactation diet; [d] effect of pre-natal life and growing diet × lactation diet × time interaction; [e] not significant

* SSS= starch-starch-starch group (3 ewes fed a starch diet during pre-natal life, growing and in mid lactation), SSF=starch-starch-fiber group (3 ewes fed a starch diet during pre-natal life, growing and a fiber diet during mid lactation), FFS=fiber-fiber-starch group (3 ewes fed a fiber diet during pre-natal life, growing and a starch diet during mid lactation) and FFF=fiber-fiber-fiber group (3 ewes fed a fiber diet during pre-natal life, growing and in mid lactation)

Table 18. Plasma insulin concentration (µg/L) in mid lactating primiparous Sarda ewes during insulin tolerance test(from 2.5 to 60 min after glucose infusion).

	Time								*P level*			
Group*	+2.5 min	+5 min	+10 min	+20 min	+30 min	+60 min	Mean	SEM[a]	PGdiet[b]	Ldiet[c]	Time	PGdiet×Ldiet×Time[d]
SSS	29.98	23.63	21.59	8.27	3.49	1.56	12.47	1.28	0.042	N.S.[e]	<0.001	<0.0001
SSF	31.52	33.62	22.07	7.96	4.27	1.62	12.08					
FFS	20.99	5.40	12.13	7.34	7.05	2.58	8.94					
FFF	17.72	23.40	19.12	6.95	3.46	2.12	11.40					

[a] Standard error of the mean; [b] effect of pre-natal life and growing diet; [c] effect of lactation diet; [d] effect of pre-natal life and growing diet × lactation diet × time interaction; [e] not significant

* SSS= starch-starch-starch group (3 ewes fed a starch diet during pre-natal life, growing and in mid lactation), SSF=starch-starch-fiber group (3 ewes fed a starch diet during pre-natal life, growing and a fiber diet during mid lactation), FFS=fiber-fiber-starch group (3 ewes fed a fiber diet during pre-natal life, growing and a starch diet during mid lactation) and FFF=fiber-fiber-fiber group (3 ewes fed a fiber diet during pre-natal life, growing and in mid lactation)

Table 19. Correlation (r vaules) among milk yield, Fat and protein corrected milk (FPCMY), body weight (BW), body condition score (BCS) and days in milk (DIM) with basal concentration of insulin and glucose during glucose tolerance test and insulin tolerance test (pooled data).

	Milk yield	Insulin	Glucose	BW	BCS	DIM
Insulin, µg/l	-0.179					
P	0.437					
Glucose, mg/dl	**-0.569**	0.155				
P	**0.009**	0.552				
BW, kg	-0.254	0.344	0.21			
P	0.231	0.127	0.374			
BCS	-0.318	0.06	-0.105	**0.468**		
P	0.130	0.796	0.659	**0.021**		
DIM	**-0.475**	**0.681**	0.096	0.03	-0.076	
P	**0.019**	**0.001**	0.686	0.889	0.723	
FPCMY, kg	**0.963**	-0.176	**-0.488**	-0.247	0.142	**-0.464**
P	**0.001**	0.445	**0.029**	0.245	0.736	**0.022**

Table 20. Correlation (r values) among milk yield, fat and protein corrected milk (FPCMY), body weight (BW), body condition score (BCS) and days in milk (DIM) with basal concentration of insulin and glucose during glucose tolerance test and insulin tolerance test. Separate analysis for diet history.

	Milk yield	Insulin	Glucose	BW	BCS	DIM	Milk yield	Insulin	Glucose	BW	BCS	DIM
	Diet SSS						Diet SSF					
Insulin, µg/l	0.108						0.624					
P	0.838						0.260					
Glucose, mg/dl	-0.713	**-0.836**					-0.526	**0.958**				
P	0.177	**0.078**					0.363	**0.042**				
BW, kg	0.198	0.320	-0.413				-0.257	0.673	0.347			
P	0.706	0.537	0.489				0.623	0.213	0.568			
BCS	-0.485	-0.109	0.264	0.523			0.337	0.020	-0.500	0.018		
P	0.330	0.838	0.668	0.286			0.513	0.975	0.391	0.973		
DIM	-0.667	0.521	-0.033	-0.175	0.072		-0.341	0.710	0.655	**0.900**	-0.263	
P	0.148	0.289	0.958	0.74	0.892		0.508	0.179	0.230	**0.014**	0.614	
FPCMY, kg	**0.991**	0.065	-0.714	0.158	-0.487	-0.723	**0.922**	0.388	-0.274	-0.501	0.253	-0.444
P	**0.001**	0.903	0.175	0.765	0.327	0.104	**0.009**	0.519	0.655	0.311	0.628	0.378
	Diet FFF						Diet FFS					
Insulin, µg/l	**-0.901**						-0.389					
P	**0.099**						0.446					
Glucose, mg/dl	**-0.886**	**0.895**					-0.648	-0.582				
P	**0.045**	**0.298**					0.237	0.304				
BW, kg	-0.637	0.751	**0.814**				**-0.721**	0.296	0.194			
P	0.174	0.249	**0.094**				**0.106**	0.569	0.755			
BCS	0.331	0.045	-0.534	0.205			**-0.719**	0.235	0.323	**0.864**		
P	0.521	0.955	0.354	0.696			**0.107**	0.654	0.597	**0.026**		
DIM	-0.730	0.660	0.368	0.245	-0.088		-0.251	**0.902**	-0.517	0.078	-0.109	
P	0.100	0.340	0.542	0.640	0.868		0.632	**0.014**	0.372	0.883	0.837	
FPCMY, kg	**0.951**	-0.754	**-0.823**	-0.437	0.411	-0.854	**0.989**	-0.326	-0.743	-0.642	-0.696	-0.181
P	**0.004**	0.246	**0.087**	0.386	0.418	0.030	**0.001**	0.528	0.151	0.170	0.124	0.731

Table 21. Plasma insulin concentration (μg/L) in lactating primiparousSarda ewes from 45 to 165 days in milk (DIM).

	DIM								*P level*			
Group*	45	65	85	125	145	165	Mean	SEM[a]	PGdiet[b]	Ldiet[c]	DIM	PGdiet × Ldiet × DIM[d]
SSS	0.19	0.55	0.23	0.44	1.31	1.05	0.70	0.15	N.S.[e]	N.S.	0.05	N.S.
SSF	0.08	0.71	0.57	0.34	0.90	0.75	0.60	0.13				
FFS	1.03	0.46	0.64	0.72	1.17	2.05	0.98	0.19				
FFF	0.17	0.26	0.77	1.55	0.96	1.09	0.80	0.19				

[a] Standard error of the mean; [b] effect of pre-natal life and growing diet; [c] effect of lactation diet; [d] effect of pre-natal life and growing diet × lactation diet × time interaction; [e] not significant

SSS= starch-starch-starch group (4 ewes fed a starch diet during pre-natal life, growing and in mid lactation), SSF=starch-starch-fiber group (4 ewes fed a starch diet during pre-natal life, growing and a fiber diet during mid lactation), FFS=fiber-fiber-starch group (4 ewes fed a fiber diet during pre-natal life, growing and a starch diet during mid lactation) and FFF=fiber-fiber-fiber group (4 ewes fed a fiber diet during pre-natal life, growing and in mid lactation

Table 22. Plasma growth hormone concentration (GH, ng/ml) in mid lactating primiparous Sarda ewes

	Days in milk (DIM)					*P level*			
Group*	85	125	145	Mean	SEM[a]	PGdiet[b]	Ldiet[c]	DIM	PGdiet ×Ldiet ×DIM[d]
SSS	0.81	1.40	1.12	1.11	0.26	N.S.[e]	N.S.	0.002	N.S.
SSF	0.80	1.29	1.19	1.09	0.16				
FFS	0.75	1.53	0.94	1.10	0.11				
FFF	1.06	1.57	1.42	1.41	0.11				

[a] Standard error of the mean; [b] effect of pre-natal life and growing diet; [c] effect of lactation diet;[d] effect of pre-natal life and growing diet × lactation diet × time interaction; [e]not significant

* SSS= starch-starch-starch group (4 ewes fed a starch diet during pre-natal life, growing and in mid lactation), SSF=starch-starch-fiber group (4 ewes fed a starch diet during pre-natal life, growing and a fiber diet during mid lactation), FFS=fiber-fiber-starch group (4 ewes fed a fiber diet during pre-natal life, growing and a starch diet during mid lactation) and FFF=fiber-fiber-fiber group (4 ewes fed a fiber diet during pre-natal life, growing and in mid lactation

Table 23. Fasting plasma growth hormone concentration (GH, ng/ml) in mid lactating primiparous Sarda ewes at 160 DIM in close blood samplings(3 samples in 10 minutes, intervals of 5 minutes).

	Time **					*P level*			
Group*	T1	T2	T3	Mean	SEM[a]	PGdiet[b]	Ldiet[c]	Time	PGdiet×Ldiet×time[d]
SSS	1.22	1.11	1.81	1.38	0.25	N.S.[e]	N.S.	N.S.	N.S.
SSF	1.21	1.20	1.19	1.20	0.13				
FFS	1.48	1.51	1.34	1.44	0.20				
FFF	1.90	1.33	1.37	1.53	0.20				

[a] Standard error of the mean; [b] effect of pre-natal life and growing diet; [c] effect of lactation diet;[d] effect of pre-natal life and growing diet × lactation diet × time interaction; [e]not significant

* SSS= starch-starch-starch group (4 ewes fed a starch diet during pre-natal life, growing and in mid lactation), SSF=starch-starch-fiber group (4 ewes fed a starch diet during pre-natal life, growing and a fiber diet during mid lactation), FFS=fiber-fiber-starch group (4 ewes fed a fiber diet during pre-natal life, growing and a starch diet during mid lactation) and FFF=fiber-fiber-fiber group (4 ewes fed a fiber diet during pre-natal life, growing and in mid lactation

** T1= 0 min; T2= +5 min; T3= +10 min

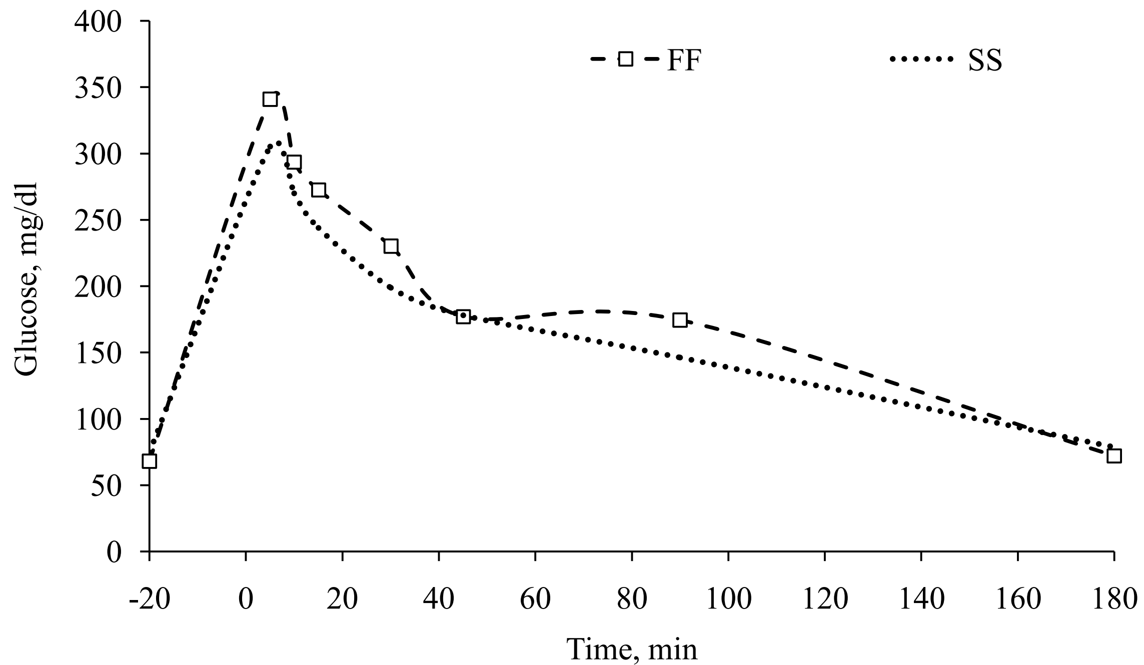

Figure 1. Evolution of plasma glucose concentration during glucose tolerance test in pregnant primiparous Sarda ewes fed a starch-starch (5 SS: fed a starch diet during pre and post-natal life or growing), and fiber-fiber (5 FF: fed a fiber diet during pre and post-natal life or growing) diet.

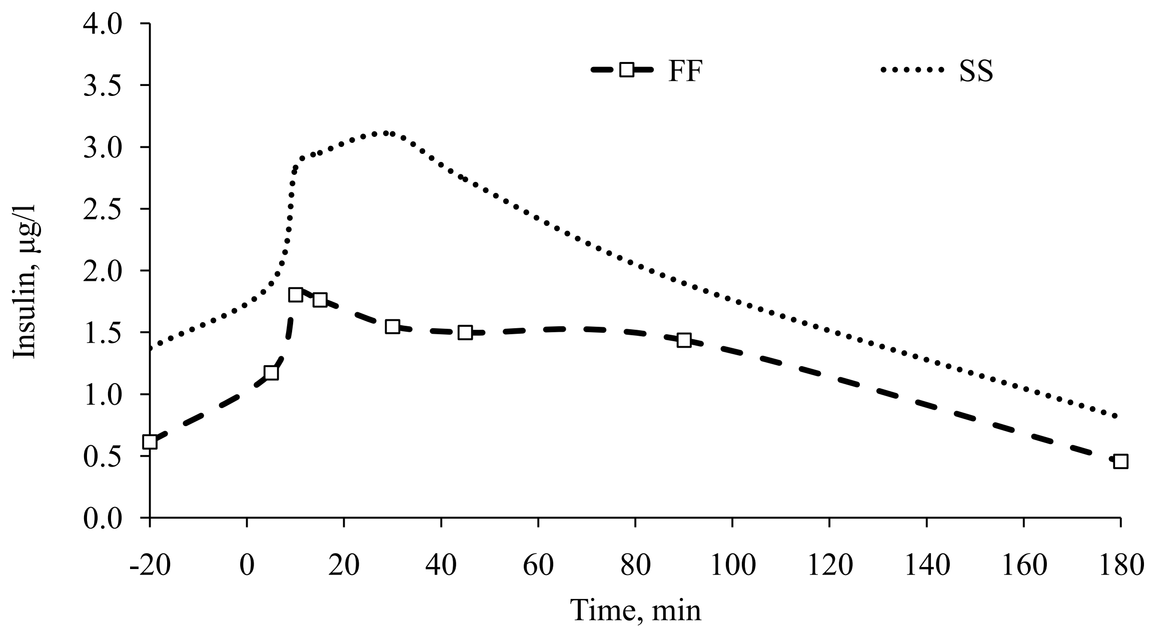

Figure 2. Evolution of plasma insulin concentration during glucose tolerance test in pregnant primiparous Sarda ewes fed a starch-starch (5 SS, fed a starch diet during pre and post-natal lifeor growing), and fiber-fiber (5 FF: fed a fiber diet during pre and post-natal life or growing) diet.

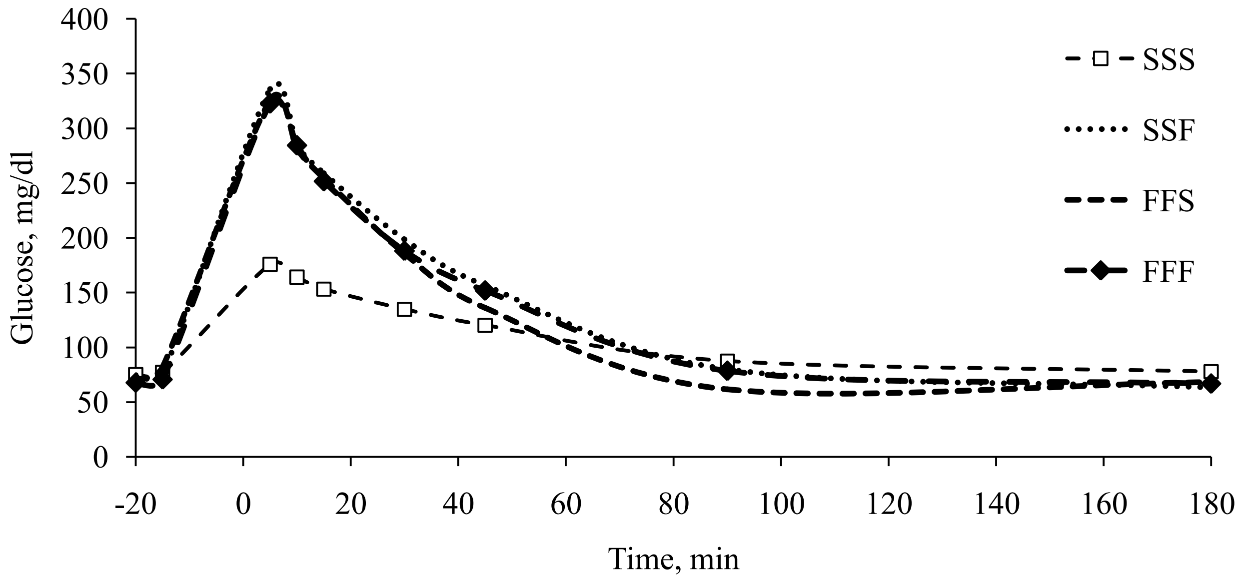

Figure 3. Evolution of plasma glucose concentration during glucose tolerance test in mid-lactating primiparous Sarda ewes fed a starch-starch-starch (3 SSS: fed a starch diet during pre-natal life, growing and in mid lactation), starch-starch-fiber (3 SSF: fed a starch diet during pre-natal life, growing and a fiber diet during mid lactation), fiber-fiber-starch (3 FFS: fed a fiber diet during pre-natal life, growing and a starch diet during mid lactation) and fiber-fiber-fiber (3 FFF: fed a fiber diet during pre-natal life, growing and in mid lactation) diet.

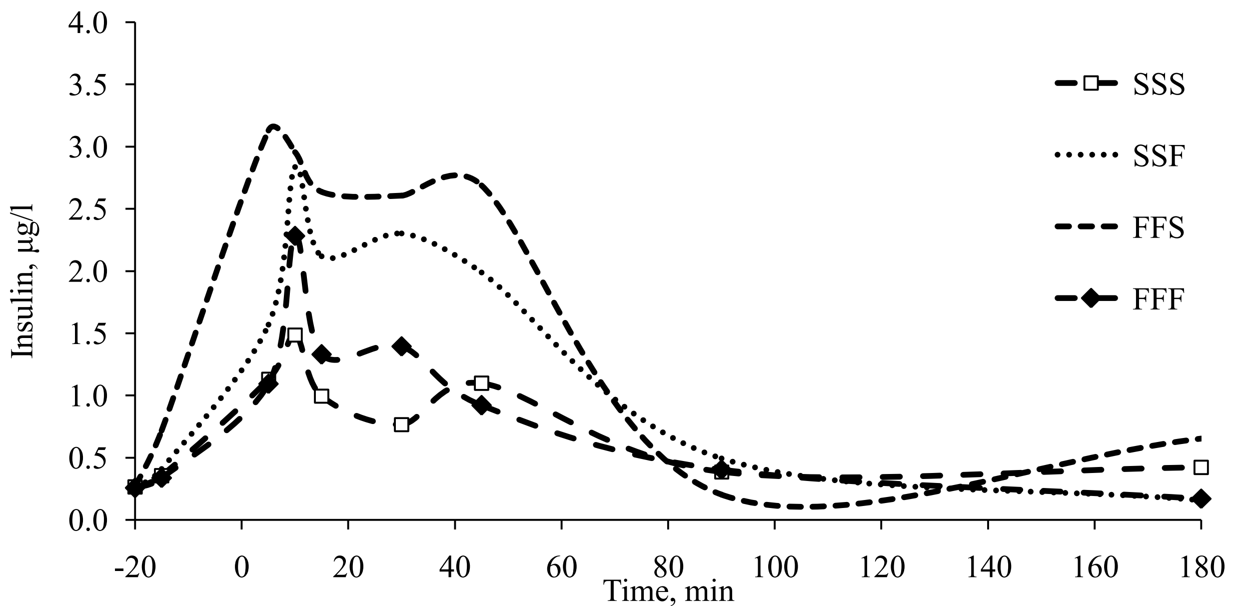

Figure 4. Evolution of plasma insulin concentration during glucose tolerance test in mid-lactating primiparous Sarda ewes fed a starch-starch-starch (3 SSS: fed a starch diet during pre-natal life, growing and in mid lactation), starch-starch-fiber (3 SSF: fed a starch diet during pre-natal life, growing and a fiber diet during mid lactation), fiber-fiber-starch (3 FFS: fed a fiber diet during pre-natal life, growing and a starch diet during mid lactation) and fiber-fiber-fiber (3 FFF: fed a fiber diet during pre-natal life, growing and in mid lactation) diet.

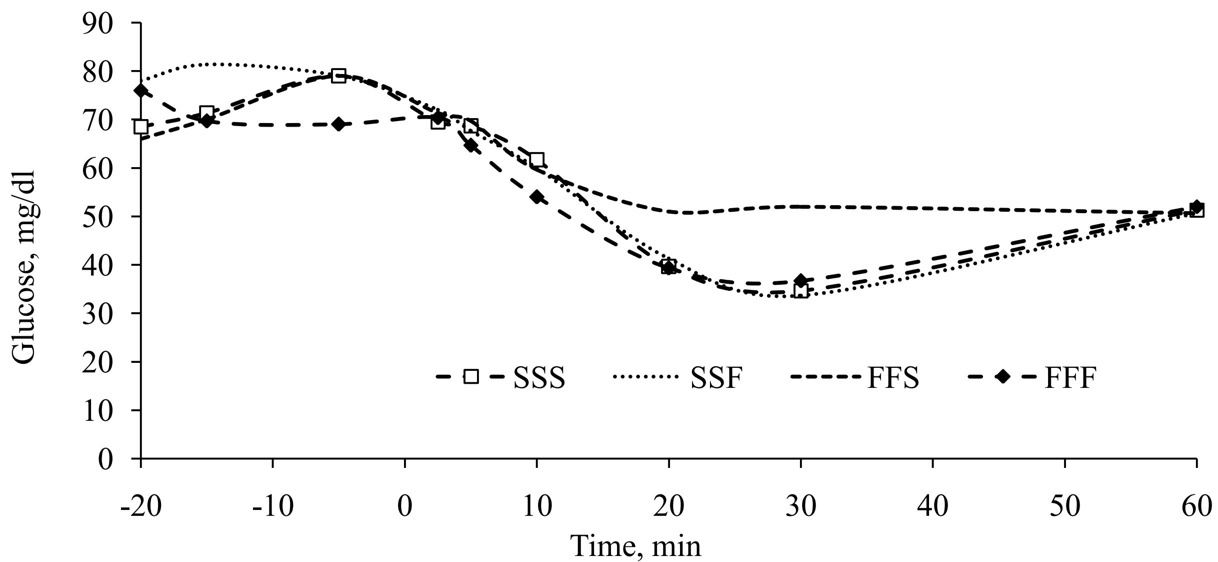

Figure 5. Evolution of plasma glucose concentration during insulin tolerance test in mid-lactating primiparous Sarda ewes fed a starch-starch-starch (3 SSS: fed a starch diet during pre-natal life, growing and in mid lactation), starch-starch-fiber (3 SSF: fed a starch diet during pre-natal life, growing and a fiber diet during mid lactation), fiber-fiber-starch (3 FFS: fed a fiber diet during pre-natal life, growing and a starch diet during mid lactation) and fiber-fiber-fiber (3 FFF: fed a fiber diet during pre-natal life, growing and in mid lactation) diet.

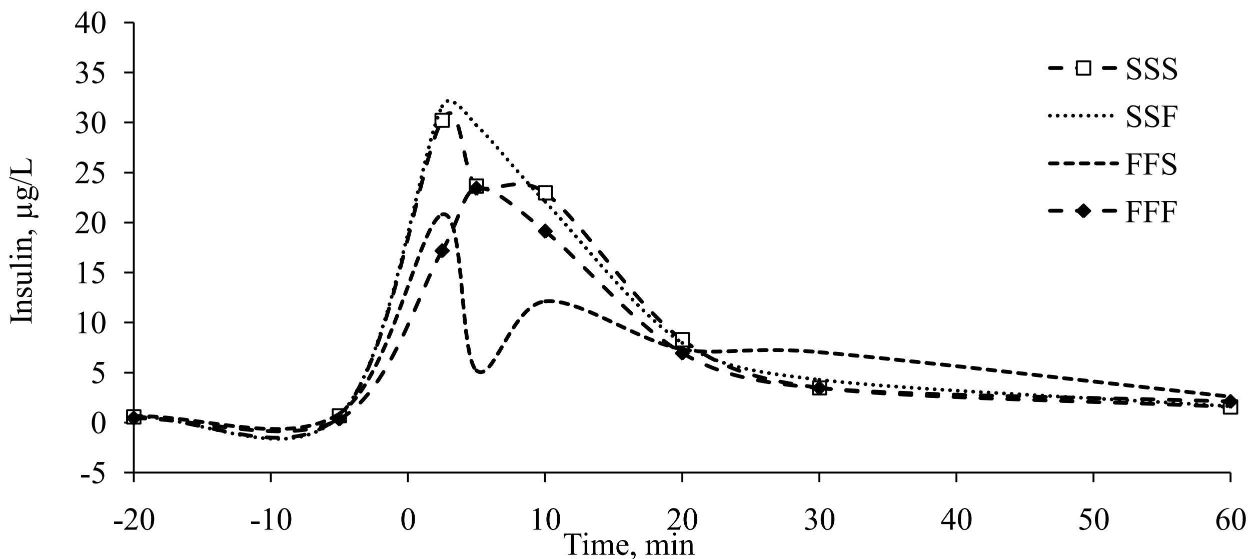

Figure 6. Evolution of plasma insulin concentration during insulin tolerance test in mid-lactating primiparous Sarda ewes fed a starch-starch-starch (3 SSS: fed a starch diet during pre-natal life, growing and in mid lactation), starch-starch-fiber (3 SSF: fed a starch diet during pre-natal life, growing and a fiber diet during mid lactation), fiber-fiber-starch (3 FFS: fed a fiber diet during pre-natal life, growing and a starch diet during mid lactation) and fiber-fiber-fiber (3 FFF: fed a fiber diet during pre-natal life, growing and in mid lactation) diet.

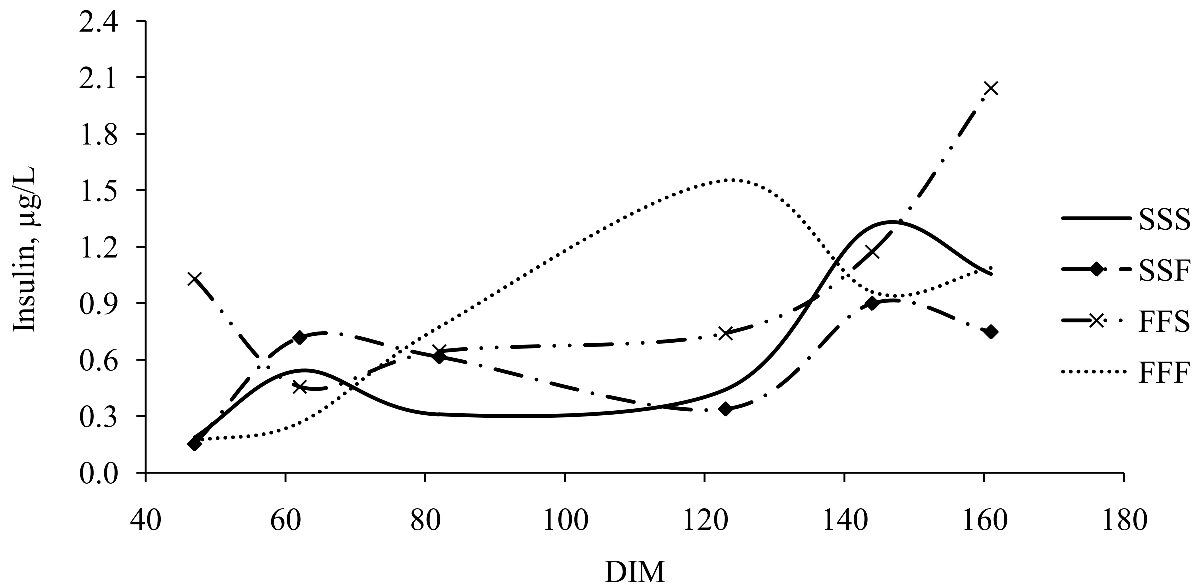

Figure 7. Evolution of insulin concentration in mid lactating primiparous Sarda ewes fed a starch-starch-starch (4 SSS: fed a starch diet during pre-natal life, growing and in mid lactation), starch-starch-fiber (4 SSF: fed a starch diet during pre-natal life, growing and a fiber diet during mid lactation), fiber-fiber-starch (4 FFS: fed a fiber diet during pre-natal life, growing and a starch diet during mid lactation) and fiber-fiber-fiber (4 FFF: fed a fiber diet during pre-natal life, growing and in mid lactation) diet.

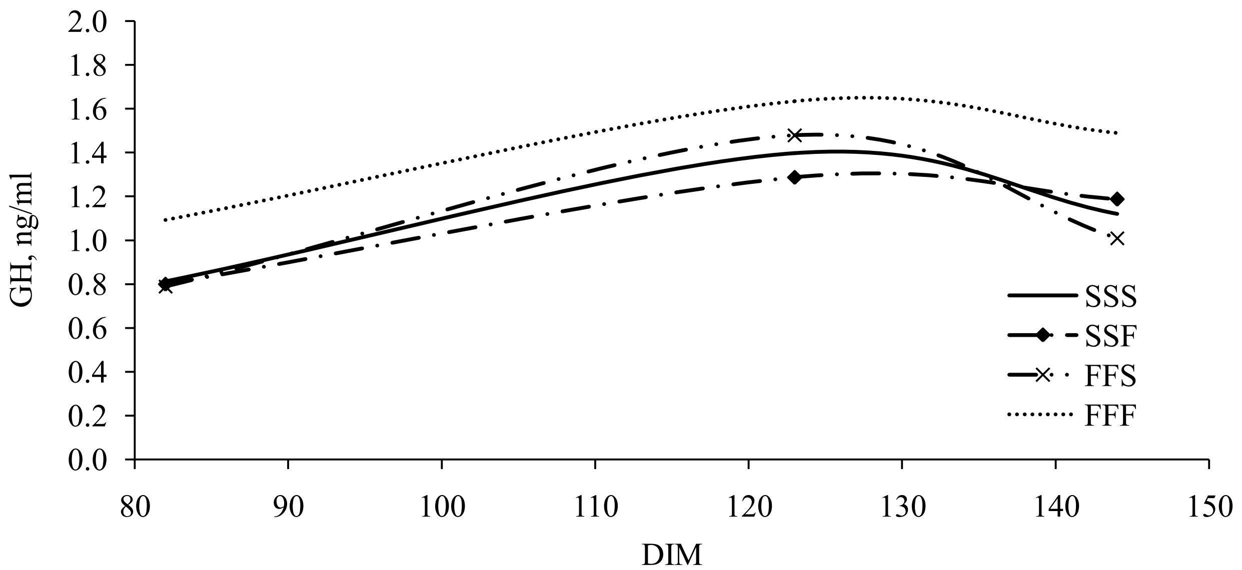

Figure 8. Evolution of growth hormone (GH) concentration in mid lactating primiparous Sarda ewesfeda starch-starch-starch (4 SSS: fed a starch diet during pre-natal life, growing and in mid lactation), starch-starch-fiber (4 SSF: fed a starch diet during pre-natal life, growing and a fiber diet during mid lactation), fiber-fiber-starch (4 FFS: fed a fiber diet during pre-natal life, growing and a starch diet during mid lactation) and fiber-fiber-fiber (4 FFF: fed a fiber diet during pre-natal life, growing and in mid lactation) diet.

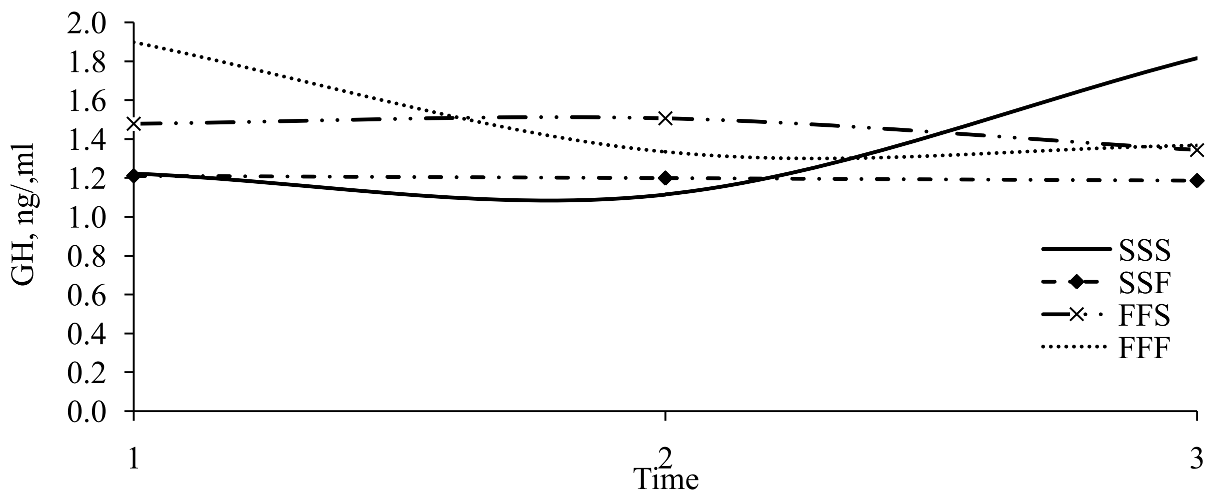

Figure 9. Evolution of growth hormone (GH) concentration from T1 to T3 minutes (T1= 0 min, T2= +5 min, T3=+10 min) in mid lactating primiparous Sarda ewes fed a starch-starch-starch (4 SSS: fed a starch diet during pre-natal life, growing and in mid lactation), starch-starch-fiber (4 SSF: fed a starch diet during pre-natal life, growing and a fiber diet during mid lactation), fiber-fiber-starch (4 FFS: fed a fiber diet during pre-natal life, growing and a starch diet during mid lactation) and fiber-fiber-fiber (4 FFF: fed a fiber diet during pre-natal life, growing and in mid lactation) diet.

CHAPTER 6

1. GENERAL CONCLUSIONS, AND PRATICAL IMPLICATIONS

The data presented in this Dissertation did not show significant evidences that an early and prolonged exposure to glucogenic or lipogenic dietary carbohydrates could induce permanent metabolic changes in dairy sheep and in a sufficient manner to economically influence the lactation persistency in dairy ewes. The experimental treatments did not always affect significantly sheep performance. Different patterns and broad numerical differences were observed among the lactation curve and fat and protein corrected milk of the experimental groups. These differences became statistically different only when a small group of animals with homogenous physiological status, in terms of stage of lactation, was considered. The performances of the experimental groups were characterized by a large individual variability that was only partially attributable to the experimental treatments.

Comparing observed results with findings from published literature, it is possible to deduce that the substitution of starch with high digestible fiber in diets of mid lactating sheep always caused numerical or significant increases of fat and protein corrected milk yield and improved partitioning of nutrients to mammary gland. The lack of statistical significance observed in many conditions indicates that this nutritional strategy might be effective only when applied in specific conditions that still need to be elucidated. The work of this Thesis partially contributed to highlight some possible element. In fact the experimental evidences of this work suggested that, early in life (prenatal and growing phase), animals fed high starch based diet developed more insulin resistance whereas those fed fiber based in the same period of life developed higher insulin sensitivity. This influence on the glucose metabolism seems also affect the nutrient partitioning in mid to late lactation. In particular, animals fed starch diets early in life, thus with glucogenic nutritional background, showed higher milk persistency when in mid to late lactation were fed a diet with high digestible fiber than animals that were fed fiber early in life (thus with lipogenic nutritional background) and either fed starch or fiber in mid to late lactation.

Thus it is not possible to completely exclude that exposure to glucogenic and lipogenic substrates could affect the metabolic regulation of lactating dairy sheep in the long term and permanently. Nevertheless, in this study the effect of the diet in respect to the metabolic programming was very small in comparison to effects of overnutrition or undernutrition on glucose and insulin metabolism already observed in literature for ruminants.

These hypothesis, need to be confirmed with deep focus on the metabolic pathways that accompanied the nutrient partitioning in each group of animals, data that are not available at the moment but will allow to valorize the effort carried out on this experiment. Further investigation might include the study of the litter size and of the growing rates of the animals in early stages of life in order to exclude confounding effects on studying their first lactation performance and their response to dietary treatments late in lactation.