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FORAGE QUALITY AND METHANE EMISSIONS

IN DAIRY SHEEP

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TABLE OF CONTENTS

CHAPTER 5 GENERAL CONCLUSIONS, SCIENTIFIC AND PRACTICAL IMPLICATIONS

LIST OF TABLES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Figure 5. Relative methane emission per g of DM (control=100 %) in the batch fermentation of the 8 treatments. CTR = Control without additives; Nano: Nanocarriers of double layered hydroxides (Nano): NAl Aluminum nitrate 2 %; A: Acrylate 2 %; NAl+A: Aluminum nitrate 1 % + Acrylate 1%; Nano NAl : nano +1 % of nitrate; Nano A: nano+1% of acrylate; iv) 2 Nano: Nano+ 0.5 % of nitrate + 0.5% of acrylate; NCa: Calcium nitrate + 2 %. 181

Figure 6. Relative methane emission per g of NDF (control=100 %) in the batch fermentation of the 8 treatments. CTR = Control without additives; Nano: Nanocarriers of double layered hydroxides (Nano): NAl Aluminum nitrate 2 %; A: Acrylate 2 %; NAl+A: Aluminum nitrate 1% + Acrylate 1%; Nano NAl : nano +1% of nitrate; Nano A: nano+1% of acrylate; iv) 2 Nano: Nano+ 0.5% of nitrate + 0.5% of acrylate; NCa: Calcium nitrate + 2 %. 182

LIST OF ABBREVIATIONS

3-NOP	3-nitrooxy-propanol
A	acrylate
A	acetic acid
A/P	acetic/propionic ratio
ADF	acid detergent fiber
ADL	acid detergent lignin
B	butyric acid
BCS	body condition score
BW	body weight
CP	crude protein
CTR	control
DIM	days in milk
DM	dry matter
DMD	dry matter digestibility
EE	ether extract
F:C	forage concentrate ratio
FA	fatty acid
FID	flam ionization detector
FPCM	fat and protein corrected milk
FW	final weight
GC	gas chromatograph
GHG	greenhouse gas
GP	gas production
GP	gas production
GWP	global warming potential

H-NDF	High NDF
Indg. NDF	indigestible neutral detergent fiber
IPCC	intergovernmental Panel on Climate Change
IVDMD	*in vitro* dry matter digestibility
IW	initial weight
kd	rate of fermentation of the substrate
LMD	laser methane detector
L-NDF	low NDF
Mcr	methyl-ComM reductase
MEF	methane emissions factor
MIR	mean infrared
MUFA	monounsaturated fatty acids
MUN	milk urea nitrogen
N	nitrogen
NAl	aluminum nitrate
NCa	calcium nitrate
NDF	neutre detergent fiber
NDFD	neutre detergent fiber digestibility
NDFdig	neutral detergent fiber digested
NDFI	neutral detergent fiber intake
NDFom	neutral detergent fiber organic matter
NE_L	net energy for lactation
NFC	non-fibrous carbohydrates
NP	nanoparticles
NSC	non-structural carbohydrates

nZnO	nano zinc oxide
OM	oganic matter
P	propionic acid
PAC	portable accumulation chamber
PEF	physically effective fiber
ppm	part per million
PUFA	polyunsaturated fatty acids
RFQ	relative forage quality
RFV	eelative feed value
RH	relative humidity
SAT	sostanze azotate totali
SCC	somatic cell count
SEM	standar error of the mean
STP	standard temeperature and pressure
T	temperature
TMR	total mixed ration
Treat	treatment
Vf	final volume
VFA	volatile fatty acid
VMR	volume mixing ratio
vol	volume
VR	ventilation rate

GENERAL ABSTRACT

The work of this thesis focused direct methane measurements in *in vivo* and *in vitro* trials to test possible nutritional strategies aimed to reduce methane emissions from dairy sheep, specifically working on their diets in term of forage quality and inclusion of additives based on nitrate compounds. Several studies in recent years have focused strategies to mitigate methane emissions from ruminants. The work of this thesis was conducted in the experimental facility of the University of Sassari in collaboration with two EU LIFE+ projects, Forage4Climate (LIFE 15 ITCCM/000039) and SheepToShip Life (LIFE 15 ITCCM/000123) which both aimed to define and diffuse good practices in sheep dairy farming to reduce methane emissions. The experimental activity also included the development of a ventilated hood system for direct measurements of methane *in vivo* on dairy sheep. This equipment represents a significant advance in the experiments of the animal nutrition research group of the University of Sassari.

The thesis consist of 4 chapters:

The first chapter includes a review of literature and the rationale background.

The second chapter presents a trial planned with the aim to evaluate the effect of hays with different NDF content on milk production, digestibility and methane emissions in dairy sheep. The experimental hypothesis assumed that a better quality of forages increases milk production and reduces methane emissions. It was observed that sheep fed hays with lower vs. higher content of NDF (54 vs. 66% of DM) produced significantly more milk (0.72 vs 0.53 kg/d respectively; $p < 0.02$), had higher DMI (1.65 vs 1.22 kg/d respectively; $P < 0.05$), lower milk urea (25.63 vs 32.97 mg/dl respectively; $p< 0.01$) and lower methane emissions per g of DMI (12.25 vs 15.62 g/kg DMI respectively; $p < 0.04$). The obtained results confirmed that the quality of forage could be a promising strategy both to mitigate methane emissions and to improve productive performance in dairy sheep.

The third chapter presents a study on the effect of haylages with low and high NDF content (37 vs. 49% of DM) on lactating dairy sheep in late lactation. The results showed that milk production and methane emissions were not influenced by the diet. Oppositely, DMI was statistically higher ($P = 0.008$) in the L-NDF vs. H-NDF group (1.81 vs 1.46 kg/d respectively). Haylage quality positively affected as expected DM

digestibility (86% vs. 79%; P = 0.03 respectively) and milk urea concentration, which was statistically lower (P <0.01) in the L-NDF group than in the H-NDF group (26.1 vs 31.4 mg/dl respectively). The H-NDF group produced more methane per unit of milk than L-NDF group, even if without significant differences (66.8 vs 38.6 gCH_4/kg milk). The results of this study did not confirm the initial hypothesis and thus further studies should be conducted to understand the dynamics of methane emission in small dairy ruminants.

The fourth chapter presents an *in vitro* experiment aimed to evaluate the effects of nanocarriers associated in different combinations to nitrate based compounds and other additives to reduce the methane emission of a generic total mixed ration. The experimental hypothesis assumed that nanocarriers might enhance additive activity in the rumen. Cumulative methane emissions at 48h (ml of CH_4 per g of DM) were significantly affected by treatments (P< 0.001). In terms of ml of methane per ml of produced gas, the use of aluminum nitrate NAl caused a reduction of emissions of 14% in respect to control without additives; whereas the combination of a nanocarrier based on double layered hydroxides and half dosis of NAl obtained a reduction of methane of 15% in respect to control (P<0.05). This work confirm the opportunity to continue research on nano materials as possible coadjutant in enhancing the effect of bioactive compounds in the rumen and to quantify the dose-response effects in ruminant diets.

CHAPTER I

Introduction and review of literature

1. METHANE EMISSIONS FROM SMALL RUMINANTS AND CLIMATE CHANGE

In recent years climate change has been recognized as one the main factors affecting sustainability of production systems and is actually linked to all the human activities including economic, energy, technological and environmental sector (Zheng *et al.*, 2019). Since agriculture is strongly influenced by environment and climate, it is also one of the first sector affected by climate change and for this reason changes in agricultural ecosystems are also used as indicator of climate change itself.

The increase in the earth surface temperature is considered an unequivocal sign of climate change. According to Intergovernmental Panel on Climate Change report (IPCC, 2018), it was estimated that anthropogenic activities contributed in the past to an increase in global warming by 1 °C, whit a continuously increasing trend that could reach 1.5 °C between 2030 and 2052. Climate change impacts on water supply and food security and therefore indirectly on the farm income, especially in those countries that present extreme temperatures, mainly developing countries with average high temperatures or very cold areas of the globe which are experiencing dramatic changes in their ecosystems due to temperature raises. Moreover, according to the latest prospects in 2055 the world population will be over 10 billion people (compared to 7.4 billion today) and will need large amount of food to be nourished (Schultz, 2017). In this context, agriculture is the sector that will have to feed the constantly growing population. A fundamental role in global warming is played by greenhouse gas (GHG) which, by trapping part of the infrared radiation emitted by the soil, cause an increase in air temperature (Peters, 1985). The GHG can be divided into two large groups: those produced naturally and partly from human activity, such as water vapor, carbon dioxide (CO_2), ozone (O_3), methane (CH_4) and nitrous oxide (N_2O), and those exclusively of human origin

including hydrofluorocarbon, perfluorocarbons and sulfur hexafluoride (Johnson *et al.*, 2007). In order to assess the effects of GHG emissions in an equal way, a single unit of measurement was introduced, the global warming potential (GWP), and the unit of measurement is the CO_2 equivalent (CO_2eq). According to Fifth assessment report of IPCC (Edenhofer *et al.*, 2014) methane has a GWP about 21-25 times greater than that of CO_2, while the nitrous oxide about 265 times greater than that of CO_2. The GHGs of the first group are those most produced and their relative quantities are: water vapor (36-70 %), carbon dioxide (9-26 %), methane (4-9%), and nitrous oxide (3-7 %) (Russell, 2007). The world's GHG are about 47 million gigagrams (Gg) of CO_2eq. The sector that produce more GHGs worldwide are energy (53 %), transport (13 %), agriculture (11 %), industry (8%), waste (3%) and other sources (12 %) (Figure 1) (FAOSTAT, 2010).

As GHG, methane (CH_4) plays an important role in global warming, and its impact comes only after that of CO_2 emissions (Xiaoli *et al.*, 2010). Chemically, methane is a simple molecule, formed by 1 atome of carbon and 4 atoms of hydrogen, and belongs to the group of alkanes. At atmospheric pressure it is found a gas, odorless, colorless and highly flammable; in fact, its concentration in the atmosphere is very low, ranging from 1.8 to 1.9 parts per million (ppm). Yusuf *et al.*, in a review of 2012 identified three major sectors that have the greatest impact on anthropogenic methane emissions: the agricultural sector, which contributes about 53 %, the energy sector, which contributes 20%, and finally the waste sector, which contributes for 19 %. The data relating to methane emissions by the agricultural sector are shown in Figure 2. According to the latest FAO data (FAOSTAT, 2017), within the agricultural sector livestock is the largest source of methane production. In fact, enteric fermentation produces about 70% of the methane deriving from agriculture, while manure management accounts for 7 %. Another source of methane emission within the agricultural

sector is the rice cultivation, which produces about 18 % of methane. Thus, we can say that livestock sector accounts for about 40% of global anthropogenic methane emissions. This is in agreement with what reported by Yusuf *et al.*, (2012), which indicates global anthropogenic methane emissions by livestock between 25.5 and 40 % of the whole emitted methane at global level.

The global ruminant domestic population is about 3,929 million heads. Cattle are the most raised ruminant (1,491 million heads, 38 % of total ruminant), followed by sheep (1,202 million heads, 31 % of total ruminants), goats (1,034 million heads, 26 % of total ruminant) and finally buffaloes (5 % of total ruminants) (FAOSTAT, 2017). Therefore, small ruminants represent about 57 % of the world's ruminant population, so their importance in terms of global methane emissions is decisive. Despite this, GHG emissions from small ruminant represent only 6.5 % (about 475 million tons CO_2eq) (Figure 3) of total emissions by livestock sector, which 299 million tons are allocated to meat production and 130 million tons to milk production (Gerber *et al.*, 2013). The various sources of GHG emissions are very similar for both meat production and milk production. In fact, as can see in Figure 4, enteric fermentations account for 55-57 % of the total GHG emissions from small ruminants, manure management for 16-17 %, the feed affects the 11-12 %, while fertilizers and crop residues for 7-9 % (Gerber *et al.*, 2013). Therefore, methane is the main GHG that is produced by ruminants.

Enteric methane emissions in the world are constantly increasing; according to a study carried out by the EPA (2011), between 1990-2000 enteric methane emissions increased by only 1.5 %, while between 2000 and 2010 they increase by 17 %. According to the latest data from FAO (FAOSTAT, 2017), the goat sector has produced about 5.17 million tons of methane from enteric fermentations (about 109 million tons of CO_2 eq), while the amount of methane

produced by enteric fermentations by sheep sector is about 6.72 million tons (about 141 million tons CO_2eq). Another activity that affect methane emissions is the manure management. Large part of excretion from small ruminants are released at pasture in aerobic conditions and emissions are generally much lower than enteric methane emission (IPCC, 2006; Gerber *et al.*, 2013). In fact, worldwide manure management in the sheep sector produces about 0.19 million tons of methane (about 4.06 million tons CO_2eq), while goats through manure produce about 0.18 million tons of methane (about 3.76 million tons CO_2eq) (FAOSTAT, 2017).

The methane measurements emitted by ruminants are important from an environmental point of view, as the livestock sector is the main producer of methane (Getabalew *et al*., 2019). Furthermore, the spread of small ruminants worldwide is concentrated in regions were climate fluctuations are greater (particularly in arid and semi-arid regions), and further increases in temperature as a result of climate change could cause problems in adapting to these species Moreover, during the process of methanogenesis a part of the gross energy (about 2-15 %) is used by methanogens for the production of methane, and consequently this lost energy lowers the efficiency of production (Guo et al., 2008).

As mentioned above, GHG emissions have a primary role in climatic change. In particular animal productions have a very important environmental impact, therefore it is necessary to look for the best strategies to mitigate this impact. The impact of livestock is not just about climate change, but also other resources such as water, land and biodiversity (Steinfeld *et al.*, 2006). The production of enteric methane and, to a lesser extent the manure management, are the main indicators of the impact of livestock farms. About 35 % of the methane produced worldwide is derived from enteric fermentations; if we add to this a further 4-5 % deriving from manure management, we can say that livestock farms account for about 40 % of global

methane emissions (Yusuf *et al.*, 2012; FAOSTAT, 2017). Knowing methane emissions precisely is fundamental to being able to implement the best strategies to reduce these emissions.

2. RUMINAL FERMENTATIONS AND METHANE STECHIOMETRY

Ruminants are mammals that thanks to their specialized digestive system have distinguished themselves from other herbivores for their ability to digest the fibrous part of feed (Van Soest, 1994). The ability of ruminants to draw energy from the fibrous parts of plants is due to their thick ruminal micro-fauna composed of bacteria (10^{10} – 10^{11} cells/ml), protozoa (10^{4} – 10^{6} ml), fungi (10^{3} – 10^{5} zoospores/ml) and bacteriophages (10^{8} – 10^{9} ml) (Kamra, 2005). These microorganisms that live inside the rumen in an anaerobic environment, are able to obtain energy through the hydrolysis of proteins, starch and the cell wall of plants that are transformed into simple products such as amino acids and sugars (Guan *et al.*, 2006).

The bacteria represent the largest group of microorganisms within the rumen. Substrates attacked by bacteria are multiple, but generally they act on molecules already hydrolyzed, such as monomers and oligomers that derive from the hydrolysis of more complex substances such as starch, proteins, cellulose and hemicellulose (Stewart *et al.*, 1997).

Another group of microorganisms present in the rumen are protozoa (Williams and Coleman, 1997). The classes of protozoa that are present inside the rumen are two: the ciliates and the flagged, even if the ciliates are by far the most numerous (Jouany, 1996). The role of protozoa in rumen fermentations is still not entirely clear. According to some researchers, protozoa would be responsible for the degradation of a significant proportion of fiber inside the rumen

(30-40 %), but also a part of non-structural carbohydrates (Williams and Coleman, 1997). The dynamics of protein and lipid fermentations by protozoa are less common.

The anaerobic fungi are the last big group of microorganism present within the rumen, and were also the last to be discovered. The rumen fungi have two life cycles: one in the form of zoospores (flagellated and mobile form) and one below the thallus form (reproductive and non-motile form) (Mountfort, 1987). Despite being the last to be discovered, several studies have shown that fungi have a crucial role in the production of enzymes that are able to degrade the structural carbohydrates present in the plant cell walls (Mountfort, 1987).

2.1 Nutrient fermentation

Carbohydrates fermentation. among the energy sources, starch and nonfibrous carbohydrates represents the most important sources present in highly productive ruminant diets (McAllister *et al.*, 1990). Starch is an organic complex belonging to the category of carbohydrates, and is formed from two different polymers: amylose and amylopectin (Chesson and Forsberg, 1997). The degradation of starch inside the rumen occurs by bacteria called amylolytic. Within this group of bacteria there are various species, among which the most studied is it *Streptococcus bovis*, which is responsible for the production of lactic acid in the rumen (Walker and Hope, 1964; Walker, 1965). However, amylolytic bacteria are made up numerous other species including: *Ruminobacter amylophilus*, *Butyrivibrio fribrisolvens*, *Bacteroides ruminicola*; these have been identified as the bacteria that most degrade starch (McAllister *et al.*, 1990). We can say that some of the ciliated protozoa (entodiniomorph) and some of fungi can degrade starch and sugar (Chesson and Forsberg, 1997). The final products of starch digestion in the rumen are volatile fatty acids (VFA), used as an energy source by microorganism that synthesize proteins (Huhtanen and Sveinbjörnsson, 2006). The main VFA

that is produced by the digestion of starch is propionate, which subsequently reaches the liver via the blood system, where it is used to produce glucose.

In addition to starch, ruminants, thanks to their complex digestive system, are able to digest the fibrous parts of plants. Fiber is defined as part of plants, food and feed that cannot be digested by the enzymatic systems of mammals (Moore and Hatfield, 1994). In the livestock sector fiber indicates the plant cell wall (Jung, 1997). The fiber plays a strategic role in feeding ruminants as, in addition to avoiding problems such as subacute ruminal acidosis and dysfermentation, they stimulate rumination, production of saliva and act as buffer within the rumen (Zebeli *et al.*, 2006). The polymer that make up the fiber are much more complex than those of starch, therefore digestion is not completely complete and is very slow (Mertens, 2002). Plant cell walls are composed mainly of structural carbohydrates, such as cellulose, hemicellulose and pectins, with a variable proportion of lignin, and a small amount of proteins and minerals (Collins and Fritz, 2003). Degradation of plant cell walls is mainly done by bacteria and fungi, while protozoa play a minor role (Dijkstra and Tamminga, 1995). The bacteria that attack structural carbohydrates are called cellulolytic, and among the most important we find: *Ruminococcus albus*, *Ruminococcus flavefaciens*, and *Bacteroides succinogenes* (Hungate, 1966). Among the fungi that degrade plant cell walls we find *Neocallimastix frontalis*, *Neocallimastix patriciarum*, *Piromyces communis and Orpinomyces bovis* (Chesson and Forsberg, 1997). Even the protozoa are able to degrade the structural carbohydrates, and in particular the genera *Diplodinium* and *Eudiplodinium* are the most active (Orpin, 1988). The degradation of structural carbohydrates by cellulolytic bacteria leads to the production of acetate. In fact, using diets rich in forages, it increases the quantity of acetate produced and moreover the acetate:propionate:butyrate ratio is shifted in favor of acetate, with typical values of 70:20:10 (France and Dijkstra, 2005).

Lipids fermentation. lipids are an important energy source in ruminant diets, and can be derived from various sources such as forages, cereals and oil seeds and they by-products (Harfoot and Hazlewood, 1997). The characteristic that distinguishes the various lipids is given by the type of fatty acid predominant in the structure. Forage lipids are rich in unsaturated linolenic acid (18:3) and linoleic acid (18:2); instead, the lipids of the oils used in the diets of ruminants are rich in linoleic (18:2) and oleic acid (18:1) (Harfoot and Hazlewood, 1997).

The first step in the digestion of lipids within the rumen is the hydrolysis of the linkages that are present in triglycerides and phospholipids (Bauman *et al.*, 2003). The hydrolysis of lipids is performed almost exclusively by bacteria, and in particular *Anaerovibrio lipolytica* and *Butyrivibrio fibrisolvens* are the two species concerned (Harfoot and Hazlewood, 1997).

The second step is the biohydrogenation of unsaturated fatty acids which, through a series of reactions and intermediate products, lead to the formation of saturated fatty acids. Among the bacteria that effect the biohydrogenation it must surely be remembered the *Butyrivibrio fibrisolvens*, that for many years was the only one to be indicated responsible for this process (Polan *et al.*, 1964).

Nitrogen compounds fermentation. the microbial population inside the rumen in order to develop itself needs both energy sources deriving from the digestion of carbohydrates and lipids and protein sources deriving from the degradation of nitrogen compounds. These requirements in ruminants are met both by digestion at the intestinal level of ruminant microbes and by protein compound that is present in the diet (Leng and Nolan, 1984). Initially the proteins are hydrolyzed into oligopeptides, which are broken down into smaller segments called peptides, and finally, following the hydrolysis of the latter, there is the formation of amino acids (Wallace *et al.*, 1997). The microorganism that are able to attack the feed

particles inside the rumen are about 70-80 %, and of these about 30-50 % possess a proteolytic activity (Bach *et al.*, 2005). A proportion of peptides and amino acids deriving from protein hydrolysis are used by microorganism to form microbial proteins, while a proportion is deaminated and the carbon structure is used to form VFA, CO_2 and ammonia (Tamminga, 1979).

The bacteria that mostly perform a proteolytic activity belong to the amylolytic group and in particular the most active species are: *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *Ruminococcus albus* and *Ruminobacter amylophulus*. The proteolytic activity of cilicate protozoa has been recognized for many years, and in particular the holotrichs and entodunuimorphs are those that have the highest proteolytic activity. Among the last we can remember *Entodinium caudatum*, *Entodinium simplex*, *Polyplastron multivesciculatum* and *Eudiplodinium medium* (Wallace *et al.*, 1997). In the various studies carried out on the proteolytic activity of the fungi, no certain results were found. Howerer, in a study of (Wallace and Joblin, 1985), they identified the species *Neocallimastix frontalis* as proteolytic.

2.2 Rumen methanogenesis

As mentioned, the rumen represents the main environment where anaerobic fermentations take place, thanks to the very rich populations of microorganisms. In addition to bacteria, protozoa and fungi, there are also other types of microorganisms such as methanogens and bacteriophages that contribute to enriching the rumen ecosystem (Morgavi *et al.*, 2010). The production of methane in the rumen represents a strategy implemented to prevent an excessive accumulation of hydrogen which prevents the normal functions of rumen microorganisms. During the fermentation process of the nutrients that come from feed, there

is the production of a large amount of VFA, CO_2 and H_2, both from those bacteria that carry out the primary fermentations, and from those that carry out the secondary fermentations (microorganism that are unable to hydrolyze polymers alone) (Morgavi *et al.*, 2010).

Mainly methane is formed following fermentation by ruminal microorganism of fibrous diet carbohydrates (Immig, 1996). The oxidation of the monomers (mainly glucose) deriving from the hydrolysis of carbohydrates occurs through Meyerhof-Parnas pathway, obtaining pyruvate as the final product. During these processes' electrons are released (in the form of H_2) which are adsorbed by the NAD^+ coenzyme to form NADH which must be re-oxided to NAD^+ to allow the continuous fermentation thus recreating an environment rich in free electrons (Immig, 1996). These alterations of the normal functions of microorganisms to transfer electrons can lead to metabolic problems such as the reduction of rumen fermentations (Morgavi *et al.*, 2010). In aerobic systems the mechanism to avoids these accumulations is to reduce the oxygen present in the air (O_2), thus forming water (H_2O) (McAllister and Newbold, 2008). Because rumen is an anaerobic environment, microorganisms within it use various strategies to re-oxidize NADH. Among the various mechanisms that have evolved, some species implement the formation of pyruvate starting from butyrate and the synthesis of propionate starting from pyruvate (Immig, 1996). However, the most widely used to re-oxidize NADH is to reduce CO_2 to methane. In the rumen environment, the main group of microorganisms that reduce CO_2 to methane are methanogens, they are prokaryotes which belong to Archea. They, in addition to carbon dioxide, are capable to transforming other substances that are produced by ruminal microorganisms into methane (Patra *et al*., 2017). Substrates that can be used by methanogens for the formation of methane are formate, acetate, methanol and mono-, di- and trymethylamine, although the species that use these substrates are very few (Wolin *et al.*, 1997). The substrates that are used as an energy source by

methanogens microorganism and the related equations for methane formation are reported in Tables 1.

Among the methanogens that use CO_2 and H_2 as an energy source *Methanobrevibacter ruminantium*, *Methanobacterium formicicum*, *Methanomicrobium mobile* and *Methanosarcina barkeri* are the most common ones. Moreover, except *Methanosarcina barkeri*, these species are also able to use the formate as a substrate (Hook *et al.*, 2010).

Methanosarcina barkeri and *Methanosarcina mazeii* can form methane starting from acetate, methanol and methylamines (Stewart *et al.*, 1997).

The role that protozoa plays in methane production is still not entirely clear. Through the degradation of the carbohydrates they produce VFA (in particular butyrate and acetate), releasing during the reaction hydrogen, in quantities respectively 2 and 4 moles per mole of degraded glucose (Guyader *et al.*, 2014). The H_2 produced is then converted by methanogenic bacteria in methane.

As for fungi, some studies have shown that there is a close relationship between methanogens and some anaerobic fungi, such as *Neocallimastix frontalis* (Bauchop and Mountfort, 1981).

3. METHANE MEASUREMENT METHODS FOR SMALL RUMINANT

Throughout the last century, various methods for measuring methane emissions have been studied and developed. In this chapter the most used will be described.

3.1 Indirect measures

In vitro measurement techniques. over the years, various techniques for measuring methane production *in vitro* have been developed, starting with the manometric ones set in the 1950s

(Bhatta and Enishi, 2007). *In vitro* gas measurement techniques are based on the study of the development of fermentation gases following incubation in gas tight culture bottles of rumen liquor and a certain known quantity of feedstuff (Goopy *et al.*, 2016). The technique to measuring methane *in vitro* is very diverse, and many aspects differ, but some features are common to all methods. The amount of feed sample used can vary from 100 mg (Pell and Schofield, 1993) up to 1250 mg (Waghorn and Stafford, 1993), but in many cases a quantity of feed used ranging from 200 to 300 mg (Menke *et al*, 1979; Gallo *et al*., 2016). The ruminal fluid collection from fistulated animals can also be done differently, and in particular can be taken before feeding (Menke *et al*., 1979; Beuvink and Spoelstra, 1992) or 2 h after feeding (Waghorn and Stafford, 1993; Cone *et al*., 1996). Before incubating, the rumen liquid is added to a buffer solution that simulates the component of saliva of ruminant. Also in this case, different proportion can be used between rumen liquid and buffer solution; for example Menke *et al*., (1979) and Beuvink and Spoelstra (1992) used a buffer-to-rumen ratio 3:1 (vol/vol), while Gallo *et al*., (2018) and Tagliapietra *et al*., (2010) used a buffer-to-rumen ratio 2:1. After the incubation (at 39 °C), the pressure of the gas produced in the head space of the bottle is measured at certain intervals (generally 2, 5, 9, 24, 36, 48 hours after the start of fermentation) or in continuous to determine the amount of produced gas from physics law of gasses and the air is sampled and analyzed over time. The analysis of the gases produced is carried out through the gas chromatography by taking a certain amount of gas present in the head space of the bottle with a syringe. An alternative method for measuring the amount of gas produced has been developed by Waghorn and Stafford (1993). Through the use of a manometric measuring device the gas production measurements are recorded every 30 minutes. The disadvantage of this method is related to the small number of samples that can be measured at the same time (Getachew *et al*., 1998). A further improvement of the in vitro

gas measurement technique has been proposed by Muetzel *et al*., (2014). In this completely automated system, the bottles with the inoculum are continuously shaken and a sensor connected to the bottles measures the pressure inside them. An automatic sampling system is connected to the gas chromatograph that analyzes the composition of the gases produced during the fermentation. The method proposed by Muetzel *et al*., (2014) is an improvement on the one developed by Pell and Schofield (1993). Their system involved the use of sensors connected to the bottles to measure continuous the pressure and then the gas production, but not automatic gas sampling. The main problem found in this system that if small feed samples (100 mg) are used, the sampling error of the gases produced can be large.

The production of methane is expressed as the amount of CH_4 per gram of dry matter (mL CH_4/g DM) per gram of dry matter degraded (DMD) or per gram of NDF degraded (NDFD) (Storm *et al.*, 2012). The main advantages of *in vitro* methane emission estimation methods are reduced times and lower costs compared to *in vivo* methods. Furthermore, a very high number of fermentations can be made. The disadvantage is that being a fermentation done in a controlled environment, all those factors that normally affect *in vivo* fermentation are neglected. Furthermore, it is not always possible to find rumen liquid.

Prediction equations. over the years, numerous methane estimation equations have been developed based on the characteristics of the feed. In France, Giger-Reverdin and Sauvant, (2000) proposed an equation available on sheep, to predict the variation in losses energy as methane, using feed components as predictors, and associating with these the digestible energy:

$$ECH_4/EB = -10.5 + 0.192\ ED/EB - 0.0567\ EE + 0.00651\ St + 0.00647\ CP + 0.0111\ NDF \quad (r^2 = 0.92)$$

where ECH_4/EB = variation in losses of gross energy as methane; ED/EB = digestible energy; EE = ether extract (g/kg DM); CP = crude protein (g/kg DM); NDF = neutral detergent fiber (g/kg DM).

More recently Muetzel and Clark (2015), have developed two equations (the firs for young sheep <1 year, the second for old sheep >1 year) which uses DMI as a predictor of methane production. These equations have been formulated on grazing sheep.

$$\ln(pCH_4) = 0.754 \ln (DMI) + 3.02 \text{ (young sheep <1 year)}$$

$$\ln(pCH_4) = 0.826 \ln (DMI) + 3.15 \text{ (old sheep >1 year)}$$

where pCH_4 = methane production (g/d); DMI = dry matter intake (kg/d)

Numerous equations for estimating methane emissions have been developed on dairy cows. For example, Mills *et al.*, (2003) developed a linear equation for estimating methane production using only dry matter intake (DMI) data:

$$CH_4 = 5.93 + 0.92 \text{ DMI } (r^2 = 0.60)$$

where CH_4 = methane production (MJ/d); DMI = dry matter intake (kg/d).

More recently, in a study carried out by Ellis *et al.* (2007) on dairy cows, they proposed a predictive equation using the quantity of forage present in the diet:

$$CH_4 = 8.56 + 0.139 \text{ forage } (r^2 = 0.56)$$

where CH_4 = methane production (MJ/d); forage = (% of forage in the diet, excluding all 100% forage data sets).

There are also other models for estimating methane production, which are based on the correlation between methane and other variables. Moss *et al.*, (2000) developed an equation that considers the concentration of volatile fatty acids (VFA) present in the rumen:

$$CH_4 = 0.45 \text{ C2} – 0.275 \text{ C3} + 0.40 \text{ C4}$$

where CH_4 = methane yield (μmol); C2 = acetate (μmol); C3 = propionate (μmol); C4 = butyrate (μmol).

Other equations like the one proposed by Dijkstra *et al.* (2011), are based on the amount of fatty acids (FA) in the milk:

$$CH_4 = 24.6 + 8.74\ C17{:}0\ \textit{anteiso} - 1.97\ \textit{trans}\text{-}10 + 11\ C18{:}1$$

$$\text{-}9.09\ \textit{cis}\text{-}\ 11 + 5.07\ \textit{cis}\text{-}\ 13\ C18{:}1\ (r^2 = 0.73)$$

where C_4 = methane yield (g/kg DM); individual FA are in g/100g FA.

Furthermore, large-scale prediction models have been developed; these are the models used to estimate methane emissions from cattle farms and which are used to estimate the production of methane at a global, national or local level. These equations are issued by Eggleston *et al.*, (2006) and are divided into 3 different levels where the first level is the simplest while the third one is the most complex. The three methods are based on the percentage of gross energy that is excreted as methane. The coefficients that are used vary according to the type of feed and the type of breed.

In recent years Bannink *et al*., (2011) have proposed a model for estimating methane emissions by cows called Tier 3. The development of the model was done in the Netherlands and is based on algorithms developed previously by Dijkstra *et al*., (1992), Mills *et al.*, (2001), and Bannink *et al*., (2008, 2010). The model represents the dynamics over time of a series of elements that influence the enteric methane emissions such as microorganisms, fermentation end products and substrates. The model is very complex and accurately describe the fermentation process that takes place inside the rumen, the degradation of feed by microorganism, the production of VFA, the utilization rates of substrates, the enzymatic kinetics, and also through the stoichiometry of VFA is estimated as enteric emissions by cattle (Bannink *et al*., 2011). The model estimates the methane production as MEF (methane

emission factor CH_4/cow/yr). The advantage of the model are linked to the large number of information that is produced and also being a tool that thoroughly analyzes the dynamics of fermentation, it can be used to understand which are the main factors that influence the production of methane (Bannink *et al*., 2011).

Among the most recent models for the estimation of enteric methane emissions, we must mention the work carried out on sheep by Bell *et al*., (2016), which highlights the strong correlation between methane production and DMI:

$$CH_4 = 18 + 22.5 \text{ DMI } (r^2 = 0.70)$$

where CH_4 = methane production (g/d); DMI = dry matter intake (kg/d)

Also, Zhao *et al*., (2016) in a work always carried out on sheep has obtained the results that demonstrate that DMI is one of the most reliable predictors regarding methane emissions. The equation developed by them is the following:

$$CH_4 = 3.1 + 16.7 \text{ DMI } (r^2 = 0.70)$$

where CH_4 = methane production (g/d); DMI = dry matter intake (kg/d)

3.2 Measures on animals

Respiration chambers. over the past 100 years, different models of respiration chambers have been used to study the energy metabolism of animals (McLean *et al.*, 1987; Johnson *et al.*, 2003). The general principle of the chambers is based on collecting the animal's gas emissions and measuring their concentration. The respiration chambers can be closed (REF) or open (REF) circuit (generally the open circuit are used in the tests).

All open circuit chambers are characterized by an incoming and outgoing air flow, so the animals breathe in a one-way air flow that passes through the chamber (Goopy *et al.*, 2016). It is necessary that the chamber is perfectly sealed so that there are no air leaks that would lead

to a wrong measurement of the quantity of methane emitted, moreover, to ensure that the air is not lost from the chamber, it is possible to use aspiration fans and discharge at different speeds thus creating a negative pressure inside the chamber (Turner and Thornton, 1966). The incoming air flow is taken from the outside or through conditioning system to control temperature and humidity. The methane emission in open circuit systems is calculated in relation to the methane concentration in the inlet and outlet air flow from the chamber (Brown *et al.*, 1984). In general, to measure the variation of methane, but also of CO_2 and O_2, gas analyzers, infrared photoacoustic monitors or gas-chromatography systems are used (Klein and Wright, 2006; Grainger *et al.*, 2007; Goopy *et al.*, 2014). The accuracy and precision of these system, and therefore their calibration, are essential. The calibration of the chambers is done by releasing inside it a certain quantity of standard gas of kwon concentration so as to measure the recovery values of these gases and to verify that there are no leaks from inside the chamber (Klein and Wright, 2006). Results of measurements are influenced by the ambient temperature, humidity, pressure and composition of the incoming air and the volume of the chamber (the greater the volume of the chamber, the lower sensitivity of the measurements) (Brown *et al.*, 1984). Furthermore, in experiments carried out on sheep, limit level has been described for temperature (< 27 °C), relative humidity (< 90 %), CO_2 concentration (< 0.5 %) and ventilation speed (250-260 L/min) (Pinares-Patiño *et al.*, 2011). The construction and operation of the chambers and related equipment is fully described in the technical manual: Technical Manual on Respiration Chamber Designs (Pinares-Patiño *et al.*, 2012). The individual chambers have two opening, the front one allows the change of feed and water, the back one is used for cleaning (elimination of urine and faces) and the entrance of the animals. The peculiarity of this system is given by the entry of the animal, in fact the respiratory chambers are fixed while the animals are enclosed inside cart metabolic cages and

are transported inside the chambers, after a certain period of adaptation. The incoming air flow is located at the front of the chamber through a common main channel for all the chambers. Fan allow the mixing of the air and the maintenance of negative pressure inside the chamber inside the chamber to prevent gas leakage outside the system. In each chamber, instrument have been included for controlling relative humidity, temperature and CO_2 concentration. The outgoing air flow is positioned in the rear part of the chamber, it passes through a filtering system for each chamber (macro and micro filtration) so as to eliminate any air impurities. The sampling takes place continuously, but before reaching the gas analyzer the humidity of the air is eliminated through two dryers, the air is cooled to 4 °C. The gas analyzer is calibrated every morning using 99.99 % N_2 and a mixture of standard gases of know concentration.

The advantage of this system is the accuracy of the measurements (all the methane emitted by the animal is measured), in fact many other systems are calibrated using the respiratory chamber data. However, high construction costs and the impossibility of estimating the emission of grazing animals make this system limited.

Ventilated hood system. the ventilated hood system uses the same operating principle as the open circuit respiration chambers. The difference between the two system is that in the ventilated hood system, the gases emitted exclusively during breathing, belching and rumination are measured therefore, the head is then isolated form the rest of the body, whereas also intestinal emissions are included in respiration chambers measurements. Modern ventilated hood systems consist essentially of the access of an animal within a metabolic cage where inside is a separation box where the animal's head is positioned (Suzuki *et al.*, 2007). To separate the two environments, the one in which the head is and the one in which the rest of the body is, a sleeve of waterproof fabric is generally used, which simultaneously closes

the animal's neck and the hole inside which it is introduced the head. This sleeve must guarantee a certain movement to the animal and, at the same time, prevent any air leaks (Bhatta and Enishi, 2007). The head boxes are equipped with fans to move the flow of the main air towards the exhaust pipe, the air filters remove humidity and the gases are led to the analyzers (Suzuki *et al.*, 2007).

This type of system has already been developed for large ruminants in Thailand (Suzuki *et al.*, 2007), USA (Place *et al.*, 2011) and Canada (Odongo *et al.*, 2007), while for small ruminants some work has been done in Australia (Takahashi *et al.*, 1999) and Spain (Fernández *et al.*, 2012, 2015). In particular, the Fernandez research group carried out various trials using ventilated hoods to evaluate methane emissions from different feed. Recently they tested emissive potential of orange leaves as a replacement for alfalfa (Fernández *et al.*, 2019) and before that they tried to replace alfalfa with alfalfa (Criscioni *et al.*, 2016). The ventilated hood was developed to replace a face mask system (described later). In the ventilated hood system, a computerized air sampling and analysis control system was used, thus replacing the collection bags and an air-cooling system to remove humidity in order to replace the silica gel filter. This improved system was used on dry sheep through an experiment that aimed to evaluate the production of methane with the use of different diets.

The advantages of this system are the low costs compared to the entire respiration chambers, therefore they represent a valid alternative, even if they have some limits. In particular, they need a longer adaptation period, which determines a prolongation of the time required to carry out the test. Moreover, compared to the respiration chambers, the total methane production of the animal is not measured but only enteric ones, leaving out those emitted through flatulence (Goopy *et al.*, 2016).

Portable Accumulation Chambers (PAC). the portable respiration chambers are transparent polycarbonate boxes in which the production of methane is measured by the increase in its concentration which occurs in an hour of stay of the animal. This method has been studied for sheep, in fact is a small box. Methane measurements are take using laser detectors (Chagunda *et al*., 2010). This technique initially gave a good comparison with the respiration chambers (Goopy *et al.*, 2009). Further studies have shown a low level of repeatability of the method, and comparing the data between PAC and the respiration chamber, differences have been found that discourage the large use of PAC system and big investments for measurements in large scale (Robinson *et al.*, 2015).

Polytunnel. polytunnels are alternative methods to the respiration chambers but which basically follow the same basic principle. They can be considered large-scale respiration chambers and are used for methane emissions measurements of grazing animals, usually sheep. These are systems that include the installation of a large inflatable tunnel or a polyethylene pavilion, above a grazing area of which the floristic composition is known, the quality and quantity of the essences, so as to be able to put in relation the characteristics of the feed intake with the production of methane. Once the forage is exhausted, or when you want to change the type of feed, the polytunnel is moved to another area of the same or another pasture plot. During grazing, the concentration of methane present inside the tunnel is detected by means of micropumps which transfer the exhaust air to a dedicated analyzer or to a gas chromatograph (Lockyer, 1997; Murray *et al.*, 2001). Murray *et al.*, (1999) conducted an experiment on sheep to compare the methane emissions detected on a tunnel system and on an open circuit respiration chamber system, noting that under the first method there was an underestimation of CH_4 emissions. Moreover, as seen from (Lockyer and Jarvis, 1995) the

fluctuations in methane emissions can depend on many factors such as the temperature of the polytunnel, the relative humidity, the ruminating etc.

Face masks. that of the masks was one of the first methods used to measure the quantity of gas emitted by the animal. This type of method was introduced by Washburn and Brody (1937), in which animals, both cattle, sheep and goats, were trained to remain in decubitus for 30 minutes every two hours. In this type of system, it is very important that the mask is well sealed to the animal's snout, which obviously produces stress. The biggest disadvantage of this practice is that the animal cannot access feed and water and the measurement time cannot be excessively prolonged. Also Fernández *et al.*, (2012) proposed the use of a respiration mask which was envisaged to collect the air exhaled by the animal (15 min/h for 15 h), an air collection system (pumps, measuring tubes flow rate, rotameter, air volume totalizer and fan) located in the lower part of a mobile trolley, waterproof air sampling bags connected to the aspiration system, after air filtration through silica gel to eliminate humidity.

Greenfeed. greenfeed is a patented device (Zimmerman and Zimmerman, 2012), which consists of an automatic feeding system which records methane emissions in the short term (on average 3-6 minutes) during feed supplementation. The system measures the emission of gas through an aspirator and sensor that induce the air flow measured beyond the animal's head, channeling the air emitted by the animal for collection and sampling. One of the advantages of this system is that it can be completely automated, with animal recognition systems, gas tracking system, a data collection and management system (Hristov *et al.*, 2015). Other advantages are also represented by the comfort and ease of use and the accuracy of the results, which are comparable with other methods such as respiration chambers and SF_6 techniques. However, there are also problems; the obligation to provide concentrates, such as "bait" to lure the animal towards the system, can influence both the estimates of the gas

emitted and the animal's diet; an adaptation period is also required for the animals to familiarize with the system. It is also necessary to consider that the measurements are carried out only while the animal is consuming the feed.

Sulfur hexafluoride tracer technique. this method was first described in 1993-1994 (Johnson *et al.*, 1994; Grainger *et al.*, 2007). Together with the open circuit respiration chamber it represents one of the main methods of direct measurement of enteric methane in individual animals, and can be used both in grazing animals and in controlled feeding situations. The idea behind the method is that methane emission can be measured by knowing the emission rate of tracer gas from the rumen. For this purpose, a stable non-toxic, physiologically inert gas is required. Furthermore, the gas should mix with rumen air in the same way as methane. Sulfur hexafluoride (SF_6) was chosen because it meets these criteria, it is economical, it has an extremely low detection limit and it is simple to analyze (Storm *et al.*, 2012). The method is then applied by inserting at ruminal level a permeable tube that gradually releases SF_6, the air expelled during breathing is continually sucked into a collection container connected to a shed equipped with a capillary tube around the neck (Johnson *et al.*, 1994). At the completion of the collection the gases are analyzed by gas chromatography and through an equation based on the CH_4/SF_6 ratio for the marker gas release rate and the sample collection time, enteric methane yield is obtained.

The main advantage of this method derives from the possibility of making measurements without having to lock up the animal; however, the high GWP of SF_6 represent an important limitation of this technique. In fact, SF_6 is more impactful than methane itself, and there is the possibility of finding residues of this gas in meat and milk. Furthermore, with this system the emissions from the gross intestine are not measured, so the measurement is incomplete compared to respiration chamber methodology.

Open path laser. this technique is based on the measurement in free atmosphere of small or large areas through laser instruments, and the results are correlated with animal emissions (Harper *et al.*, 2011). This technique is currently being developed, because it still does not provide accurate estimates of animal emissions, but the possibility of obtaining data regarding the interactions between farms and environment is very interesting.

Spot sampling with lasers. this technique is based on the use of a laser methane detector (LMD); a detector to estimate the methane content in the air. This tool is based on the use of infrared absorption spectroscopy to establish methane concentrations (ppm). This technology has been used by Chagunda *et al.*, (2009) in a test with cows comparing the emissions deriving from the use of two different diets, obtaining satisfactory results; however, further studies are needed to have more data repeatability (Chagunda *et al.*, 2009).

3.3 Emerging and future technologies

Blood methane concentration. this technique is based on the determination of enteric methane (or tracing gases such as SF_6) which passes into the bloodstream through the ruminal walls (Ramírez-Restrepo *et al.*, 2010). However, this is a technique that requires further study (Goopy *et al.*, 2016).

Intraruminal telemetry. it is a system to measure the methane produced in the rumen through a ruminal bolus which records even small variations of methane (Gibbs, 2008).

Milk analysis. as mentioned in the paragraph on the estimation of emissions using predictive equations, some of these use VFA in milk as predictors. Dehareng *et al*., (2012) used the milk mid-infrared spectra as a tool to estimate methane emissions in cows. The data obtained with the spectra were then compared with the emissions measured through the use of sulfur hexafluoride. The results obtained have shown how the MIR spectra technique can be useful

tool for estimating methane emissions and in addition it was more precise then point measurements. This study confirmed the results obtained by Chilliard (2009), highlighting the close relationship between fatty acid in milk and methane production.

4. FORAGE QUALITY AND METHANE EMISSIONS

As previously mentioned, ruminants have developed a digestive system with which they manage to digest the fibrous parts of plants. Being the forages the main feed of ruminant diets, whether they are used in the form of pastures or used in the form of hay and silage, knowing their quality is fundamental in order to reach high production levels.

The fibrous part of the plants is formed by three fractions: cellulose, hemicellulose and lignin. The fiber content and its fractions in the forage are influenced by various factors but mainly by the maturity stage of the plant (Stokes and Prostko, 1998). During the early stages of growth, the plants are characterized by a high protein content and digestibility reaches maximum levels. As the growth process progress, the more fibrous parts like the stems develop, leading to a decrease in digestibility and therefore in quality (Newman *et al.*, 2006). Advancing in the maturity stage of the plant as well as increasing the fibrous fractions there is also a deposit of lignin (substance indigested by ruminants) in the cell walls. In this case there is a collapse of the digestibility of the plant.

The level of nutrients in forage is very variable, and depends mainly on the species and climate. For example, the crude protein can vary in legumes between 12-25 %, in cool-season grasses between 8-23 % and in warm season grasses between 5-18 %. Also, the fiber (NDF) has very different levels among the various plants; in grasses will be higher (60-65%) than for legumes (45-55 %) (Newman *et al.*, 2006).

Several indexes have been sought for several years to evaluate the quality of the forage, but these are not very indicative if they are used alone (Mertens and Ely, 1979).

One of the parameters most used to define forage quality is the relative consumption potential. This parameter is mainly influenced by the maturity, coarseness and quantity of fibrous materials in the forage (Welch and Smith, 1970).

According to Collins and Fritz, (2003) Forage quality is defined as "*the potential of a forage to produce the desired animal response"*. The chemical composition is the main factor that influences the quality of the forage and therefore its intake and digestibility. In fact, the most important parameter for evaluating a forage is the voluntary intake by the animal. The intake of forages is closely correlated with the digestibility of the fiber, which is influenced by the rate with which it is removed from the digestive tract (Mertens and Ely, 1979). Poor quality forages require a very long digestion time inside the rumen, consequently lowering the dry matter intake (Collins and Fritz, 2003). Generally, forages with high quality are consumed in greater quantity than forage with bad quality.

An additional index that can be used to assess the quality of forage is the Relative Feed Value (RFV), which takes into consideration the correlation between content of NDF and intake and content of ADF and digestibility (Newman *et al.*, 2006). This index considers intake potential and digestibility of DM of alfalfa at full bloom (RFV = 100). For example, if alfalfa it is in pods phase, the RFV is between 75-86 % (Stokes and Prostko, 1998).

Recently a new method has been developed for estimating the quality of forage that is called RFQ (Relative Forage Quality). Unlike the previous index, more nutritional parameters of the feed are taken into consideration in this system (CP, NDF, ADF, fat and ash), resulting more accurate than RFV (Moore and Undersander, 2002).

The quality of forage affects both the quantity and quality of animal production. In particular, increasing the digestibility of NDF contained in forages has positive effects on DMI and milk production (Oba and Allen, 1999). Furthermore, several studies have shown that the quality of forages is positively correlated with the milk fat content (Kalscheur *et al.*, 1997) and the quality of milk fat (Kalac and Samkova, 2010).

Lately, the production of enteric methane represents one of the most discussed topics in the livestock sector. As mentioned in the paragraph on rumen fermentation and enteric methane production, fiber digestion is the main mechanism for methane production. As forages rich in fiber, it is the feed mainly attributed to the production of methane by ruminants. For several years, studies been carried out concerning the quality of forages and the production of methane.

Moe and Tyrrell, (1979) in a study carried out on dairy cows, observed that the production of methane per gram of digested cellulose was five times that of soluble residue digested and three times that per gram of hemicellulose digested. These results are mainly given by the speed with which the various components of the plant are digested. Cellulose and hemicellulose have slower fermentation rates compared to soluble carbohydrates; therefore, stopping for longer in the rumen, producing more methane per unit of digested substrate (McAllister *et al.*, 1996).

Similar results were obtained from Chagunda *et al.*, (2010); in their study carried out on dairy cows, they observed that rations with low forages quality produce between 16-33 % of methane/kg of milk more than diets containing forage with good quality.

In conclusion, increasing the quality of forages leads to an increase in the digestibility of the diet, greater production and consequently lower methane emissions per kg of product (Beauchemin *et al.*, 2009).

Several works have been carried out to evaluate the emissions of small ruminants using different measurement methods. Lockyer *et al*., (1997) performed work using methane measurement through the polytunnel, to assess the emissive potential of grazing sheep. The results obtained showed a large emission range ranging from 8 to 20.9 gCH_4/d per sheep. Another work carried out on grazing sheep by Zhai *et al*., (2015) using face mask as a measurement system, showed how intensive grazing has an emissive potential about 3 times higher than light grazing (42.26 vs 12.92 gCH_4/d respectively).

As previously anticipated, bier has a fundamental role in the production of methane, and one of the strategies to reduce them could be to use feed with a better quality of fiber. Fernandez *et al*., (2019) tested the use of orange leaves as substitute for alfalfa in dairy goats and evaluated their emissive potential. Methane emissions were lower in animals fed with orange leaves than those fed alfalfa (12.3 vs 18.1 gCH_4/d respectively). The orange leaves used had a lower amount of NDF and lignin than alfalfa (37.6 vs 49.4 and 6.7 vs 7.1 % on DM respectively), therefore in this case the quality of the fiber seems to have brought positive results in the mitigation of methane emissions. Molano and Clark (2008) have also studied the effects of forage quality on methane emissions in sheep using two different ryegrass hay, one cut during vegetative phase (NDF = 46.9 % DM basis) and the other in reproductive phase (NDF = 51.5 % DM basis). In their study there were no significant differences in methane emissions, although from numerically the sheep fed with the forage cut during the vegetative phase emitted less methane than those fed with reproductive forage (22.9 vs 23.7 gCH_4/kg DMI respectively). This indicates that one of the strategies to improve the quality of forages and reduce methane emissions could be to choose the right cutting age.

Archimede *et al*., (2011), performed a review to understand the emissive potential in terms of methane of diets with C3 vs C4 plants. The results obtained have shown that the use of C3

plants produces 17 % less methane (per kg of OMI) than C4 plants; the authors concluded that the emissive differences are related to the fact that the C3 plants possessed a lower NDF level compared to C4 plants (55.7 vs 64.6 on DM basis respectively). Still talking about the quality of the fiber, Criscioni *et al*., (2016) in a study carried out on goats, tried to replace the hay of alfalfa (low NDF) with that of maralfalfa (high NDF). The results obtained showed that animals fed alfalfa hay produced more methane than those fed with maralfalfa hay (28.5 vs 25.9 gCH_4/d respectively), but the differences became not significant when expressed on DMI (15.8 vs 16.2 gCH_4/kg DMI respectively).

Therefore, improving fiber quality, for example using C3 cycle species, making cuts at the best time or using species that naturally possess a better fiber are among the strategies that can be used to improve the quality of forages and mitigate methane emissions in small ruminant sector. Table 2 shows the methane emission values of different workscarried out on sheep using different measurement techniques and different types of diets.

5. ADDITIVES AND METHANE EMISSIONS

As already seen in the previous paragraphs, enteric methane in ruminants is produced by methanogenic bacteria, which using CO_2 and H_2 as a source of energy, releasing in the rumen CH_4. As methane is one of the main greenhouse gases, in recent years research has researched strategies to mitigate these emissions. These strategies can be grouped into 3 broad categories: i) feed, feeding management and nutrition; ii) rumen modifies; iii) improving animal production through genetics (Tate *et al.*, 2015). Among the strategies that affect feed and diets, the use of additives is one of the most recently used and applicable at farm level in the short term. Additives are used in ruminant diets for different purpose: to improve feed quality,

to reduce nutritional deficiencies, to promote animal growth and to compensate for energy wastes by intervening in this regard also on methane emissions (Durmic *et al.*, 2014). A large number of additives have been tested to try to reduce methane emissions in ruminants, but only a few have produced satisfactory results.

Among the main objectives is included to inhibit methanogenesis, decreasing the amount of OM digested inside the rumen and therefore producing lower quantity of hydrogen (McGinn *et al.*, 2004). On the other hand, decreasing the digestion of OM (mainly of the fiber) produces a shortage of energy that could be available to the animal. Therefore, other strategies should be preferred that instead of decreasing the digestibility of nutrients, and focusing on the possibility of finding substances that bind to hydrogen, thereby inhibiting the activity of methanogens (Asanuma *et al.*, 1999). Asanuma *et al.*, (1999) in an *in vitro* study have tried the use of fumarate as an additive as it is indicated as possible electron acceptor. The results obtained by them have shown how the use of fumarate reduces methane production and increase the quantities of propionate in the rumen. However, other studies carried out using fumarate as an additive did not produce the same results in terms of methane production (Callaway and Martin, 1996).

McGinn *et al.*, (2004) have tested the use of different types of additives (sunflower oil, monensin, enzymes, yeast, and fumaric acid) to decrease the production of methane. The results obtained showed how the addition of sunflower oil and monensin decreased the production of methane in animals fed with diets rich in forages. However, the use of enzymes, fumarate and yeast did not produce significant results in the reduction of methane.

A way to mitigate methane production by methanogens is to act on enzymes that mediate the pathways of methanogenesis, such as the Methyl-ComM reductase (Mcr) (Patra *et al.*, 2017). In these sense, different compounds are useful to inhibit the activity of Mcr as an example

several halogenated sulfonated compounds such as 2-bromohethanesulfonate and 2-chloroethanesulfonate (Nollet *et al.,* 1997).

The secondary plants metabolites, possess an antimicrobial activity towards different microorganism, and among these also the methanogens. Tannins, for example, being compounds that decrease fiber digestibility, consequently reduce the amount of free hydrogen that is used by microorganism. Furthermore, they also have an inhibitory action against protozoa. Puchala *et al*., (2012) tested the use of a condensed tannin containing Sericea lespedeza for several days on goats to understand the effect on methane production. The results showed that using tannins in the diet every day, the emissions were about half compared to their use once every 8 days (6.3 vs 12.0 gCH_4/d).

Among the additives that for some years have been introduced in ruminant diets to lower methane emissions are included nitrate salts. The great affinity that nitrates have with hydrogen (greater than that with CO_2), and the consequent formation of ammonia, inhibit the production of enteric methane (Nolan *et al.*, 2010). The problem of using nitrates as an additive in ruminant diets is related to their toxicity. As suggested by (Bruning-Fann and Kaneene, 1993), excess nitrates can be lead to various problems such as: depressed feed intake and production, no weight gain, susceptibility to infection, reproductive failure, brown mucous membrane discoloration, respiratory distress, coma, cyanosis, and even death. However, in a study by van Zijderveld *et al.*, (2010) carried out on sheep, they saw a decrease in methane production using nitrates as an additive in diets. Li *et al.*, (2012) in a study carried out on lambs compared two different diets containing urea (1.5 %) and calcium nitrate (3 %). Results obtained have shown how calcium nitrate can be used as an additive in ruminant rations, without producing toxicity problems or decreasing nitrogen digestibility.

Among the most recent applications in livestock sector, nanotechnologies have been used to create nanoparticles that can be used as additives in ruminant diets to improve digestibility and also to improve food safety (Bunglavan *et al.*, 2014). Nanoparticles are a class of compounds formed by different materials, which are always smaller than 100 nm in size (Khan *et al.*, 2017). For example, the use of zinc nanoparticles (ZnO NP) can bring advantages as they improve growth performance, reduce the somatic cell counts, increase the growth rate of microorganisms in the rumen (Swain *et al.*, 2016). Recently, ZnO NP have been used to carry out *in vitro* studies on the mitigation of methane emissions by ruminants. Sarker *et al.*, (2018) tested four different level of ZnO NP (110, 200, 500 and 1000 μg/g) added to two different feed (corn silage and alfalfa), to evaluate the effect on methane production. The results obtained showed that as the quantity of ZnO NP added increases, both the production of enteric methane and CO_2 decreases. However, it must be said that zinc, if administered in too high doses, can be toxic to the animal.

Other compounds, such as halogenates, have been tested on ruminant diets as methane inhibitors, but have achieved poor results mainly due to their toxic nature. However, (Martinez-Fernandez *et al.*, 2016) have shown that the use of chloroform as an additive to ruminant diets can have positive effects on methane emissions (< 30%) without toxicity problems. Again, (Martinez-Fernandez *et al.*, 2018) tested the use of a new product, the 3-nitrooxy-propanol (3-NOP) on diet for dairy cows rich in forages, obtaining as result a decrease in methane emissions very similar to the results obtained with chloroform.

Concluding, we can say that a large range of products have been tested as additives in ruminant diets to lower enteric methane emissions and every day new ones are tested to counteract the problem of climate changes, and in particular to mitigate the environmental impact of the livestock sector.

6. STRATEGIES FOR REDUCING METHANE EMISSIONS IN RUMEN

The reduction of enteric methane emissions also has an effect on the energy available to the animal as this energy tends to increase with the reduction of emissions (Gorh and Baruah, 2019). The production of methane depends on digestibility, on the type and quantity of feed, on forage:concentrate ratio and above all on the quantity of hydrogen released during rumen fermentations (Janssen, 2010; Kim *et al*., 2013). Among the strategies to reduce methane emissions, the most used are those that have an action at the level of the diet, both to decrease methane emissions and to increase the productivity of the animals themselves (Teklebrhan *et al*., 2020).

Feed treatments. Feed treatments that allow an increase of the rumen escape of digestible fractions in the rumen lead to a lower production of hydrogen formed by fermentations, thus reducing methane emissions (Janssen, 2010). Changing the physical structure of the feed, such as cut and pelleting, can lead to a 20-40% reduction in methane losses per unit of DMI in the case of ad libitum feeding (Johnson *et al*., 1996). One solution could be to adopt short fiber forages as it increases the rate of passage (*kp*) of feed in the rumen and therefore they remain in the rumen for less time, undergoing less degradation by the rumen microflora. In this case, however, care must be taken not to encounter rumen acidosis problems, caused by too short a fiber length (Keunen *et al*., 2002). The cut of forages, however, is a practice little used by farmers for various reasons: the technique is expensive, and it also leads to an increase in the incidence of rumen acidosis and reduction of milk fat, problems caused by the lack of physically effective fiber (pef) (Boadi *et al*., 2004).

Type of carbohydrates. Several studies have shown how methane production tends to decrease with the use of non-structural carbohydrates (NSC), compared to the use of

structural carbohydrates (SC) (Albenzio *et al*., 2018). This is mainly related to the fact that, using feeds with a high level of NSC or concentrates, there is a greater production of propionic acid and a lower production of acetic and butyric acid which are the main responsible for the production of H_2 which is used for the methane production (Janssen, 2010; Knapp *et al*., 2014).

By increasing the NSC in the ration, the rumen pH is lowered thus inhibiting the development of methanogenic bacteria and protozoa, considered to be the main culprits of methane emissions (Van Kessel and Russell, 1996; Hegarty, 1999).

Therefore, a useful strategy to reduce methane emissions may be to use more digestible feeds (<NDF; >NSC) as they have a higher kd and kp than fiber-rich feeds and in addition lead to greater milk production. Various authors suggest the use of C3 cycle plants (autumn-winter cereals e.g. wheat, barley) compared to C4 cycle plants (cereals from tropical climates, e.g. corn, sorghum) as the latter have a more lignified fiber and longer ruminal retention time (Pinares-Patiño *et al*., 2009; Archimède *et al*., 2011).

In the choice of carbohydrates, the quality of the starch used in feed is also very important, as the degradation of the starch depends on the structure of the molecule, that is, on the relationship between the amylose and amylopectin chains (Albenzio *et al*., 2018). With more slowly degradable starch at ruminal level (corn), methane production is lower than when a higher fermentability starch (wheat) is used. However, a recent study by Teklebrhan *et al*., (2020) has shown how concentrated feeds with a higher level of starch (such as corn meal) produce more methane in the rumen than concentrated feeds with a higher NDF content (such as corn gluten). As explained by the authors, this is probably linked to the quantitative difference in the number of hydrogen acceptors and the two diets which drives the decrease in

methane production. Another explanation could involve the total amount of degraded substrates at rumen level.

Forage quality. The increase in the forage quality represents one of the key factors for the reduction of methane emissions by ruminants (Lee *et al*., 2017) The methane production is influenced by the age of the plant, by the treatments the forage is subjected to and by the conservation methods. It is possible to intervene on the quality of the forages by using less mature essences in the diet, whether they are harvested or used for grazing. Or by adopting good storage and ensiling techniques in order to conserve nutrients (Xue *et al*., 2020). A study done by Sundstol (1981) has shown that the use of silage forage leads to a lower production of methane than the use of hay forage.

The silage especially in the dairy cow sector represents the main component of the rations as it is the least expensive feed. To reduce methane emissions, therefore, is possible to intervene by choosing the most suitable species for ensiling (Warner *et al*., 2017). Corn silage, having a higher starch content (30% DM basis) compared to other silages (e.g. barley, 9% DM basis) is less impactful. Therefore, giving more starch with rations without causing rumen acidosis and without compromising the quality of the productions (% of fat in the milk) leads to lower methane production (Mills *et al*., 2003). It has been observed with this study that by using more starch-rich silages there is a higher milk production, and consequently lower emissions per quantity of milk produced.

A study carried out in New Zealand has shown that condensed tannins present in legumes have led to a reduction in methane production on cows raised on pasture of *Hedysarum coronarium* (Woodward *et al*., 2002).

Lipid integration. The use of feed-based fats is among the most effective nutritional strategies in reducing enteric methane emissions. The addition of lipids to the diet of ruminants provides

a certain amount of metabolizable energy (ME), thus decreasing the digestion of the other nutrients and therefore a lower production of hydrogen which is transformed into methane inside the rumen (Kliem *et al*., 2019). Moreover, being the lipids rich in monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids, they are an alternative source of hydrogen conversion inside the rumen (Fievez *et al*., 2003). Patra *et al*., (2013) demonstrated that the use of diets with a fat content close to 6%, compared to diets with a low-fat content, as well as improving milk production significantly reduces the intestinal methane emission. However, if the 6% threshold is exceeded despite a continuous reduction in emissions, milk production decreases (Patra *et al*., 2013) mainly due to impaired ruminal equilibrium and reduction of diet digestibility.

The use of various lipid sources (whole cottonseed, rumen by-pass fat, soybean oil) to reduce methane emissions in ruminants has been recently studied by Beck *et al*., (2019). In their work they have shown how the use of lipids decreases methane emissions as fats decrease the digestibility of fibrous fractions (NDF and ADF) and inhibit methanogenesis.

Introducing essential oils into the diet as an energy source, there is a reduction in the production of VFA and consequently a lower production of methane. Furthermore, dietary fats lead to a reduction in the number of protozoa that are physically associated with methanogens (Beauchemin *et al*., 2008).

From another study conducted on beef cattle by Beauchemin *et al*., (2007), it has been seen that the use of some lipid sources such as sunflower and tallow oil in nutrition have led to a reduction in methane emissions. However, it is necessary to consider the possibility that the addition of fats may negatively affect the cost of feed and also on production (Beauchemin *et al*., 2007).

Use of additives. Recently, to reduce methane emissions, the use of nitrates has been of particular interest as they could act as hydrogen acceptor, which is used for the formation of methane (Lee and Beauchemin, 2014). In a study carried out on cattle and sheep has shown that with the increase in the use of feed nitrates per kg of BW there is a reduction in methane (Lee and Beauchemin, 2014).

A problem related to the use of nitrogen sources is their toxicity since nitrates and nitrites could accumulate in the rumen liquid and cross the rumen wall and end up in the blood. This problem is particularly important with nitrite as it binds to red blood cells by hindering oxygen transport (Alemu *et al*., 2019).

Among the commercially available additives, the yeasts that are usually used in the feeding of ruminants can also be used to reduce methane emissions. In fact, they increase production performance, improve feed utilization efficiency, reduce rumen imbalances and improve animal health (Lascano *et al*., 2011). Among the additives commonly used in ruminant diets we find enzymes (such as cellulase and hemicellulase). These can increase the digestibility of the fibers, and reducing the acetate: propionate ratio also decreases the production of enteric methane (Haque, 2018). A further category of products that have a methane reduction action are the secondary metabolites produced by plants, such as tannins and saponins (Eugène *et al*., 2019). The mechanism by which tannins act on methane production is not yet entirely understood, but is thought to have a direct inhibitory action against methanogens and protozoa (Aboagye and Beuchemin, 2019). The role that polyphenols (like tannins) have on fiber degradation and methane production is explained in a recent review by Vasta *et al*., (2019), and in particular the study highlights how tannins inhibiting the production of enzymes that attack the cell wall of plants decrease its digestion and therefore also the production of methane. One of the most effective strategies for decreasing methane production is to find

alternative compounds that act as hydrogen acceptors which is the main precursor of methane production (Adejoro *et al*., 2020). Several studies have been conducted in this sense and the products that have had the best results have been nitrate and sulfur compounds (van Zijderveld *et al*., 2010).

7. OBJECTIVE OF THE RESEARCH

The general objective of this Thesis was to focus on good practices and nutritional strategies that could be applied at farm level in order to reduce methane emission from dairy sheep. The objective of the work included the development of a ventilated hood that allow to measure methane emission in small ruminants combined with a metabolic cage for digestibility measurements inspired to a similar equipment proposed by Fernandez et al., (2015).

In particular, three experimental trials were carried out: two *In vivo* with direct methane measurements on sheep using the ventilated hood; one *In vitro* with a gas production test on ruminal fermentations carried out in presence of feed additives with high mitigation potential in previous studies.

The specific objectives of the different trials were listed below following the order of presentation in the Thesis:

Trial 1. rationale, methods and results of this trial were presented in Chapter II. This trial was executed within the activities of the project EU LIFE+ FORAGE4CLIMATE"*Forage systems for less GHG emission and more soil carbon sink in continental and Mediterranean agricultural areas* (LIFE15_CCM/IT/000039) which aimed to stimulate the definition of good practices to mitigate methane emissions in dairy sheep.

The specific objectives of the trail were: i) to develop and test the application of a ventilated hood for direct methane measurements on small ruminants, and define a methodological protocol of the use the hood in combination with digestibility trials; ii) to evaluate the effect of forages with different quality in terms of NDF content on DM intake, milk yield, milk composition, digestibility and methane emissions in Sarda dairy ewes.

Trial 2. rationale, methods and results of this trial were presented in Chapter III. This trial was executed within the activities of the project EU LIFE+ FORAGE4CLIMATE"*Forage systems for less GHG emission and more soil carbon sink in continental and mediterranean agricultural areas* (LIFE15_CCM/IT/000039) which aimed to stimulate the definition of good practices to mitigate methane emissions in dairy sheep. The specific trial was conducted: i) to improve the analytical performance of sampled methane from a ventilated hood used to measure direct methane measurements on small ruminants described in trial 1; ii) to evaluate if Sarda dairy ewes fed with two different haylages of different quality, in terms of high and low NDF content, could have different response in milk production, diet digestibility and enteric methane emissions.

Trial 3. rationale, methods and results of this trial were presented in Chapter IV. This trial was executed in collaboration with the activities of the project EU LIFE+ SheepToShip Life "*Looking far an eco-sustainable sheep supply chain: environmental benefits and implications*", (LIFE15_CCM/IT/000123) which aimed to quantify effects of different possible mitigation strategies for the dairy sheep sector in Sardinia. The specific objective of this work was to evaluate, with *in vitro* tests, the effects of original additives, such as nanoparticles carriers of double layered hydroxides, associated to nitrate based compounds and other additives in different combinations to reduce the emission potential of a generic animal diet. The experimental hypothesis assumed that additives associated with nanocarriers might have similar effects than additives alone by using half concentration of the active molecule.

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8. TABLES

Table 1. Substrates and related reactions for the production of methane in rumen.

Substrates	**Reactions**
H_2 and CO_2	$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$
Formate	$4HCO_2H \rightarrow CH_4 + 3CO_2 + 2H_2O$
Methanol	$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$
Methanol and H_2	$CH_3OH + H_2 \rightarrow CH_4 + H_2O$
Methylamine	$4CH_3NH_2Cl + H_2O \rightarrow 3CH_4 + CO_2 + 4NH_4Cl$
Dimethylamine	$2(CH_3)_2NHCl + 2H_2O \rightarrow 3CH_4 + CO_2 + 4NH_4Cl$
Trimethylamine	$4(CH_3)_3NCl + 6H_2O \rightarrow 9CH_4 + 3CO_2 + 4NH_4Cl$
Acetate	$CH_3CO_2H \rightarrow CH_4 + CO_2$

Table 2. Methane production in sheep fed with different types of diets and measured with different techniques

References		Treatments	Nutrients			DMI	CH_4 (g/d)	P	CH4 (g/kg/DMI)	P
			DM	NDF	CP					
Bhatt, 2019	SF6	TMRm (mash)	89.6	59.5	11.1	0.93	27.9	NS	30.3	0.003
		TMRb (block)	90.1	59.7	10.9	1.25	26.9		21.4	
Knight *et al.,* 2008	RC	Maintenance level: 0.8	17.4	44.7	15.5	0.355	8.95	<0.001	35.25	<0.001
		Maintenance level: 1.2				0.554	13.20		23.82	
		Maintenance level: 1.6				0.702	16.22		23.10	
		Maintenance level: 2.0				0.871	18.05		20.77	
Pinares *et al.,* 2003	SF_6	Pasture October	-	36.5	24.2	-	23.0-37.3	NS	-	-
		Pasture November	-	41.3	22.1	-	32.8-37.3		-	-
		Pasture January	-	37.5	22.5	-	31.3-32.0		-	-
		Pasture February	-	39.7	29.3	-	27.3-36.4		-	-
Jinker *et al.,* 2016	RC	Corn silage % diet = 25	42.9	43.9	12.7	1.15	23.7	<0.05	20.6	<0.05
		Corn silage % diet = 50	45.3	42.2	14.0	1.13	26.3		23.3	
		Corn silage % diet = 75	47.4	43.4	11.3	1.16	25.5		22.1	
		Corn silage % diet = 100	49.0	42.4	11.5	1.06	22.3		21.0	

Table 2. (continued).

References		Treatments	Nutrients			DMI	CH_4 (g/d)	P	CH4 (g/kg DMI)	P
			DM	NDF	CP					
Archimède *et al.,* 2018	SF_6	C3 high quality	-	74.2	12.0	1.008	23.4	<0.001	23.9	0.01
		C3 low quality		74.2	6.9	1.160	21.7		19.0	
		C4 high quality		58.6	13.4	0.830	19.9		24.0	
		C4 low quality		62.3	8.3	0.675	10.8		16.9	
McGeough *et al.,* 2019	RC	Low fiber digestibility, low fat	95.0	34.4	17.0	2.60	31.5	-	12.1	<0.01
		Low fiber digestibility, high fat	95.2	34.1	16.8	2.52	30.0		11.9	
		High fiber digestibility, low fat	95.1	31.5	17.1	2.08	29.9		14.4	
		High fiber digestibility, high fat	95.3	30.8	16.8	2.19	34.4		15.7	

[1] SF_6 = sulfur hexafluoride; RC = respiration chamber

[2] DM = dry matter; NDF = neutral detergent fiber; CP = crude protein

[3] DMI = dry matter intake

9. FIGURES

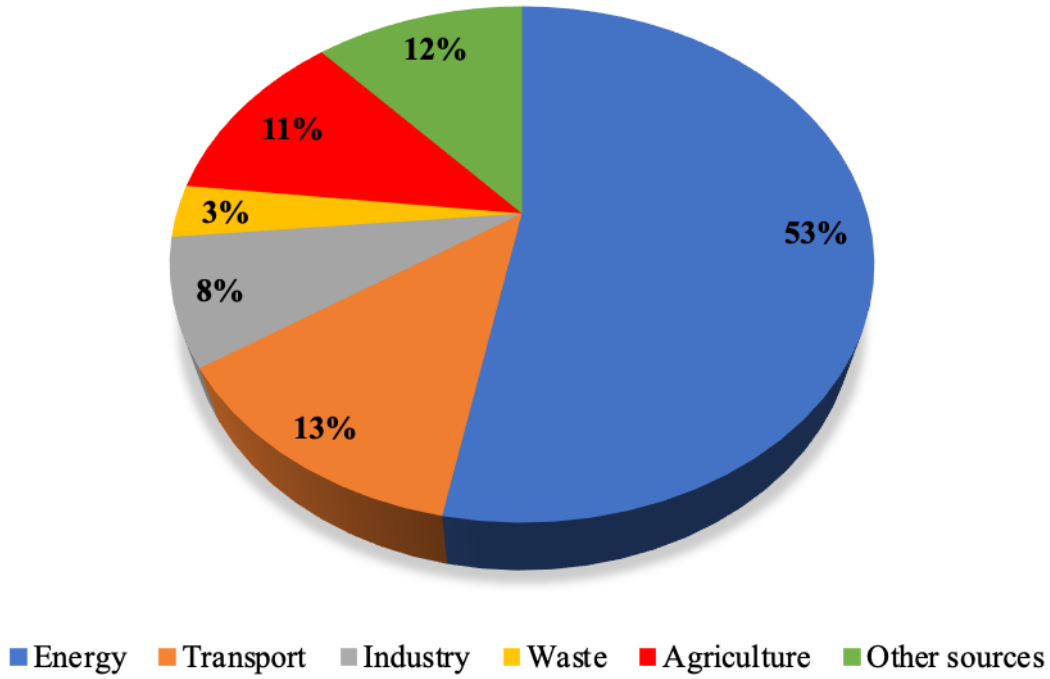

Figure 1. GHG emissions by sector (% of total GHG emissions) (FAOSTAT, 2010).

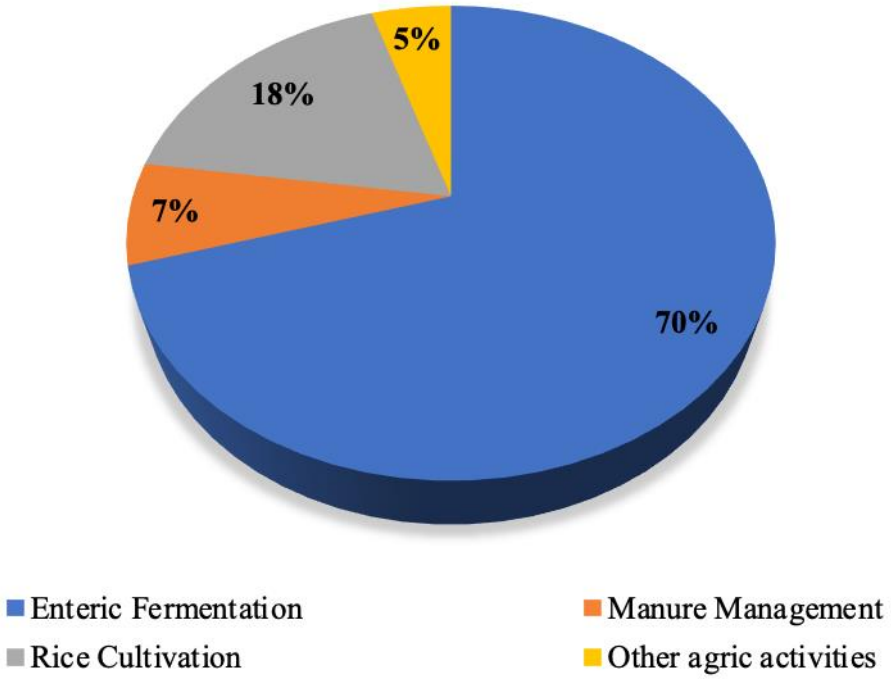

Figure 2. Methane emissions from agriculture (FAOSTAT, 2017).

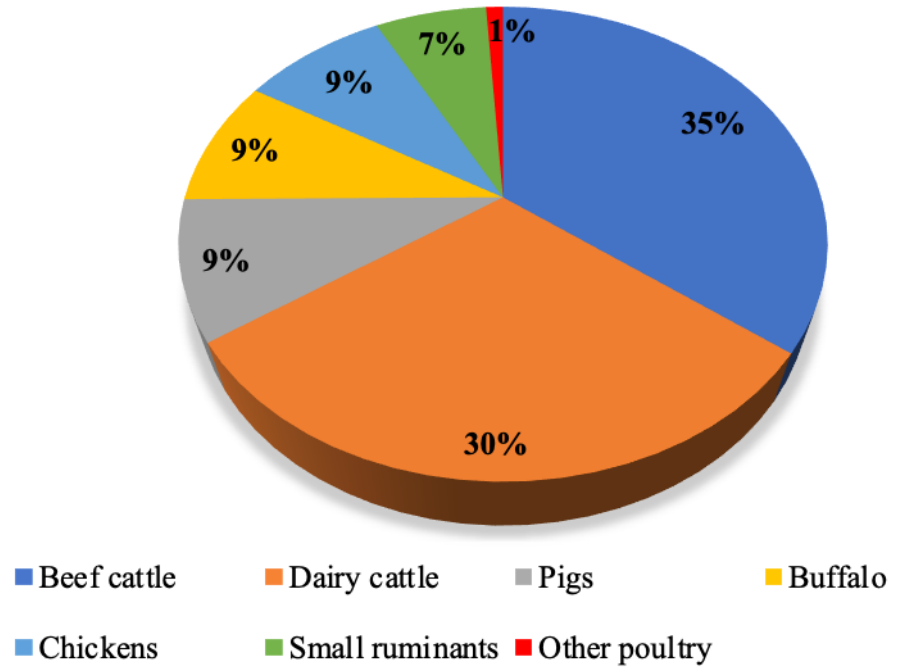

Figure 3. Global estimates emissions by species (% of total emissions of GHG) (Gerber *et al.,* 2013).

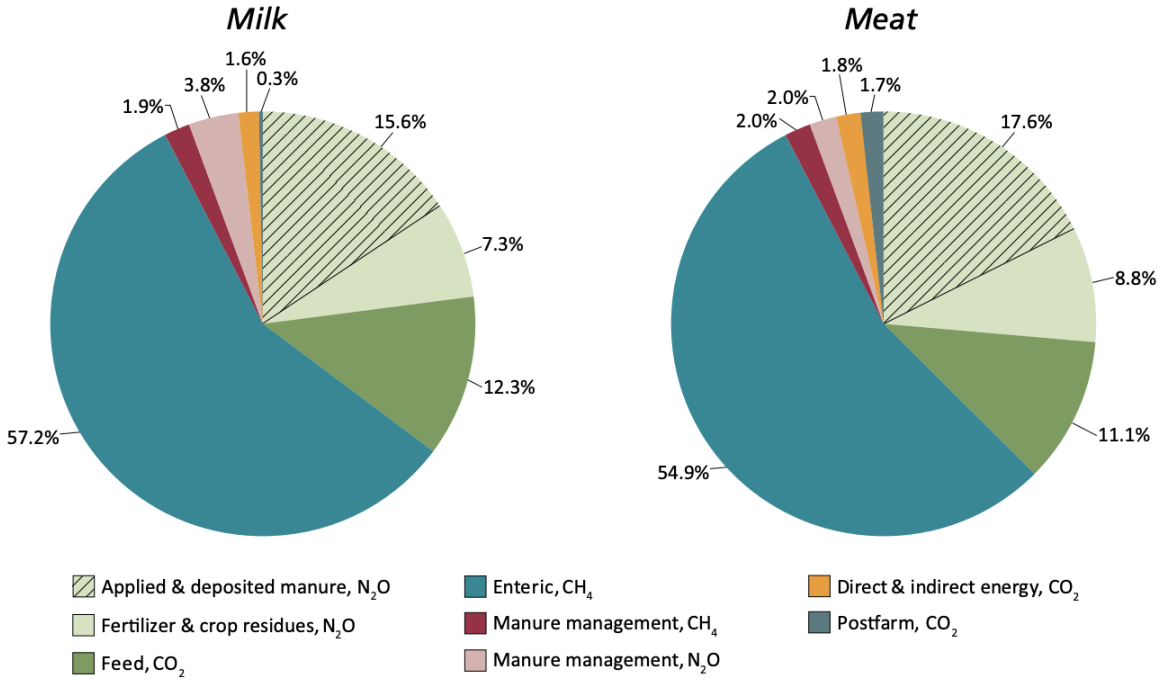

Figure 4. Global emissions from small ruminant milk and meat supply chains, by category of emissions (Gerber *et al.,* 2013)

CHAPTER II

Effect of NDF content of hays on production performance and methane emission of dairy sheep

ABSTRACT

Several studies in recent years have focused strategies to mitigate methane emissions from ruminants. The aim of the following study was to evaluate the effect of the forage quality on milk production, digestibility and methane emissions in dairy sheep. The experimental hypothesis assumed that a better quality of forages increases milk production and reduces methane emissions. The experimental activities were carried out on Sarda dairy sheep housed in individual pens (n=16). Digestibility and methane emissions were measured in dry sheep in metabolic cages (n=8) and ventilated hood (n=4), respectively. Animals were divided in two groups and individually fed two diets containing 770 g of DM concentrates (soybean meal, corn flakes and corn grain, offered in equal doses for both groups) and two different forages offered ad libitum. One group was fed a forage with a lower NDF content (54.3% on DM basis; L-NDF) and one with a higher NDF content (66.1% on DM basis; H-NDF). Furthermore, the intake of water was also evaluated. Methane measurements were carried out using an open-circuit system with a ventilated hood. The test consisted of 8 hours of adaptation to the ventilated hood and 16 hours of emission measurements. Methane samples were collected every hour for 16 consecutive hours. Milk production was influenced by the type of diet (P = 0.02), L-NDF group produced more milk than the H-NDF group (0.72 vs 0.53 kg/d respectively) which was in line with their DMI recorded by the L-NDF group despite the H-NDF group (1.65 vs 1.22 kg/d respectively). Also, milk urea content was influenced by diet (P <0.01) and lower in the L-NDF than in the H-NDF (25.63 vs 32.97 mg/dl respectively). This result was probably related to the best quality of fiber in the L-NDF. Nutrient digestibility showed no significant differences between the two groups. Methane emissions per g of DMI were significantly lower (P = 0.036) in the L-NDF group compared to the H-NDF group (12.25 vs 15.62 g/kg DMI respectively). Methane emissions on milk production were also estimated based on the average intake recorded in lactating sheep and resulted lower in the L-NDF group than in the H-NDF group (28.49 vs 35.69 g/kg of FPCM respectively). The results obtained in this study preliminary show that the quality of forage could be a promising strategy both to mitigate methane emissions and to improve productive performance in dairy sheep.

1. INTRODUCTION

The importance of forages in ruminants feeding has evolutionary roots. Forage evaluation, and therefore the distinction between high or low forage quality, has always been made by the man since the domestication of animals (Combs, 1936). Forage is defined as 'edible parts of plants, other than separated grain, that can provide feed for grazing animals or that can be harvested for feeding (Allen *et al*., 2011). In recent years, the use of green forages has been a basic strategy to allow dairy farms to make profits (Kumar *et al.*, 2019). The production of high-quality forages is important for two reasons: i) increase the profits of the farmers; ii) decrease health problems (which lead to substantial economic losses). The main factor that reduces forage quality are mineral deficiencies, toxicity and nutrient imbalance (Allen and Segarra, 2001). The most important results linked to the low forage quality lead to a loss of milk yield, reproduction, morbidity and mortality, which consequently turn into large losses for the farmers both in the sheep and cow dairy sectors (Allen and Segarra, 2001).

The quality of forages is determinate by the level of available nutrients to the animal, which influences the digestibility, the breakdown of the nutrients in their fundamental units inside the digestive tract, the forage intake and, consequently, the productive performances of the animals (Dumont *et al*., 2015). To determine the nutrient level of forages, chemical analysis is used to quantify: the level of ash and nitrogen (N), structural carbohydrates such as neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL), the content of non-structural carbohydrates (NSC) and starch. In addition, biological methodologies are also carried out to estimate the digestibility *in vitro* of the dry mater (DM) and organic matter (OM), through the use of ruminal fluid (Tilley and Terry, 1963).

One of the main parameters used to evaluate the quality of the forage is the digestibility of NDF, because the rumen degrades the NDF contained in the forage in an extremely different way (Nocek and Russell, 1988). The level of NDF in forage is extremely variable, and depends on various factors such as the cultivated species, the phenological stage, and the environment in which the plant growth (Oba and Allen, 1999).

Several studies have shown that by increasing the quality of the forage there are increases in productive performances of the animals and, consequently, a decrease in greenhouse gas (GHG) emissions (in particular methane). For example, a study by Keady *et al.*, (2012) carried out using silage with different quality on ruminants, has shown that the 1% increase in digestibility of OM has brought benefits on productive performance both for cow (in terms of milk yield and daily carcass gain) and sheep (increase in daily carcass gain, birth weight and post lambing weight).

Furthermore, forages play a strategic role on the qualitative characteristics of milk. Diet poor in forage and rich in concentrate have a negative effect on fat milk % (Kalscheur *et al*., 1997). Forage are not important for increasing the amount of milk fat, bat they improve the quality of the fat itself. Despite not having high amounts of fatty acid (FA, 20-50 g/kg DM), forage are the safest health and least expensive source of FA in ruminant diets (Kalač and Samková, 2010).

Using high quality forages in ruminant diets is also important for mitigating environmental impact, as fiber is the main substrate that is used for methane production (McAllister *et al*., 1996). In fact, as demonstrated by Moe and Tyrrell, (1979), the methane emissions deriving from fermentation of cellulose are three times greater than those deriving from the fermentation of hemicellulose. This is confirmed by a study carried out on sheep by Hammond *et al*., (2011) to assess the effect of forage quality on methane production. In their

study they showed that using forages with low NDF content produces less enteric methane than those with higher NDF.

Livestock sector is one of the main producers of methane on global level, it is important to precisely know and quantity these emissions. Over the years different methods for methane measurement by ruminants have been developed, but the most precise system is undoubtedly that of the respiration chamber, which however have the disadvantage of being very expensive. Recently it has been proposed by Fernandez *et al.*, (2015) a new system very similar to the respiration chamber, which takes the name of ventilated hood. This system, which is much less expensive than the respiration chamber, only measures the gases emitted through rumination, belching and breathing; despite everything it has proved to be an excellent alternative to respiration chamber.

The objective of this study was to evaluate the effect of diets containing forages with different quality (in terms of NDF) on DM intake, milk yield, milk composition, digestibility and methane emissions in Sarda dairy ewes fed

2. MATERIALS AND METHODS

The experiment was conducted at the experimental farm of the University of Sassari, located in Ottava (Sassari), in the north-west of Sardinian, Italy, and was divided into 2 different parts: i) a lactation trial; ii) a digestibility trial. All procedures involving animals were fully in compliance with the European Community (86/609) and Italian regulations (DPR 27/1/1992, Animal Protection Regulations of Italy) on animal welfare and experimentation.

2.1 Lactation measures

Animals, experimental design and treatments. Sixteen Sarda ewes in late-lactation (184 ± 15 DIM, mean ± standard deviation) were allocated to two experimental groups, homogeneous for milk production (0.82 ± 0.20 kg/d per head), body weight (44.94 ± 5.58 kg) and body condition score (BCS 2.84 ± 0.16). Animals were confined in a barn and kept in individual boxes (about 2 m^2), where the daily amount of feed was offered individually and fresh water was always available. The experimental diets (used for both the two parts of the experiment) consisted of concentrate (Table 1) and forages. The concentrate was fed in the same amount to both groups and the experimental treatment was based on the forages offered to the animals. It was hypothesized that animals will vary their daily intake on the basis of the quality of the forage offered.

The forages for the two groups were selected to have high difference in NDF content (Table 2). Forage 1 was used in the diet containing hay with low level of NDF (L-NDF; 54.3 % of NDF); forage 1 and forage2 were both used, in combination, for the diet containing hay with high level of NDF (H-NDF), with a ratio of 34/66, respectively, obtaining a forage with 66.1% of NDF. In particular, forage1 was composed by a mix of Italian ryegrass (var. Attain) and clover (*T. squarrosum Savi*) and was characterized by a low level of NDF (54.34 % DM basis); forage2 was a mix of Italian ryegrass and oat, and had a very high level of NDF (72.10 % DM basis). Diets contained 16% CP (DM basis) and were formulated to meet animal requirement using the Small Ruminant Nutrition Model (Tedeschi *et al*., 2010).

During the two-daily milking (at 7:00 and 16:00), the animals were fed with 220 g/d per head of corn grain, the amount was split in the two milking. The remaining part of the amount of concentrate was divided into 3 equal meals, offered at 7:30, 12:00 ad 16:30. In order to guarantee an *ad libitum* consumption of the hay, the daily amount offered was calculated on

the basis of the hay intake registered in the previous day (+ 20 % of the hay consumed). The hay was offered once a day in the morning and was available throughout the day.

Samples collection. Samples of feeds ingredient were collected one time a week and stored until chemical analysis. Animals were milked at 7:00 and 16:00; milk yield of each experimental group was measured every day, while individual milk yield was measured two times a week and samples of two milking (morning and afternoon) were collected for analysis.

2.2 Digestibility measures

Animals, experimental design and treatments. For this part of the trial 8 animals (4 per group) of the total 24 were selected and confined into metabolic cages, making 2 subgroups homogeneous for BW (44.53 ± 3.83 kg) and BCS (BCS 2.92 ± 0.21). The trial consisted of 5 days of measurements and data collection, preceded by a 7 days period of adaptation to the metabolic cages. The concentrate was offered in a fixed amount, equally divided in three meals: at 23:00, at 7:00 and a 15:00. The daily amount of hay was prepared daily for *ad libitum* consumption (calculated on the hay intake of the day before); it was offered, once a day, in the morning and was available during all day.

Samples collection. Feed orts were collected, measured and sampled every day. Water was offered and consumption measured at 23:00, at 7:00 and at 15:00. Feces were collected, weighted and sampled every day, at 10:30.

2.3 Methane measurements

To measure methane emissions from sheep, an open-circuit system with a ventilated hood was used. The system, schematized in Figure 1, consists of two parts: i) a head hood suspended on

the front part of a classic metabolic cage for small ruminants, and ii) an air sampling system, which contains instruments for air evacuation and sampling from the hood. Details about materials and dimensions used for the construction of the ventilated hood and for the air sampling system are described in the following sections.

Hood. The head hood (Figure 2) was built at the Department of Animal Science of the University of Sassari, Italy. the hood was designed following the example of Fernandez et al. (2015), whereas some elements and components were different, and several modifications were made. The frame of the hood was made by galvanized angle iron (30 × 30 × 3 mm) and was 50 cm × 125 cm × 35 cm (L × H × W), resulting in a volume of 218 liters. The top and the bottom of the hood were closed by soldering 3 mm iron galvanized plats. The front and the two sides of the hood were closed by 4 mm clear polycarbonate plates, in order to allow, as much as possible, a full vision for the animal inside. This is of particular importance, as it allows the animals to see each other and to acclimatize, as soon as possible, to the system. The polycarbonates plates were laid and glued with silicon on the inside of the angle iron frame; moreover, the three plates were soldered each other by silicon. In the front part, in the bottom, the hood has a drawer made by 3 mm galvanized plat (41.5 cm × 30 cm × 33.5 cm, L × H × W, respectively), and equipped with a handle. The drawer can be easily opened and closed, hallowing to place feed and water and to take possible residues. The airtight closing of the drawer is ensured by rubber seal tape and by two lateral locks. The back of the hood was closed by a 4 mm galvanized iron plate, that was clamped to the frame by a number of stainless-steel bolts and nuts; an adequate seal was ensured by a rubber tape placed on the edge of the plate. On this plate, a 45 cm × 45 cm (H × L) opening was cut, in order to allow the head to enter the hood. In order to avoid gas leakage, the rear opening is closed on the neck of the animal by a slave made by airtight material; the end of the neck slave was

provided of a wood frame (that fits on the edges of the hood's rear plate opening) that hallows the fixing to the hood by a series of stainless-steel bolts and nuts. The part of the neck slave to be placed on the neck of the animals has a maximum diameter of 26 cm and can be adjusted to the animal neck dimension by nylon drawstring. The material of the neck slave was chosen to ensure airtight but also for its robustness that allows to avoid the use of neck lace and chain. Moreover, it allows the animal to stand, to reach the feed and water and to lie down. On the top of the hood, two opening (50 mm diameter) were used as the air inlet and outlet. The outlet opening was equipped with PVC nipple and connected by a PVC spiral pipe (50 mm internal diameter) to the gas sampling system. Another opening (8 mm diameter) allows to connect an instrument (TIM 12 CO_2 meter, USA) used for the continuous measurement of CO_2 concentration, temperature and humidity inside the hood. During the adaptation and measurement periods, the CO_2 meter continuously measures CO_2, temperature and relative humidity inside the hood. The instrument gave and recorded measures every 20 seconds. The continuous monitoring of CO_2 is essential to avoid accumulation of this gas in the hood, which may be dangerous over certain levels. Values of temperature and humidity are also important information, as they must be considered for the calculation of the amount of air drawn by system.

Gas sampling system. Air inside the head hood is drawn by a centrifugal fun (CST 60 Solar Palau Inc., Barcelona, Spain), which can supply approximately 310 m^3/h air flow rate as maximum capacity. The fun is located at the end of the air circulation system, with free escape of the air; it works continuously allowing a negative pressure in the hood head and ducting, thus avoiding any leakage out of the system. Air flowing out from the head hood is first filtered to keep away the dust, by a F198 air filter (PVR srl, Valmadrera, Lecco, Italy). The air flow is measured by a flowmeter (M123, Rometec srl, Roma, Italy), having a range of

1.5 to 1000 L/h, and is regulated at 3000 L/h by a two PVC manual valves system: one is placed between the flowmeter and the fun, serves as inlet to receive air from the hood head and allows to regulates the air flow of the system; the other valve regulates the inlet of the air from the ambient, necessary to avoid stress to the fun, as the air arriving from the hood head is only 1/100 of the operating fun capacity. Connections between the dust filter, the flowmeter, and the manual valves are with PVC spiral pipe (30 mm internal diameter).

The outlet air from the hood head system is continuously sampled, immediately after the dust filter, into 10 L non-diffusing collection gas bags (Supelco, Supel™-Inert Multi-Layer Foil, Sigma Aldrich, Italy). The gas is delivered and stored into a 10 L non-diffusing bag, at a flow rate of 125 cc/min (total of 7.5 L/h) by a micro pump (N89KNE, KNF Italia srl, Milano, Italy. Before entering the pump, the air is dried on a silica gel filter. The volume of gas to be collected in the bag is regulated by a micro valve and controlled by a 30-200 cc/min flowmeter (RMA-11, Rometec srl, Roma, Italy).

After bag collection, every hour, the air was subsampled into a vacutainer (10 mL) with no additive, by a sterile syringe (30 ml) and kept in the dark and at room temperature (18 °C) until analysis.

Animals, experimental design and treatments. 4 animals (2 per group) of the 8 that were used in the digestibility trial were selected and kept in the metabolic cages to performed methane measurements. The trial consisted of 12 days of measurements and data collection (3 days per animal) of feed intake and enteric methane. Feed was offered in the same way described above for the digestibility trial considering 3 intervals of 8 hours. At the beginning of each period (23:00, 7:00 and 15:00 hours) the feed was offered considering that methane emission patterns are highly dependent on the feeding frequency.

Samples collection. Feed orts were collected, measured and sampled every day. Water was offered and consumption measured at 23:00, at 7:00 and at 15:00. Feces were collected, weighted and sampled every day, at 10:30 as done in the digestibility trial.

Gas sampling was conducted using the ventilated hood described above and following this scheme: animals were introduced in the hood systems at 23:00 and the first 8 hours were considered as adaptation. Then, the remaining two period of 8 hours from 7:00 to 23:00 the air drawn during each hour was sampled for chemical determinations.

Air was sampled for 16 continuous hours for each day of experiment and emissions of the remaining 8 hours were estimated considering a simple time proportion. Daily emissions were converted from L/day to g/day using the conversion: 1 g CH_4 = 1.3962 L CH_4.

2.4 Laboratory analysis

Feed, residues and feces. The following analysis were conducted, in duplicate, for feed ingredients, residues and feces collected during the two experimental trials. Dry matter (DM) was determined by oven-drying samples at 105 °C for 24 h. An Ankom 220 fiber analyzer (Ankom™ technology, Fairport, NY, USA) was used to determine neutral detergent fiber (NDF) and acid detergent lignin (ADL), following the method of Van Soest *et al.,* (1991). The NDF content was determined using heat stable amylase and expressed exclusive of residual ash (NDFom); for ADL determination, concentrated sulphuric acid was used to solubilize the cellulose. Crude protein (CP) content was measured according to the Kjeldahl method (proc. 988.05; AOAC, 2000), extract ether (EE) by the Soxhlet method (proc. 920.39; AOAC, 2005) and ash by using a muffle at 550°C (proc. 942.05; AOAC, 2000). N_{EL} was calculated using the Small Ruminant Nutrition Model (Tedeschi *et al.,* 2010). The following

equation (Weiss, 1999) was used to calculate the concentration of non-fiber carbohydrates (NFC):

$$NFC\ (g/kg\ DM) = 100 - (NDF + CP + ash + EE)$$

Milk. Milk analyses were performed on individual milk samples in the morning and afternoon milking. The values of fat, protein, casein, lactose and milk urea (MU), have been determinated using a Milkoscan 6000 instrument (Foss Electric, Hillerød, Denmark); somatic cell count (SCC) have been analyzed using a Fossomatic 360 instrument (Foss Electric), in the ARAS (Sardinian Regional Farmer Association) laboratories.

Gas Analysis for methane. The determination of methane emission was carried out by a gas chromatographic (GC) method. The GC used was a Dani Master GC (DANI Corporation, Milan, Italy), equipped with a Valco-plot capillary column (30 m × 0.53 mm id × 10 μm; Valco, Poulsbo, WA, USA) and a flam ionization detector (FID). Helium was used as carrier gas. The split mode was 1:50. Analysis was operated in 2 minutes, in isothermal conditions with an oven temperature of 130 °C. Sample injection was carried out, manually, by a syringe for gas. Quantitative analysis was carried out by a calibration curve obtained with injections of methane standard at different concentrations.

VFA. supernatant that was injected into a HPLC system (Varian Inc., Palo Alto, California, USA) after filtration (PTFE 0.45 μm, 13 mm). 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 column was set at 55°C; the eluent was H_2SO_4 0.008 N and the flow rate 0.6 mL/min. 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), was used to estimate concentrations of VFA. The standard solutions were prepared 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 single VFA were expressed as mmol/L and mol/100 mol of total VFA, respectively.

2.5 Digestibility calculation

Individual feed intake (kg/d) and its composition was estimated as the difference between diet offered and orts, correcting for the chemical composition of the orts of each animal.

The digestibility coefficients of DM and of each chemical component of the diet (OM, ash, CP, NDF, ADF, ADL, EE and NFC) were calculated as:

Digestibility (%) = ((nutrient intake - nutrient excreted)/nutrient intake) * 100

2.6 Methane calculation

Calculation of enteric emissions of CH_4 was carried out according to Pinares and Waghorn (2012). Accurate measurements of the head hood wet ventilation rate (Wet VR), the net concentration of gas in dry sample, and the percentage of gas recovery in the entire system were considered. The calculation of Pinares and Waghorn (2012) starts with the result obtained from the gas analyzer, for each point of measurement, expressed as CH_4 ppm. Differently, in our work, a point of measurement is represented by the result of the analysis of an air sample accumulated in one hour of sampling in the bag. This difference must be considered for the calculation of daily emission.

The wet ventilation rate (Wet VR) has to be adjusted to dry standard temperature and pressure ventilation rate (Dry STP VR). For a given point of measurement, the emission of CH4 is calculated as follows:

CH_4 emission (L/min) = (Dry STP VR × ([CH_4 ppm]/1000000)) / gas recovery rate

The calculation of dry STP ventilation rate (Dry STP VR) requires data for relative humidity (%), temperature (°C) and pressure (hPa) specific for each head hood system.

Dry STP ventilation rate (L/min) = [(Air pressure × Dry gas VR) / (Chamber T + 273.15)] × 273.15/1013.25

where pressure is hPa, Dry gas VR is L/min, Chamber T is the chamber temperature in °C.

Dry gas VR (L/min) = Wet VR × [(100 – VMR)/100]

where Wet VR is the ventilation rate recorded from the flow meters in L/min, VMR is the Volume Mixing Ratio of moisture (%).

Volume mixing ratio (VMR) (%) = 100 × PWP/air pressure

where PWP is the partial water pressure (hPa), and the air pressure in hPa.

The partial water pressure (hPa) is obtained using the Wexler equation

Partial water pressure (hPa) = $(6.1117675 + 0.4439\ T + 0.014305\ T^2 + 0.000265\ T^3 + 0.00000302\ T^4 + 0.0000000204\ T^5 + 0.00000000006388\ T^6) \times RH/100$

where T is head hood temperature (°C) and RH is the chamber relative humidity (%).

As we sampled air for 16 continuous hours for each day of experiment, emissions of the remaining 8 hours were estimated considering the mean value of methane emission for each animal in a considered day. Daily emissions can be converted from L/day to g/day using the conversion: 1 g CH_4 = 1.3962 L CH_4.

2.7 Statistical Analysis

Data of BW, BCS, milk yield, milk composition, digestibility and methane production were analyzed with Anova, and means of groups were considered statistically different at $P < 0.05$ (Tukey test). For this analysis we used the PROC MIXED procedure of SAS (Version 9.0, SAS Institute Inc., Cary, NC) with repeated measurements.

In particular, a mixed model was used to test the differences between the diets as reported in the following model:

$$Y_i = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \gamma + \pi_{ij} + \varepsilon_{ij}$$

where Y_{ij} is the dependent variable, μ is the general mean, α_i is the effect of diet (i=H-NDF, L-NDF), β_j is the effect of period (j=1-9 Milk; j=1-3 BCS and BW, 1-5 Digestibility, 1-12 Methane), $\alpha\beta_{ij}$ is the diet × period interaction (i=H-NDF, L-NDF; j=1-9 Milk; j= 1-3 BCS and BW, 1-5 Digestibility, 1-2 Methane), γ is the random effect of animal and ε_{ij} is the residual error. SCC was previously log transformed.

3. RESULTS AND DISCUSSION

3.1 Lactation trial

Data of feed intake, energy intake, body weight, and body condition score of sheep of the two experimental groups are reported in Table 3; data on milk yield and composition are reported in Table 4. The figures 3 and 4 shown the evolutions of DMI and milk yield, respectively, of the sheep during the experiment. The most important results are represented by the higher level of milk production of sheep of L-NDF group than sheep of H-NDF group (0.74 vs. 0.53 kg/d ± 0.02 (mean ± SEM); $P < 0.01$) (Table 4), measured throughout the experiment despite the ewes were in late-lactation phase. Similar proportions were observed in terms of fat and protein corrected milk (FPCM). The higher production of sheep of H-NDF group was related to the higher level of intake showed by the same group in comparison to sheep of L-NDF group. In fact, throughout the experimental period the DMI was higher in the L group than the H group (1.64 vs. 1.22 kg/d ± 0.02) (Table 3). Although not significantly, similar results were obtained by Rodriguez *et al*., (2019) in a study carried out on ewes fed with grass of different

quality in terms of fiber (NDF content). In their study animals fed with plantain and chicory (diet NDF = 24.6% DM basis) compared to animal fed grass-based permanent sward (GBS) produced about 20% more milk (1.56 vs 1.31 kg/d respectively). The authors explained the phenomenon as driven by an increase in nutritional value, correlated to a lower neutral detergent fiber content which led to an increase in the DMI and consequently to a greater production of milk in the animals fed with the best quality grass. The results on milk production are in agreement with those obtained from Adewumi and Ahmed (2013), which have seen that diets with a low fiber content have positive effects on milk production in sheep. Although the L-NDF group was fed with a forage with a low NDF content, the total NDF intake (kg NDF) were numerically greater than in the H-NDF group (0.52 vs 0.42 kg/NDF respectively) but this difference was not statistically significant (Table 3). On the other hand, even expressing the NDF intake as % on the DMI, the values of the two groups were very similar (Table 3) and the differences were not statistically significant. Several studies carried out on dairy sheep in late lactation, shown how the use of diets with a higher level of NDF, in substitution of starch, had positive effects on milk production in dairy sheep (Cannas *et al.,* 1998; Cannas *et al.,* 2004; Cannas *et al.,* 2013; Kochendoerfer and Thonney, 2018). The highest milk yield registered for the L-NDF group has been likely favored by the high DMI, promoted by the highest quality of the forage (low NDF and high CP). As well know, DMI is the main factor that influences milk production also for small ruminants (Rapetti *et al.,* 1995). Differences observed in DMI are in agreement with Pasha et al. (1994) and Molano and Clark (2008). In both these studies it was noted that the DMI of forage is negatively correlated with the content of NDF. However, several studies also have shown that there is no relationship between the NDF level of the diet and the DMI. Weiss and Wyatt (2002), who carried out a study on dairy cows substituting silage with a high NDF content

with silage with a low NDF content, have not observed differences on DMI. Findings of this experiment in respect to DMI confirmed that low NDF forages are eaten in higher amounts and even when associated to lower consumption of concentrates allow to reach higher animal performances in respect to the inclusion of high NDF forages in the diets. Similar evidences are observed in dairy cows (Alstrup *et al.,*2016) and sheep (Jalali *et al.,* 2012; Cannas *et al.,* 2002; 2004; 2013). It was related to the fact that feeding low-digestible forage need higher chewing time per unit of DM and NDF intake and more intense rumination cycles in comparison with higher digestible forages (Jalali *et al.,* 2012).

The figures 5 to 9 shown the evolutions of the nutrients in the milk (fat, protein, lactose, SCS and urea).

Milk fat content was lower in the sheep of L-NDF group that sheep of H-NDF group (7.27 vs. 7.59 % ± 0.08) but this difference was not significant (Table 4). These results were unexpected because as it is commonly known that the degradation of the fiber releases acetic acid, which is the main precursor of milk fat (Linington *et al.,* 1998). Pulina and Rassu (Pulina and Rassu, 1991) found a positive relationship between the fiber level of the ration and the fat content in the milk (r^2 = 0.48). However, the positive relationship that exist between the level of NDF of the diet and the concentration milk fat is not easy to interpret, because, by increasing the % of the fiber in the diet there is a reduction in the DMI and digestibility. This leads to a lower milk production and a higher milk fat content due to a concentration effect (Bencini and Pulina, 1997). Milk protein concentration did not differ between the two diets but this was numerically lower in the L-NDF group than in the H-NDF group (6.43 vs. 6.51 % ± 0.06) (Table 4). This result is in contrast to what was seen by Lynch *et al.,* (1991), Cannas *et al.,* (1998) and Pulina *et al*., (1990), who noticed a positive relationship between the protein level of the diet and the milk protein concentration. The

results obtained in our study could arise from a dilution effect of the milk protein in animals of L-NDF group, determined by a greater DMI and consequently by a greater milk yield. Similar results have been proposed by Frey et al. (1991) and Robinson et al. (1974) in sheep. Similar results were found for SCS which were lower in the L-NDF group than the H-NDF group (2.17 vs. 2.40 Log SCC ± 0.03) (Table 4) but the difference was not significant. Milk lactose concentration did not differ between the two diets but was numerically greater in the animals of L-NDF group than those fed H-NDF group (4.42 vs. 4.28 % ± 0.04) (Table 4). The concentration of urea in milk (MUN) was different between the two diets. In fact, in the L-NDF group the MUN was on average 23.3% lower than the H-NDF group (25.63 vs. 32.97 mg/dl ± 0.76) (Table 4). This trend was seen throughout the duration of the experimental trial (Figure 9). This result was unexpected, because the CP in the diet of the L-NDF group was higher than that of the H-NDF group (Table 2). The mechanism that explain these results could be linked to the energy level of the diet. In fact, the energy intake was greater in the L-NDF group compared to the H-NDF group (2.62 vs. 1.97 Mcal/d ± 0.02) (Table 3). The greater energy inside the rumen could have led to a better utilization of the ammonia by the rumen microflora and therefore to a lower production of urea by the liver. Similar results were observed by (Frey *et al*., 1991), who studied the effect of diets with a different energy level on the MUN concentration. As in our study, in that case the MUN concentration was lower for animals fed with diets richer in energy. Other studies such as those carried out by Bonanno *et al.*, (2008) and Broderick and Clayton (1997), showed a negative correlation between the energy concentration of the diets and the Urea level in the milk.

In a study conducted on dairy sheep fed diets containing different energy levels by Cannas *et al*., (1998), no significant differences were found regarding the concentration of MUN. In this case the higher passage rate of the feed due to a higher DMI by the group fed with the more

energetic diet, could have increased the protein escape from the rumen, increasing the amount of protein absorbed from the intestine and consequently the concentration of MUN (Cannas *et al*., 1998).

3.2 Digestibility trial

Data of DMI and nutrient intake of two experimental group are reported in Table 5. Data of digestibility of sheep of the two experimental groups are reported in Table 6 and in Figure 12. DM apparent digestibility was not affected by dietary treatments. However, coefficient of DM digestibility was slightly higher in sheep of H-NDF group than sheep of L-NDF group (78.40 vs. 75.57 % ± 0.81) (Table 6). These results were unexpected because the group fed with the best quality hay should have had higher digestibility coefficients. Matejovsky and Sanson, (1995), in a work done to evaluate the effect of forage quality on DMI and digestibility, saw how as the quality of the forage increases both DMI and DM digestibility increase. The higher DMI of sheep fed L-NDF diet (Table 5) could have increased the passage rate (*kp*), leading to a decrease in the DM apparent digestibility (Matejovsky and Sanson, 1995). The results obtained in our study are in line with those obtained by Archimède *et al*., (2018) in a study carried out on sheep to evaluate the effect of grasses quality. Also, in this case grasses with a lower quality in terms of NDF content (in specific C4 cycle plants) were more digestible than plants with a lower fiber content (in specific C3 cycle plants). According to the authors these results depend on the greater DMI of the animals fed with C3 grasses, which led to a greater rate of passage (*kp*) inside the rumen and consequently the ruminal microorganisms had a shorter time to digest the nutrients. This is in agreement with the results obtained by Blaxter *et al*., (1956) that, in a study carried out on sheep they have seen how as the level of feeding increases the DM digestibility decreases. Similar results on sheep were obtained from

Waghorn *et al*., (2002), which in a study concerning methane emission by various forages, found a negative relationship between DMI and DM digestibility. Similarly, NDF true digestibility was not influenced by diet, but was numerically higher in the H-NDF group than the L-NDF group (65.29 vs. 60.00 % ± 1.75) (Table 6). The results obtained are in agreement with a work done by Qiu *et al*., (2003), who tested diets containing different NDF levels to evaluate NDF true digestibility on dairy cows. Also, in that case there were no differences in terms of NDF true digestibility among the forages used.

With regard to water consumption, significant statistical differences between the two groups have emerged (P = 0.06; Table 5). In particular, the L-NDF group ingested about 3.04 L/kg of DMI whereas the H-NDF group ingested 2.64 L/kg of DMI. The results obtained confirmed that water intake is positively correlated to water consumption. DMI has been indicated as one of the major factors influencing water intake in sheep (Hadjigeorgiou *et al*., 2000; Araújo *et al*., 2010; Hussein *et al*., 2018) however it was expected that water intake per kg of DMI would be higher in the H-NDF group due to the positive effects of NDF on rumination activity.

3.3 Gas measurement trial

Data of body weight, body condition score and intake of sheep of the two experimental groups are reported in Table 7. The BW and BCS of the animal of the two groups were similar because the 4 sheep were in order to have balanced BW and BCS.

Feed intake was slightly higher in the L-NDF group, due to the higher, even if not significant, intake of hay compared to H-NDF group. This first result might confirm that high quality forages stimulate intake. The concentrate was completely eaten by all animals throughout the experiment. Generally, nutrient intake was higher in L-NDF vs. H-NDF group, except for

NDF that was only numerically higher (Table 7). Higher nutrient intake was related to the lower content of forage NDF in the L-NDF group compared to the H-NDF group, considering that NDF content of feed intake was very similar in both groups (29.6 and 28.1 % of DM for L-NDF and H-NDF, respectively).

The different composition of the hay in the diet tended to influence the apparent digestibility of DM, OM and CP (Table 8), with the L-NDF group having the lowest values. The effect was significant for fiber (NDF), that resulted to be less digestible for the L-NDF group. This result was quite unexpected as the L-NDF group was characterized by a higher quality hay, in term of a lower NDF content. One explanation for the lower digestibility of the diet of L-NDF group can be found in the different intake of the two groups. In fact, higher intake can be related to a higher rate of passage of feed from the rumen to the intestine; the consequent lower time spent from the feed into the rumen, could have negatively affected its fermentation rate (Lunesu, 2016). In cows, reduction in hay digestibility was related to shortened ruminal residence time occurring at high intake (Shaver *et al*., 1986; Jentsch *et al*., 2007). It has to be noticed that total dry matter intake under methane measurements was highly and negatively correlated to CP digestibility ($r = -0.80$; $P < 0.02$) which is also consistent with high passage rate and reduction of CP digestibility in percentage terms.

Data on ruminal pH and VFA in sheep fed the different experimental diets are reported on Table 9. No diet effect was found for the analyzed parameters of ruminal metabolism, included the estimation of methane emission, obtained from the equation of Moss et al. (2000), based on the production of ruminal VFA. The lack of differences in term of VFA produced between the two groups agrees with the similar value of estimated methane, as its production is strictly related to the VFA metabolism (Moss *et al*., 2000).

This observation was confirmed by the *in vivo* measurement of methane in the sheep of the two experimental groups. Inclusion of forages with different NDF contents in the sheep diet did not affect the daily emission of methane among groups (Table 10). The mean of daily amount of methane produced by sheep involved in the experiment was 13.6 g/d, ranging from 10.71 g/d and 20.11 g/d. Opposite results were obtained by Criscioni *et al*., (2016) in a study carried out on dairy goats using two forages with different NDF level. In this case, the group fed with a forage with a higher NDF content produced more methane than the group fed with the best quality forage (25.9 vs 28.5 g/d respectively), but expressing the emissions on the DMI (g/kg DMI) the differences were not significant. Contrasting results have been obtained by Jonker *et al*., (2018) in a study carried out on sheep fed with pastures of different quality (high and low fiber content). The results of this study have shown how animals fed with good quality pastures (NDF = 51.5%; ADL = 2.1% DM basis) compared to animals fed with low quality pastures (NDF = 63.1%; ADL = 3.4% DM basis) produced about 20% more methane (422.7 vs 353.1 g/d CO_2 eq respectively). According to the authors the results can be explained by the greater digestibility of both DM and NDF in animals fed with good quality pastures compared to animals fed with low quality pastures (DMD = 73.9% vs 51.9%; NDFD = 76.0% vs 53.8%, respectively); probably the greater digestibility of the fiber led to a greater production of acetate compared to propionate, releasing greater quantity of H^+ inside the rumen that was used by methanogens to produce methane. Figure 13 reported the evolution of methane emission during the experimental days. Accumulation of CH_4 is evident in the hour immediately after the two meals (at 7:00 and 15:00); it is also evident a decrease in the methane emission at the last hours of the day (from 19:00 to 23:00). It consists with the pattern of methane emissions previously observed (Crompton *et al.*, 2011). In Figure 14 are reported the values of methane emission measured in single time spans between the daily

meals (7:00, 15:00 and 23:00); the mean value measured in the first part of the experimental day (from 07:00 to 15:00) was higher ($P < 0.05$) than that measured in the second part (from 15:00 to 23:00); this difference was significant ($P < 0.05$) for H-NDF group, and tended to be significant ($P < 0.10$) in L-NDF group. The sheep of H-NDF group showed higher value than L-NDF group from 07:00 to 15:00, whereas, from 15:00 to 23:00 sheep of the two diets presented similar values.

The mean of CH_4 (g/d) value was similar to that observed in other works on sheep. Lockyer and Jarvis (1995), using a polytunnel system, reported values (ranging 7.7 g/d to 18.7 g/d) obtained from 4 different studies, with mean value of 14 g/d. On sheep, mean value measured in respiration chambers was 13.9 g/d (Pinares *et al*., 2011). Also, Fernandez *et al*., (2019) in a work carried out on goats reported similar values of methane emissions (ranging from 12.3 to 18.1 g/d). Other works on sheep report mean values higher than these found in the present experiment. Results of our work are, in part, the emission range and trends observed by Molano and Clark (2008). Molano and Clark (2008) investigated the effect of herbage quality (NDF levels of the diet (obtained by using different stage of physiological maturity, vegetative vs reproductive of ryegrass) and found values ranging from 10.5 g/d to 35.9 g/d in sheep.

Differences between the two groups of animals can be observed when the daily amount of methane is reported as proportion of DMI and intake of NDF and CP, with the L-NDF group having lower values than H-NDF group (12.3 vs. 15.6 g/Kg of DMI, respectively). Decrease of CH_4 production per unit of intake related to increase of feed intake was previously reported (Ramin and Huhtanen, 2013). Three mechanisms were proposed by these authors, to explain it: the first (i) is related to the faster passage rate of feed, occurring as intake increase; this can reduce diet digestibility and the amount of fermented feed per unit of intake. This is in

accordance with our work, where nutrient digestibility was lower in the group with higher intake. Similar result was reported by Jentsch *et al.*, (2007). Other explanations regard: ii) the increase of efficiency of microbial synthesis which can improve the hydrogen sink of microbial growth; iii) change in rumen fermentation pattern with reduction of acetate to propionate ratio and, consequently, of the amount of H_2 available for CH_4 production. Also, McGeough *et al*., (2019) in a study carried out on lambs to evaluate the effect of diets with different fiber digestibility have obtained very similar results. In this case the most digestible diets turned out to be those that produced more methane, and the results are attributable to the increased production of acetate which led to greater methane production.

The methane emission was negatively associated with diet digestibility CH_4 (g/d per head) = 37 - 0.299 × DM digestibility (%) ($r^2 = 0.36$ P = 0.05). It was attributed on the negative effect of passage rate on DM digestibility being DMI negatively associated to digestibility (r = -0.75; P < 0.01) whereas methane emission tended to be positively associated to DMI (r = 0.56; P = 0.08).

An important point, related to the GHG reduction in dairy sheep farms, is the relationship between methane emission and milk production level. Even if in this work the measures of methane emission were carried out in dry ewes, some interesting considerations can be made when the daily amount of methane is extended to lactating animals fed the same diet. This estimate (Table 10, *estimated* g/d) was made multiplying, within each group the DMI observed in lactating sheep (Table 3) times the CH_4 emitted per kg of DMI measured in the hood and then dividing the daily emissions per kg of fat and protein corrected milk produced feeding sheep with L-NDF or H-NDF diets (Table 10). Mean of milk production was 0.58 and 0.70 kg of FPCM for sheep H-NDF and L-NDF, respectively (Table 4). Sheep fed L-NDF vs. H-NDF were expected to have a lower value of estimated CH_4 per kg of produced milk (28.5

vs 35.7 g of CH_4 per kg of FPCM) due to both the higher milk yield and the lower emission intensity for unit of DMI. A lower estimated value of CH_4 produced per kg of milk is also in line with then need to improve forage quality and get important benefits for production efficiency and to reduce GHG emission. In fact the mail goal of GHG mitigation strategies is not to reduce the emission for the unit of produced milk output more than to reduce methane emitted per animal on daily or annual basis. In applicative terms, it can be an important evidence to farmers that good nutritional practices could be a great opportunity for farm efficiency and incomes as well an important contribution to the global warming reduction.

4. CONCLUSIONS

This study has shown that lowering NDF content of forages of diets allow to increase DMI and improve productive performance in dairy sheep. In particular, dairy sheep fed with high quality hay and lower NDF (L-NDF) produced more milk (about + 35 %) and had longer milk persistency than those fed with poor quality hay. Forage quality also influenced the level of MU, which was lower in the L-NDF group. It was probably due to the greater energy intake which facilitates the use of the ammonia by the rumen microflora. On the other hand, forage quality did not increase the total digestibility of nutrients because compensated by higher passage rate (*kp*) of the feed in the rumen when associated to higher DMI.

The daily amount of methane (g CH_4/d) was no significantly different among treatments, but if methane emission was expressed per unit of DMI it resulted 20 % lower in animals fed L-NDF vs. H-NDF forage. It confirmed that forage quality improvement can be a strategy to mitigate methane emissions in the dairy sheep sector. This study was limited by the complex procedure for air sampling and gas composition analysis in respect to have continuous on-

time measurements. Other limits were represented by the fact that measurements were carried out in dry animals with low energy requirements and DMI: Thus, these results could be considered as preliminary and further studies are needed to confirm the main findings of this work.

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6. TABLES

Table 1. Ingredients and chemical composition of concentrate used in sheep diet

Ingredients	DM, %
Soybean meal	0.266
Corn	0.198
Flacked corn	0.172
Composition[1]	
DM, %	88.34
CP, % DM	21.01
NDFom, % DM	24.23
ADF, % DM	6.47
ADL, % DM	0.30
Ash, % DM	3.30
NE_L, Mcal/kg[3]	1.86

[1] DM: dry matter; CP, crud protein; NDF, neutral detergent fiber determined on an organic matter bsis; ADF, acid detergent fiber; ADL, acid detergent lignin.

[3] NE_L, energy was calculated using the Small Ruminant Nutrition System equations (Tedeschi *et al.,* (2010).

Table 2. Chemical composition of forages used in the trial

	Forages[1]		
	Forage 1 (L-NDF)	Forage 2	H-NDF[2]
DM, %	91.01	92.44	91.95
CP, % DM	11.37	5.67	7.61
NDFom, % DM	54.34	72.10	66.06
ADF, % DM	39.17	43.84	42.25
ADL, % DM	5.43	5.95	5.77
Ash, % DM	10.74	6.24	7.77
NE_L, Mcal/kg[3]	1.41	1.30	1.34

[1] Forage 1: used in the diet of L-NDF group (containing forage with low level of NDF) and in the diet of H-NDF group in combination with forage 2 (34:66, respectively).

[2] H-NDF: forage used in the H-NDF dieted obtained by the combination of the Forage 1 and Forage 2 in proportion of 34:66, respectively; used in the diet H, containing forage with high level of NDF.

[3] NE_L, energy was calculated using the Small Ruminant Nutrition System equations (Tedeschi *et al.,* (2010).

Table 3. Effect of diet, time and their interaction on intake, body weight (BW) and body condition score (BCS) of dairy sheep (n=16) during the lactation trial.

	Diet[1]		SEM[2]	P		
	H-NDF	L-NDF		Diet	Time	Diet × Time
Feed Intake, kg/d	1.36	1.83	0.018	< 0.001	< 0.001	< 0.001
Hay intake, kg/d	0.58	1.07	0.190	< 0.001	< 0.001	< 0.001
DMI, kg/d	1.22	1.64	0.017	< 0.001	< 0.001	< 0.001
NDFI, kg/d	0.42	0.52	0.039	NS	-	-
NDF, % of DMI	35.6	33.6	2.190	NS	-	-
NE_L intake, Mcal/d[3]	1.97	2.62	0.025	< 0.001	< 0.001	< 0.001
BW, kg	44.4	47.8	1.001	< 0.001	< 0.001	0.99
BCS	2.84	2.91	0.025	0.27	0.53	0.53

[1] H-NDF, sheep fed diet containing forage with high level of NDF; L-NDF sheep fed diet containing forage with low level of NDF.

[2] SEM, standard error of the mean.

[3] NE_L, energy was calculated using the Small Ruminant Nutrition System equations (Tedeschi *et al.,* (2010).

Table 4. Effect of diet, time and their interaction on milk yield and composition of dairy sheep (n=16)

	Diet[1]		SEM[2]	P		
	H-NDF	L-NDF		Diet	Time	Diet × Time
Milk yield, kg/d	0.53	0.72	0.019	0.02	< 0.001	< 0.01
FPCM, kg/d[3]	0.59	0.79	0.020	NS	<0.001	0.01
Fat, %	7.59	7.27	0.081	NS	< 0.01	0.05
Protein, %	6.51	6.43	0.056	NS	NS	NS
Lactose, %	4.28	4.42	0.037	NS	< 0.01	NS
Log SCC (x1000 cell/mL)	2.40	2.17	0.031	NS	NS	NS
Urea, mg/dl	32.97	25.63	0.760	< 0.01	< 0.001	< 0.001

[1] H-NDF, sheep fed diet containing forage with high level of NDF; L-NDF sheep fed diet containing forage with low level of NDF.

[2] SEM, standard error of the mean.

[3] FPCM, fat and protein corrected milk with 6.5% of fat and 5.8% of protein calculated as FPCM kg/d = milk yield * (0.25 + 0.085G + 0.035SAT) (Pulina and Nudda, 2001).

Table 5. Nutrient intake (expressed in kg of DM) and water intake (L/d) of the two experimental groups (L-NDF and H-NDF), of the digestibility trial.

	Diet[1]		SEM[2]	P		
	H-NDF	L-NDF		Diet	Time	Diet x Time
DM	1.18	1.34	0.022	NS	< 0.01	NS
OM	1.12	1.25	0.025	NS	< 0.01	NS
CP	0.22	0.25	0.003	< 0.001	< 0.01	0.054
NDF	0.43	0.46	0.015	NS	< 0.01	NS
NFC	0.44	0.51	0.008	< 0.001	< 0.01	0.060
Ash	0.06	0.09	0.004	< 0.01	NS	NS
ADF	0.26	0.31	0.011	NS	< 0.01	NS
Fat	0.02	0.03	0.000	0.025	< 0.01	NS
Water	3.12	4.08	0.113	0.006	0.042	NS

[1] H-NDF, sheep fed diet containing forage with high level of NDF; L-NDF sheep fed diet containing forage with low level of NDF.

[2] SEM, standard error of the mean.

Table 6. *In vivo* digestibility coefficient (expressed in % on DM) of the two experimental groups (L-NDF and H-NDF).

	Diet[1]		SEM[2]	P		
	H-NDF	L-NDF		Diet	Time	Diet x Time
DM apparent digestibility	78.40	75.57	0.806	NS	0.049	NS
OM apparent digestibility	80.41	77.50	0.753	NS	0.032	NS
CP apparent digestibility	79.43	76.83	0.799	NS	NS	NS
NDF true digestibility	65.29	60.00	1.750	NS	0.086	NS
NFC apparent digestibility	94.90	92.85	0.363	0.027	0.030	0.017
Ash apparent digestibility	42.27	50.29	2.120	NS	NS	NS
ADF true digestibility	54.83	53.02	2.190	NS	0.063	NS
Fat apparent digestibility	76.72	75.15	0.906	NS	0.017	NS

[1] H-NDF, sheep fed diet containing forage with high level of NDF; L-NDF sheep fed diet containing forage with low level of NDF.

[2] SEM, standard error of the mean.

Table 7. Body weight (BW), body condition score (BCS) and intake of sheep in the gas measurement trial

	Diet[1]		SEM[2]	P		
	H-NDF	L-NDF		Diet	Dime	Diet × Time
BW, kg[3]	41.35	41.60	0.802	-	-	-
BCS	2.78	2.78	0.132	-	-	-
Feed intake, kg/d	1.02	1.19	0.042	0.048	NS	NS
Hay intake, kg/d	0.30	0.50	0.058	0.075	NS	NS
Nutrient intake,						
DM, kg/d	0.93	1.11	0.037	0.021	NS	NS
OM, kg/d	0.89	1.04	0.032	0.026	NS	NS
NDF, kg/d	0.26	0.33	0.020	NS	NS	NS
CP, kg/d	0.20	0.23	0.005	0.001	NS	NS
Indig. NDF (ADL x 2.4), kg/d	0.15	0.18	0.007	NS	<0.01	NS
DM, % of BW	2.25	2.67	0.011	0.033	NS	NS

[1] H-NDF, sheep fed diet containing forage with high level of NDF; L-NDF sheep fed diet containing forage with low level of NDF.
[2] SEM, standard error of the mean.
[3] body weight measured at the end of the trial.

Table 8. Digestibility of sheep in the gas measurement trial

	Diet[1]		SEM[2]	P		
	H-NDF	L-NDF		Diet	Time	Diet × Time
DM apparent digestibility	81.73	78.76	1.525	0.071	0.929	0.352
OM apparent digestibility	83.45	80.38	1.418	0.057	0.923	0.348
NDF true digestibility	66.58	58.37	2.587	0.042	0.896	0.56
CP apparent digestibility	82.00	79.49	1.634	0.078	0.952	0.259

[1] H-NDF, sheep fed diet containing forage with high level of NDF; L-NDF sheep fed diet containing forage with low level of NDF.
[2] SEM, standard error of the mean.

Table 9. Ruminal pH, total VFA, proportion of single VFA and estimated methane emission, in sheep fed L-diet and H-diet

Item	Diet[1]		SEM[2]	P-value
	H-NDF	L-NDF		
Rumen pH	6.50	6.33	0.146	0.675
Total VFA, mmol/L[3]	100.94	109.41	10.05	0.757
VFA, mol/100mol				
Acetic acid	57.26	57.02	0.996	0.929
Propionic acid	14.31	13.89	0.540	0.780
Iso butyric acid	2.09	1.96	0.118	0.673
Butyric acid	20.32	21.06	0.681	0.684
Iso valerianic acid	1.90	2.20	0.310	0.714
Valerianic acid	4.12	3.86	0.498	0.849
Acetic:propionic ratio	4.02	4.18	0.148	0.809
Methane, mol/mol of VFA[4]	29.96	30.26	0.362	0.757

[1] H-NDF, sheep fed diet containing forage with high level of NDF; L-NDF sheep fed diet containing forage with low level of NDF.

[2] SEM, standard error of the mean.

[4] VFA: volatile fatty acids.

[3] Estimated according to Moss et al. (2000) CH_4 (mol/mol of VFA) = 0.45 × C2 − 0.275 × C3 + 0.4 × C4, where C2, C3 and C4 are acetate, propionate and butyrate, respectively, expressed as mol/100mol of VFA.

Table 10. Methane emission from dairy ewes fed diets containing forages with low and high level of NDF (L-NDF and H-NDF, respectively).

Methane emission	Diet[1]		SEM[2]	P		
	H-NDF	L-NDF		Diet	Time	Diet × Time
Measured						
g/d	14.81	13.59	0.795	NS	NS	NS
g/kg of DMI	15.63	12.25	0.930	0.033	NS	NS
g/kg of NDFI	53.98	41.90	3.600	0.091	NS	NS
g/kg of NDFdig	75.62	74.64	5.730	NS	NS	NS
g/kg of CPI	73.11	59.17	4.175	0.034	NS	NS
Estimated for lactating animals						
g/d[3]	21.24	22.42	-	-	-	-
g/kg of FPCM[4]	35.69	28.49	-	-	-	-

[1] H-NDF, sheep fed diet containing forage with high level of NDF; L-NDF sheep fed diet containing forage with low level of NDF.

[2] SEM, standard error of the mean.

[3] methane estimated considering the average DMI, measured during the lactation trial (DMI_L) and methane emissions in g/kg of DMI measured in the gas measurement trial as follow: CH_4 g/d in lactation = [(measured CH_4 g/d) / DMI_G] * (DMI_L), where DMI_G and DMI_L are the DMI measured during the gas emission trial and lactation trial, respectively per each group.

[4] calculated dividing the estimated emission in lactating sheep (g/d of methane) by the fat and protein corrected milk (FPCM) produced during the lactation trial as follow: g of methane/kg of milk = CH_4 g/kg of DMI_L / FPCM (kg)].

7. FIGURES

Figure 1. Hood head system suspended on the front of a metabolic cage for small ruminants. Metabolic cage (**A**); head hood (**B**); drawer (**C**); neck slave (**D**).

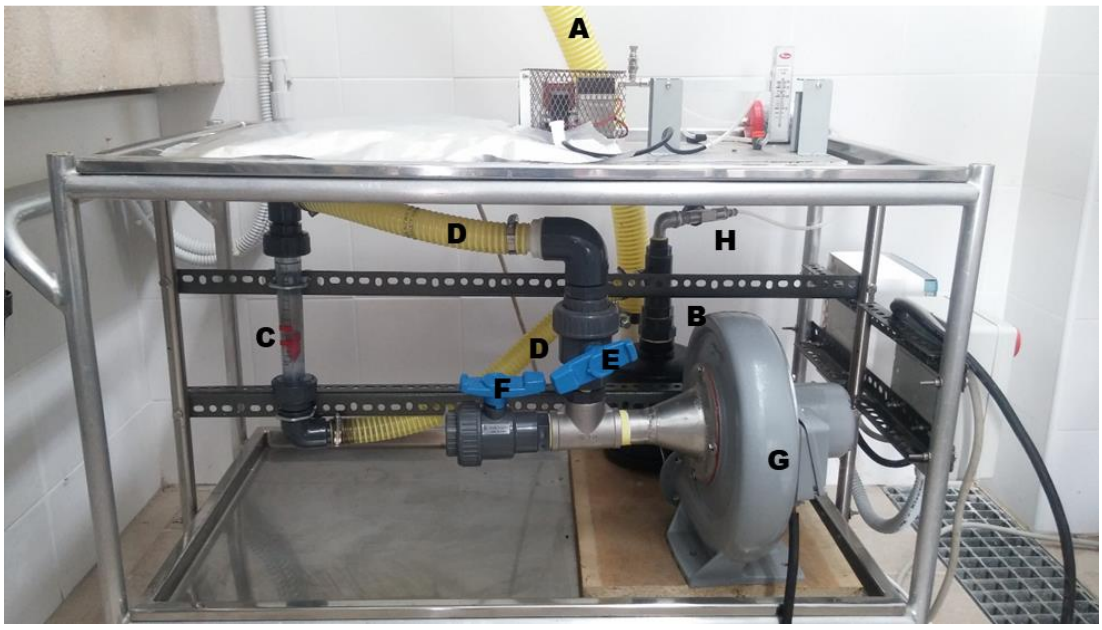

Figure 2. Air drawing equipment. Pipe line from head hood (**A**); dust filter **(B**); flowmeter (**C**); pipe lines for connections (**D**); regulation valves (**F, E**); centrifugal fun (**G**); connection to the upper sampling line (**H**).

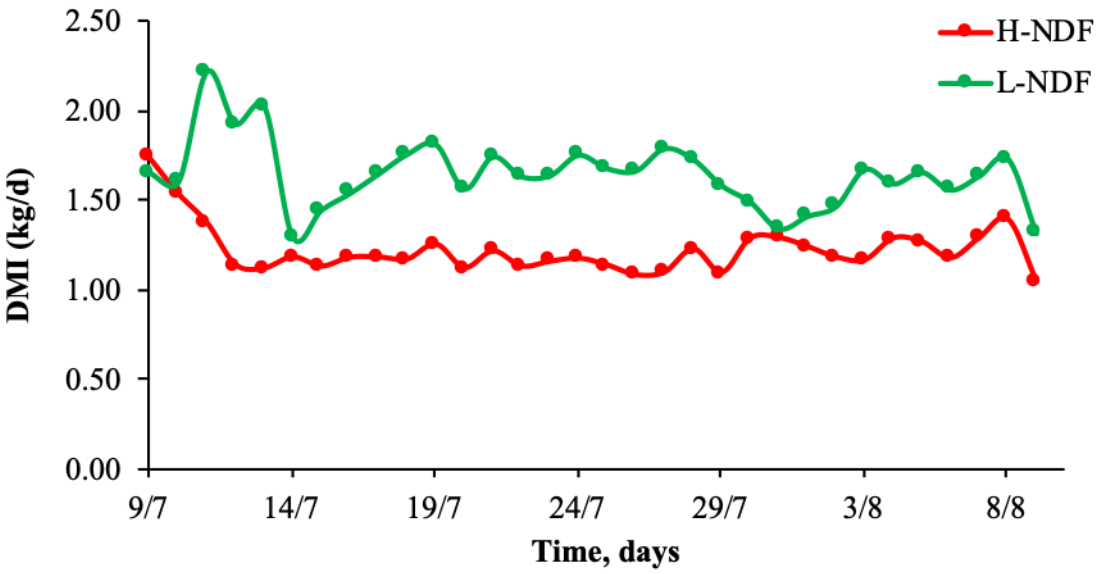

Figure 3. Evolution of DMI of sheep (n=16) of the two experimental groups (red line= H-NDF: sheep fed diet containing forage with high level of NDF; green line = L-NDF: sheep fed diet containing forage with low level of NDF).

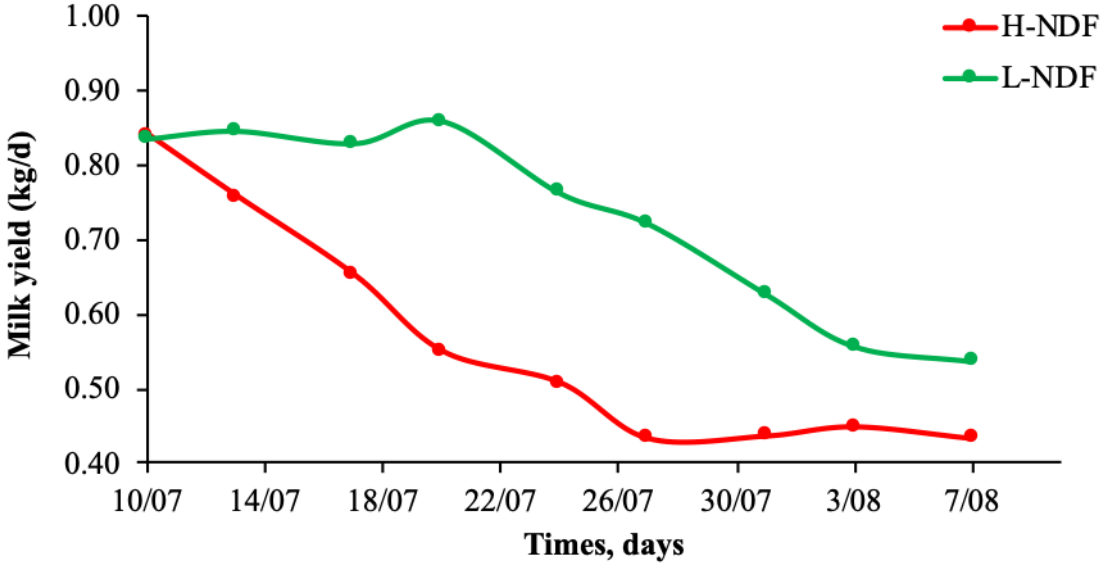

Figure 4. Evolution of milk yield of sheep (n=16) of the two experimental groups (red line= H-NDF: sheep fed diet containing forage with high level of NDF; green line = L-NDF: sheep fed diet containing forage with low level of NDF).

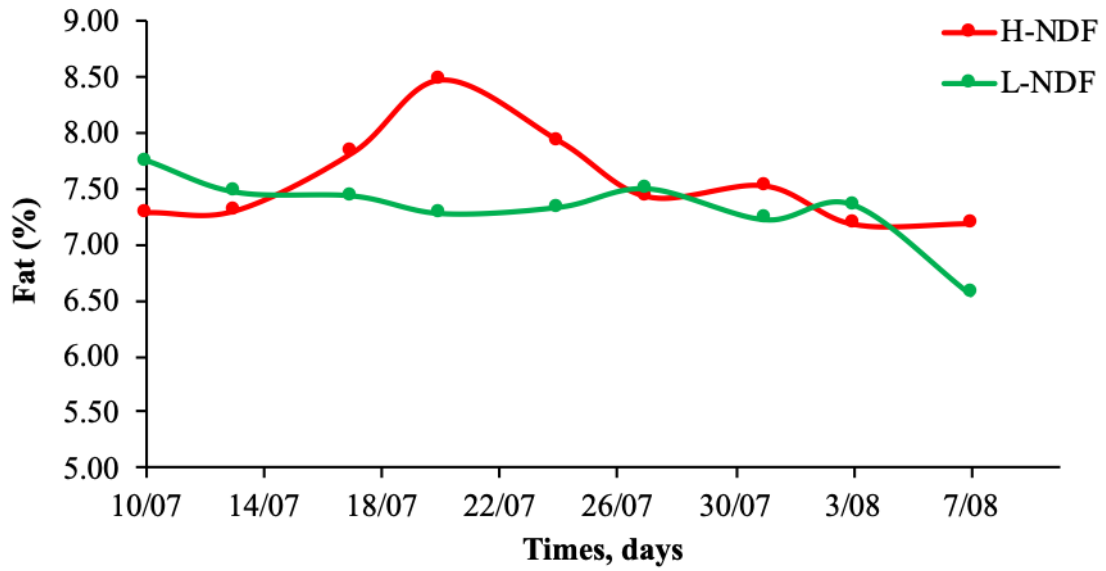

Figure 5: Evolution of milk fat of sheep (n=16) of the two experimental groups (red line= H-NDF: sheep fed diet containing forage with high level of NDF; green line = L-NDF: sheep fed diet containing forage with low level of NDF).

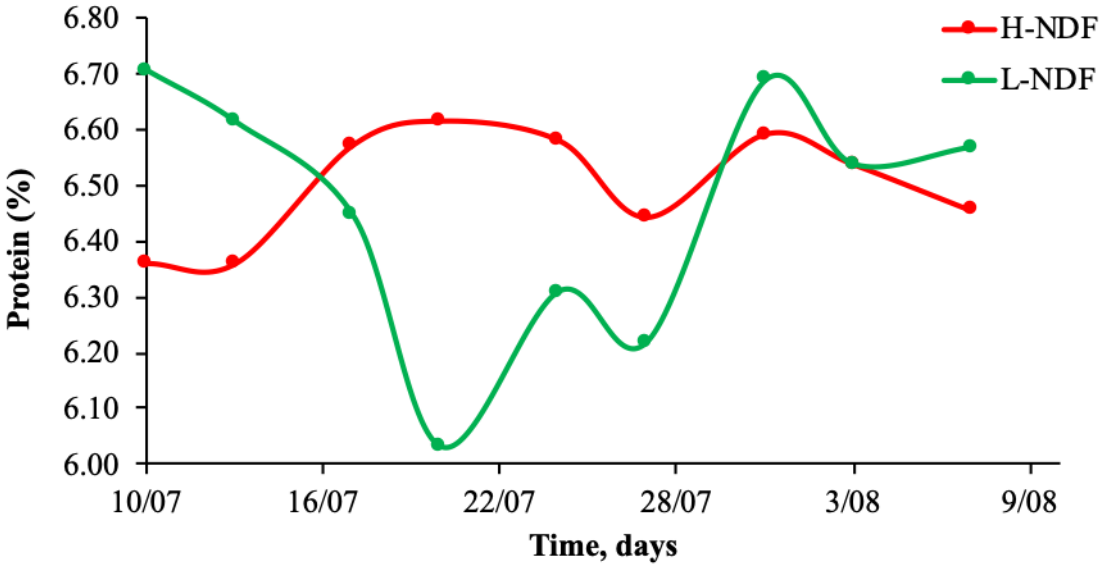

Figure 6: Evolution of milk protein of sheep (n=16) of the two experimental groups (red line= H-NDF: sheep fed diet containing forage with high level of NDF; green line = L-NDF: sheep fed diet containing forage with low level of NDF).

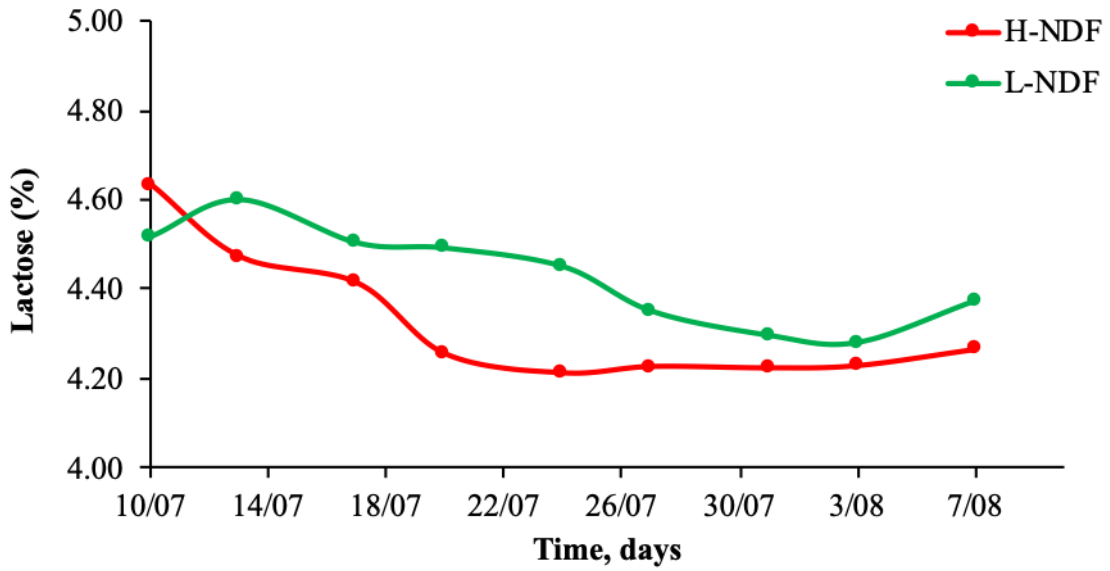

Figure 7: Evolution of milk lactose of sheep (n=16) of the two experimental groups (red line= H-NDF: sheep fed diet containing forage with high level of NDF; green line = L-NDF: sheep fed diet containing forage with low level of NDF).

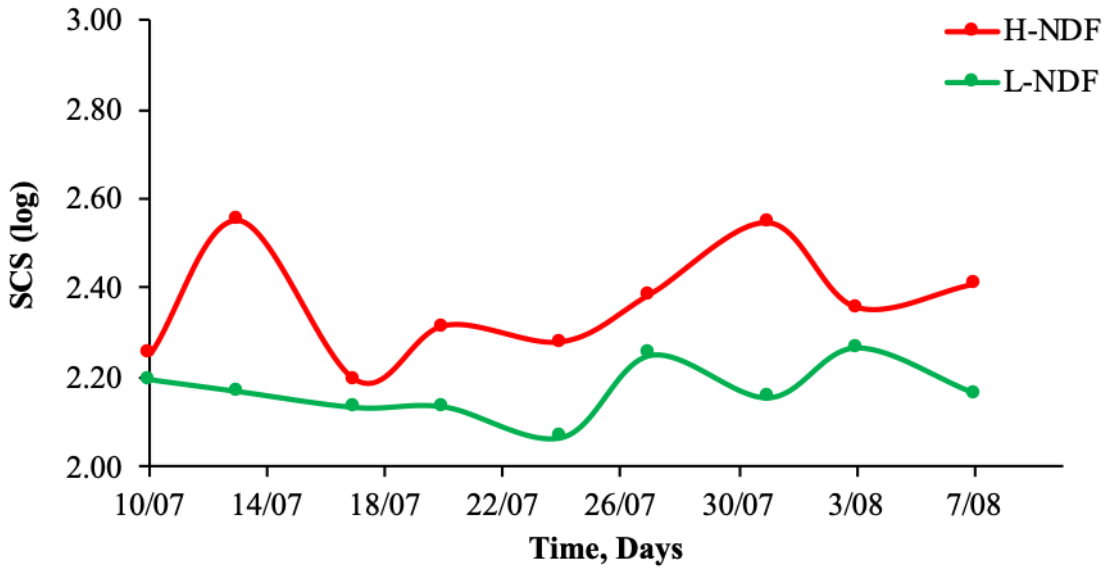

Figure 8: Evolution of milk SCS of sheep (n=16) of the two experimental groups (red line= H-NDF: sheep fed diet containing forage with high level of NDF; green line = L-NDF: sheep fed diet containing forage with low level of NDF).

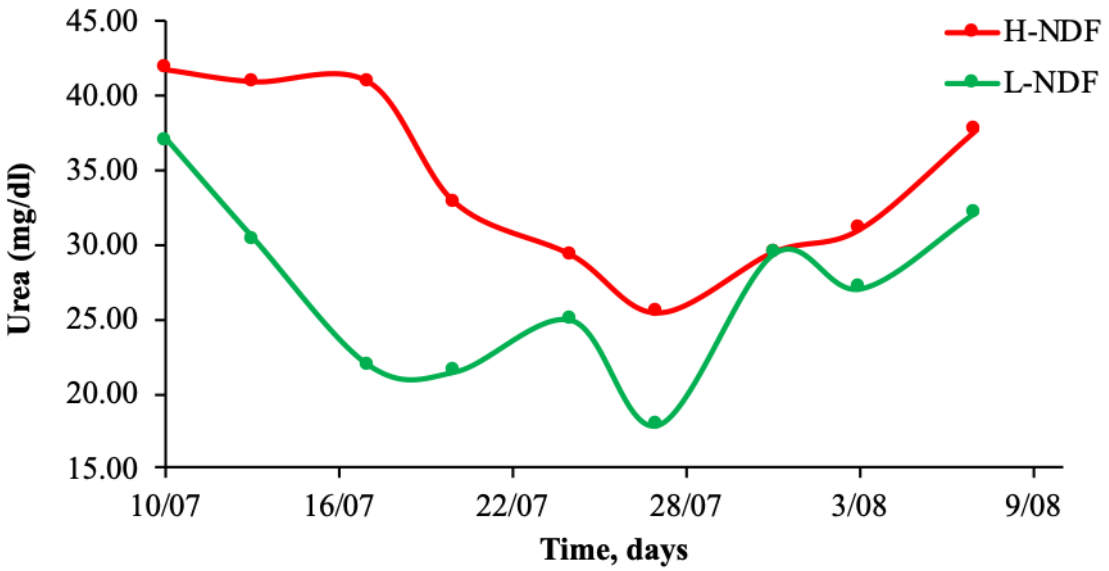

Figure 9: Evolution of milk urea of sheep (n=16) of the two experimental groups (red line= H-NDF: sheep fed diet containing forage with high level of NDF; green line = L-NDF: sheep fed diet containing forage with low level of NDF).

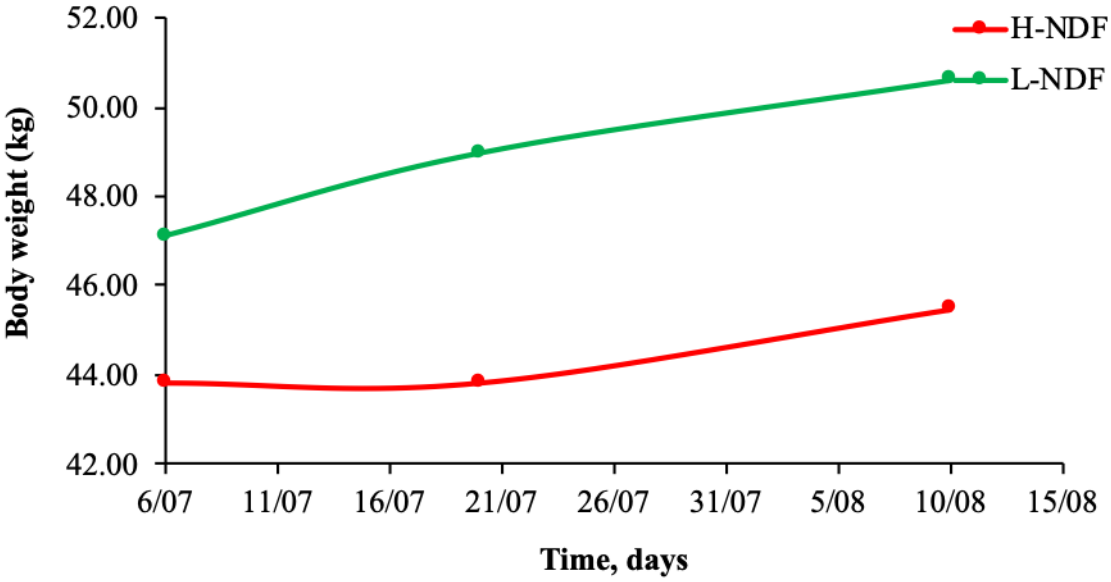

Figure 10: Evolution of body weight of sheep(n=16) of the two experimental groups (red line= H-NDF: sheep fed diet containing forage with high level of NDF; green line = L-NDF: sheep fed diet containing forage with low level of NDF).

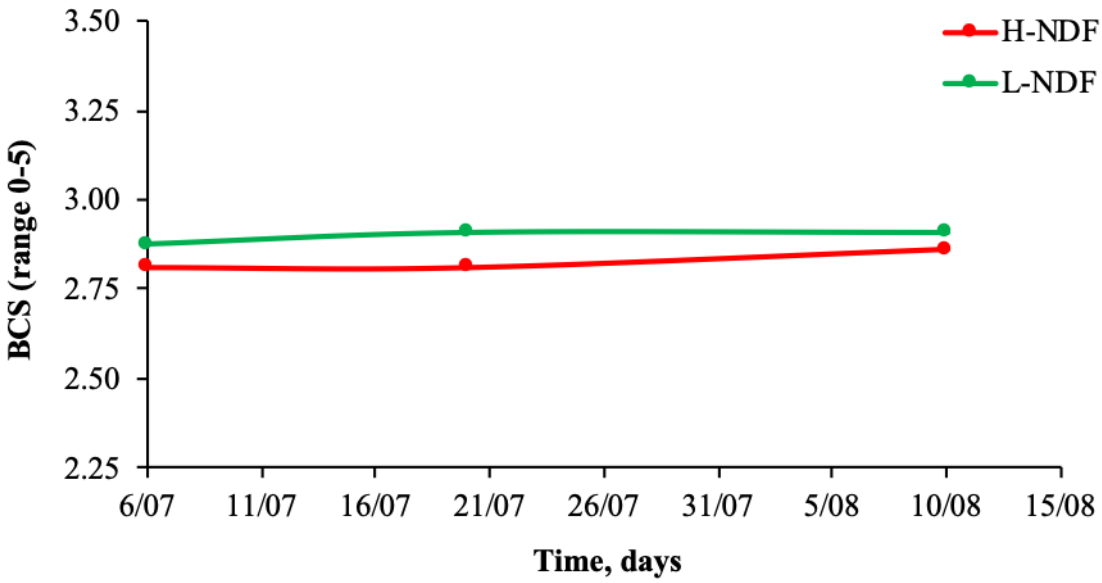

Figure 11: Evolution of BCS of sheep (n=16) of the two experimental groups (red line= H-NDF: sheep fed diet containing forage with high level of NDF; green line = L-NDF: sheep fed diet containing forage with low level of NDF).

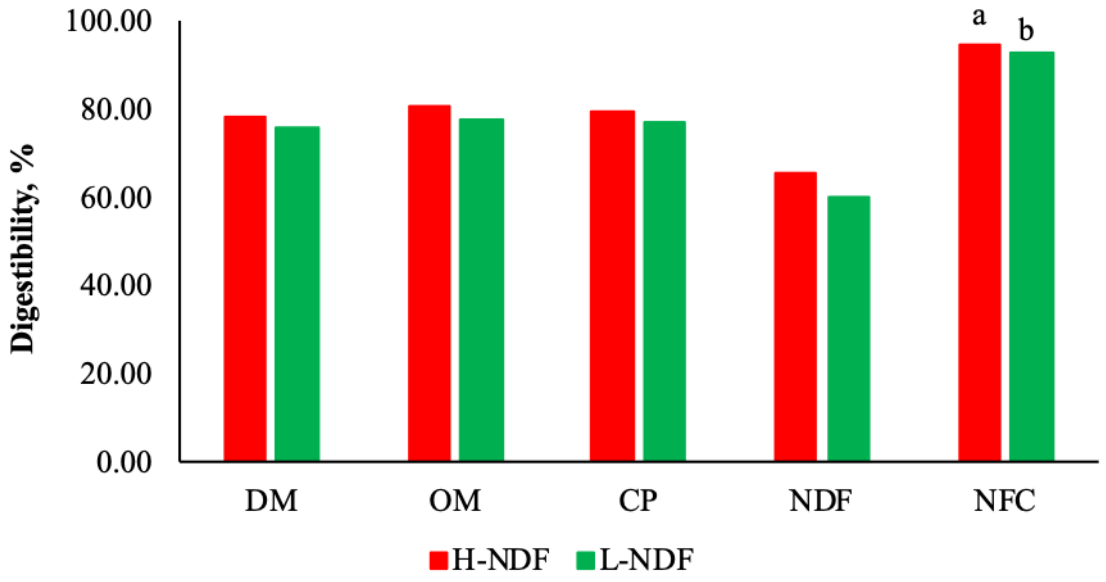

Figure 12: *In vivo* digestibility coefficient (expressed in % on DM) of the two experimental groups (L-NDF group and H-NDF group); letters a and b indicate significant differences (P <0.05).

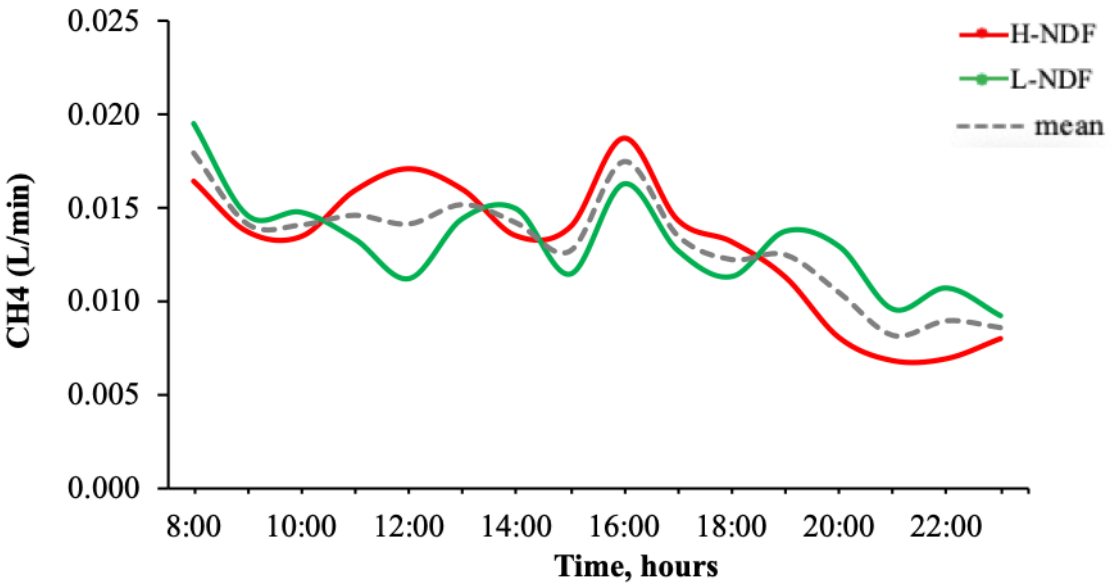

Figure 13. Evolution of methane emission during the experimental days, in the two experimental groups. (red line= H-NDF: sheep fed diet containing forage with high level of NDF; green line = L-NDF: sheep fed diet containing forage with low level of NDF). Feed was supplied at 7:00 and 15:00 hours.

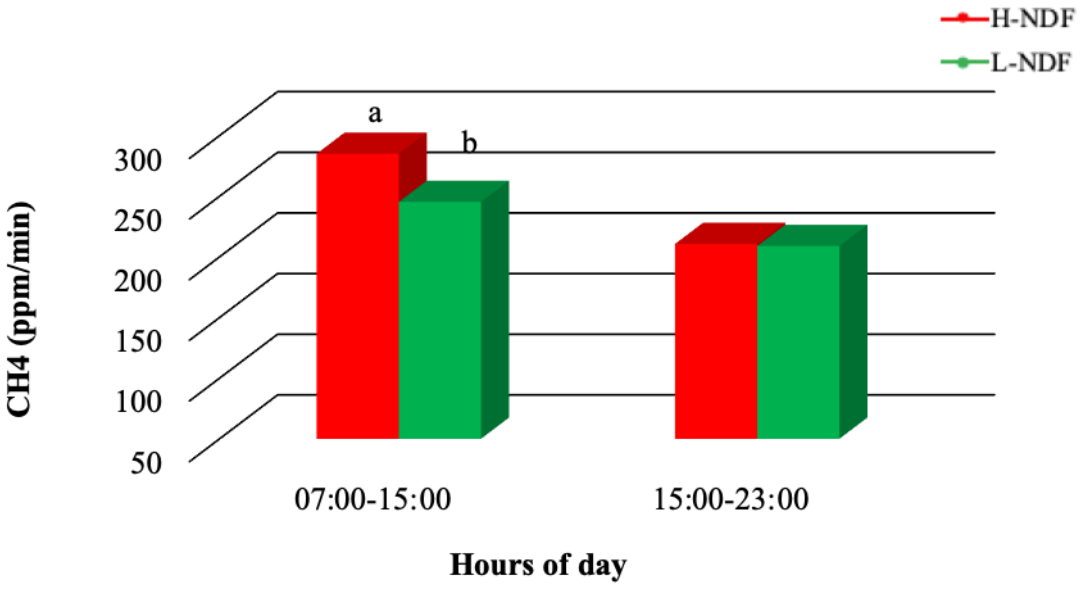

Figure 14. Differences in methane production between two different times within a day for sheep fed diets including hay with high or low content of NDF (H-NDF and L-NDF, respectively); letters a and b indicate significant differences ($P < 0.05$).

CHAPTER III

Effect of NDF content of haylages on production performance and methane emission of dairy sheep

ABSTRACT

Ruminant feeding is of considerable importance and increasing production performance through the optimization of the use of forages is a useful strategy to improve production and to mitigate the environmental impact of the livestock sector. Increasing the use of haylages could be an effective way to improve the quality of forages in dairy systems of Mediterranean areas. The objective of the following study was to evaluate the effect of the use of fodder on productive performance and methane emissions in dairy sheep.

The experimental, carried out on Sarda dairy sheep on late lactation, was divided into 3 parts: lactation trial, digestibility trial and methane emissions trial. Animals were divided into two groups and fed which two unifeed containing concentrates (soybean meal, corn meal and corn grain) and two different haylage: one with a low NDF content (37.12 % DM basis) and one with a high NDF content (48.73 % DM basis). The diets were isoproteic, isoenergetic, and had the same content as NDF. The L-NDF group (low NDF) was fed with a haylage with a low level of NDF, while the group H-NDF (high NDF) was fed with a haylage with a high level of NDF. In animals used during the lactation trial (15 sheep, located in individual box) the DMI, milk production and composition were measured (two weekly measurements). The digestibility trial was carried out with 8 sheep (4 per group) in metabolic cages, and feed and feces residues were collected and analyzed to perform the digestibility calculations. In addition, the milk production was measured and the milk composition analyzed. Methane emission measurement was carried out with 4 animals (2 per group) of the 8 that had previously been used during the digestibility test. The measurements were carried out using two open-circuit system with a ventilated hood in which the emissions of two animals of the two different groups were measured. The trial consisted of 8 hours of adaptation to the ventilated hood and 16 hours of emission measurements; measurements were performed using a continuous analyzer. Also in this case milk production was measured and the samples were analyzed.

DMI was statistically higher ($P = 0.008$) in the L-NDF group compared to the H-NDF group (1.81 vs 1.46 kg/d respectively). Milk production was not influenced by the diet, despite the different quality of the forages used. Also, in the milk composition there were no differences

between the groups, except for the milk urea concentration, which was statistically lower ($P < 0.01$) in the L-NDF group than in the H-NDF group (26.06 vs 31.43 mg/dl respectively). Probably in this case the quality of the fiber has determined a better synchronization of the nutrients inside the rumen. The quality of the forage appears to have had an effect on the digestibility of the DM ($P = 0.028$) and of the OM ($P = 0.044$), which were both greater in the L-NDF group. NDF digestibility was also superior in the L-NDF group compared to the H-NDF group, although not significantly (88.72 vs 66.99% respectively). As regards methane emissions, there were no significant differences between the two groups, although numerically the H-NDF group produced less than the L-NDF group (17.5 vs 23.0 gCH_4/d respectively). On the other hand, by expressing methane emissions on milk production, the L-NDF group emitted less than the H-NDF group (66.8 vs 38.6 gCH_4/kg milk).

The results of this study have shown in part how the use of high quality haylage can be an alternative for mitigate methane emissions in dairy sheep.

1. INTRODUCTION

Feeding is one of the main techniques to increase animal production performance, in terms of milk production, prolificity, growth rate or disease resistance, (Ulyatt and Waghorn, 1993). Among feeding strategies, the optimization of forage consumption is the most promising way to improve feed efficiency in ruminants. The consumption of forages by ruminants is influenced by various factors, but the fiber content (NDF) is that most influences ingestion (Harper and McNeill, 2015). As confirmed also by Herrero *et al*., (2015) the chemical composition of forage influences various aspects such as palatability, quality and quantity of milk produced and productive performance of ruminants.

The new mechanization techniques and the constant risk of adverse weather conditions have favored the replacement of the hay with the haylage within the livestock sector (Martin *et al*., 2004). In particular the climatic conditions are the main limiting factor for the production of quality forages. For example, the forages that are frequently produced during the summer period, where the weather conditions are more stable, are qualitatively worse than forages collected during the winter period (Kering *et al*., 2011) due to their high lignification and lower digestibility. Charmely (2001) has indicated the main advantages in the production of haylage compared to a hay, and in particular has emphasized how the shorter production time, the lesser dependence on the climatic conditions are among the main strengths of the haylage. Furthermore, the same machines that are used for the production of hay can be used, with in addition only a wrapping machine (Borreani *et al*., 2007). The main difference between silage and haylage lies in the content of DM; in fact, while silage generally has a quantity of DM between 30-40 % the haylage have a dry DM content that generally ranges from 40-50% (Luchini *et al*., 1997; McCormick *et al*., 2001). Furthermore, compared to traditional hay, the

haylage being produced at the best time for harvesting has a better quality in terms of nutrients and therefore a better digestibility.

In fact, poor quality forages has a DM digestibility minor, which is associated with a decrease in production performance by animals (Lee *et al*., 2017). Borreani *et al*., (2007) in a study carried out on dairy cows evaluated the effect of using hay or haylage on milk production. The main results obtained were: i) the quality of the haylage was greater than that of hay (lower content of NDF); ii) the animals fed with haylage produced more milk than those fed with traditional hay.

Several studies have also shown how the use of high-quality forages has a positive influence in mitigating the environmental impact of the livestock sector, and in particular in the reduction of enteric methane emissions from ruminants.

Recently, Fernandez *et al.,* (2019a) carried out a study on dairy goats to evaluate methane emissions and milk production using two alfalfa forages (a hay and a silage). The results showed that animals fed silage produced less methane (g/d) than hay-fed animals (21.5 vs 26.6 g/d respectively). With regard to milk production, on the other hand, no significant differences emerged, probably due to the fact that animals fed with hay had higher DMI than animals fed silage (1.72 vs 1.25 kg/d of DM respectively).

Waghorn *et al*., (2002) evaluated sheep enteric methane emissions using forages with a different fiber quality (NDF content); the forages used had an NDF content (DM basis) ranging from 44.0 % to 22.5 %. The results obtained showed that forages with lower NDF content produced less methane (about 20 % less) than forages with a higher NDF content. Yet, Fernandez *et al*., (2019b), in a study carried out on dairy goats have shown how using food with a better quality fiber decreases enteric emissions.

The objectives of the following work were i) to improve the analytical performance of sampled methane from a ventilated hood used to measure direct methane emission on small ruminants described in trial 1; ii) to evaluate if Sarda dairy ewes fed with two different haylages of different quality, in terms of high and low NDF and CP content, could have different response in milk production, diet digestibility and enteric methane emissions.

2. MATERIALS AND METHODS

The study, conducted in July and August at the experimental farm of the Department of Agriculture of the University of Sassari, in Ottava (Sassari), of the total duration of 30 days was divided into three phases: 1) lactation trial lasting 30 days including 12 days of adaptation (to new feed and stall boxes) and 18 days of measurements; 2) 10 days digestibility test, 5 days of adaptation to metabolic cages and 5 days of measurements; 3) 4 days methane emission trial. All procedures involving animals were fully in compliance with the European Community (86/609) and Italian regulations (DPR 27/1/1992, Animal Protection Regulations of Italy) on animal welfare and experimentation.

2.1 Lactation measures

Animals, experimental design and treatments. Fifteen Sarda ewes in late-lactation (140 ± 17 DIM, mean ± standard deviation) were allocated to two experimental groups, homogeneous for milk production (1.00 ± 0.19 kg/d per head), body weight (46.21 ± 5.60 kg) and body condition score (BCS 2.71 ± 0.17). Animals were confined in a barn and kept in individual boxes (about 2 m^2), where the daily amount of feed was offered individually and fresh water was always available. As for feed management, the administration was carried out once a day,

in the form of unifeed, which was prepared every day immediately after the morning milking. In order to guarantee an *ad libitum* consumption of the unifeed, the daily amount offered was calculated on the basis of the unifeed intake registered in the previous day (+ 20% of the unifeed consumed). The unifeed was offered once a day in the morning and was available throughout the day.

Haylage used to fed L-NDF group was characterized by a low content of NDF (37.12 % on the DM); haylage used to fed H-NDF group had a high NDF level (48.73 % on the DM). The composition of the haylages used during the experimental trial is shown in Table 1. The diets used during the experimental test consisted of a mix of concentrates and two different haylage of ryegrass and clover. During the diet adaptation phase, the animals of the two groups were fed with the same type of unifeed, characterized by the presence of two haylage wrapped in a 1:1 ratio and a mix of concentrates. The diets of the two groups used during the experimental trial were isoproteics (17 % of PG on DM) and isoenergetics (NEL = 1.71 Mcal/kg DM) and were formulated using the Small Ruminant Nutrition System software (Tedeschi *et al*., 2010). The concentrates used were soybean meal, corn meal and corn grain. The corn grain was offered during the two milking while the other concentrates were mixed together with the haylages.

Samples collection. Samples of feeds ingredient were collected one time a week and stored for the analysis.

Animals were milked at 7:00 and 17:00; milk yield of each experimental group was measured every day, while individual milk yield was measured two times a week and samples of two milking (morning and afternoon) were collected for analysis.

2.2 Digestibility measure

Animals, experimental design and treatments. For this part of the trial 8 animals (4 per group) of the total 23 were selected and confined into metabolic crates, making 2 subgroups homogeneous for BW (47.40 ± 5.05 kg) and BCS (BCS 2.81 ± 0.22). The trial consisted of 5 days of measurements and data collection, preceded by a 5 days period of adaptation to the metabolic crates. The daily amount of unifeed was prepared daily for *ad libitum* consumption (calculated on the hay intake of the day before; 20% orts); it was offered, once a day, in the morning and was available during all day.

Samples collection. Refusals were collected, measured and sampled every day in the morning. Feces were collected, weighted and sampled every day, at 10:30.

Animals were milked at 7:00 and 17:00; milk yield of each experimental group was measured every day, while individual milk yield was measured two times a week and samples of two milking (morning and afternoon) were collected for analysis.

2.3 Methane measurements

The system used in this trial (Figures 1 and 2) was an update of that described in the chapter 2. The most relevant differences, compared to the first system, were represented by the use of a second hood head and the of a gas analyzer. In particular the second head hood was built with the same materials and measures of the first, in order to conduct measurement on two animals in the same day; the gas sampling equipment was modified in order to draw and sample air from the two hood heads. The whole system, schematized in Figure 3, consists of two parts: i) two head hoods suspended on the front part of classic metabolic cages for small ruminants, and ii) an air sampling system, which contains instruments for air evacuation and

sampling from the hoods. Details about materials and dimensions used for the construction of the ventilated hood and for the air sampling system are described below.

The uploaded sampling system is reported in Figures 1 and 2. Air inside the two head hoods is drawn by a centrifugal fun (CST 60 Solar Palau Inc., Barcellona, Spain), which can supply approximatively 310 m^3/h air flow rate as maximum capacity. The fun is located at the end of the air circulation system, with free escape of the air; it works continuously allowing a negative pressure in the two hood heads and ducting, thus avoiding any leakage out of the system. Air flowing out from the two head hoods is first filtered to keep away the dust, by two F198 air filters (PVR srl, Valmadrera, Lecco, Italy). The air flows are measured by two (one for each head hood) flowmeters (M123, Rometec srl, Roma, Italy), having a range of 1.5 to 1000 L/h, and is regulated at 3000 L/h by a three PVC manual valves system: two are placed between the two flowmeters and the fun, serve as inlet to receive air from the two hood heads and allow to regulates the air flow of the system; the other valve regulates the inlet of the air from the ambient, necessary to avoid stress to the fun, as the air arriving from the two hood heads is only 1/100 of the operating fun capacity. Connections between the dust filters, the flowmeters, and the manual valves are with PVC spiral pipe (30 mm internal diameter).

Differently from the first system (chapter 2), where the outlet air from the hood head systems was continuously sampled, in this uploaded system a multi valves manual switch allows to select the air from one of the head hoods or from ambient. From the switch the air is delivered to the gas analyzer (GMS810 SICK S.p.A., Vimodrone, MI, Italy; range: CH_4 0-200 ppm; CO_2 0-2500 ppm; O_2 0-25 %vol), equipped with an internal micro pump and an auto calibration system that allows to keep the system calibrated for a long time. Data from the analyzer are than acquired by a software (SOPAS Engineering Tool. SICK S.p.A., Vimodrone, MI, Italy) installed on a PC.

2.4 Laboratory analysis

Feed, residues and feces. The following analysis were conducted, in duplicate, for feed ingredients, residues and feces collected during the two experimental trials. Dry matter (DM) was determined by oven-drying samples at 105 °C for 24 h. An Ankom 220 fiber analyzer (Ankom™ technology, Fairport, NY, USA) was used to determine neutral detergent fiber (NDF) and acid detergent lignin (ADL), following the method of Van Soest et al. (1991). The NDF content was determined using heat stable amylase and expressed exclusive of residual ash (NDFom); for ADL determination, concentrated sulphuric acid was used to solubilize the cellulose. Crude protein (CP) content was measured according to the Kjeldahl method (proc. 988.05; AOAC, 2000), extract ether (EE) by the Soxhlet method (proc. 920.39; AOAC, 2005) and ash by using a muffle at 550°C (proc. 942.05; AOAC, 2000). N_{EL} was calculated using the Small Ruminant Nutrition Model (Tedeschi *et al*., 2010). The following equation (Weiss, 1999) was used to calculate the concentration of non-fiber carbohydrates (NFC):

$$NFC\ (g/kg\ DM) = 100 - (NDF + CP + ash + EE)$$

Milk. Milk analyses were performed on individual milk samples in the morning and afternoon milking. The values of fat, protein, casein, lactose and milk urea (MUN), have been determinated using a Milkoscan 6000 instrument (Foss Electric, Hillerød, Denmark); somatic cell count (SCC) have been analyzed using a Fossomatic 360 instrument (Foss Electric), in the ARAS (Sardinian Regional Farmer Association) laboratories.

2.5 Digestibility calculation

Individual feed intake (kg/d) and its composition was estimated as the difference between diet offered and orts, correcting for the chemical composition of the orts of each animal.

The digestibility coefficients of DM and of each chemical component of the diet (OM, ash, CP, NDF, ADF, ADL, EE and NFC) were calculated as:

Digestibility (%) = ((nutrient intake - nutrient excreted)/nutrient intake) * 100

2.6 Methane calculation

The calculations for the determination of methane (L/d, g/d) were performed in the same way as described in chapter 2. The only difference is that in this case at each hour of measurement about 20 methane measurement were taken for each animal. To complete the measurement time, these were then averaged with readings for the next hour.

2.7 Statistical Analysis

Data of BW, BCS, milk yield, milk composition digestibility and methane production were analyzed with Anova, and means of groups were considered statistically different at $P < 0.05$ (Tukey test). For this analysis we used the PROC MIXED procedure of SAS (Version 9.0, SAS Institute Inc., Cary, NC) with repeated measurements.

In particular, a mixed model was used to test the differences between the diets as reported in the following model:

$$Y_i = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \gamma + \pi_{ij} + \varepsilon_{ij}$$

where Y_{ij} is the dependent variable, μ is the general mean, α_i is the effect of diet (i=H-diet, L-diet), β_j is the effect of period (j=1-9 Milk; j=1-3 BCS and BW, 1-5 Digestibility, 1-4 Methane), $\alpha\beta_{ij}$ is the diet × period interaction (i=H-diet, L-diet; j=1-9 Milk; j= 1-3 BCS and BW, 1-5 digestibility), γ is the random effect of animal and ε_{ij} is the residual error. SCC was previously log transformed.

The DMI data were analyzed with the same model previously described, but in this case the covariate effect was added (considering the data of the survey preceding the subdivision of the groups as covariate).

Data of digestibility and methane production were analyzed with Anova, and means of groups were considered statistically different at $P < 0.05$ (Tukey test).

3. RESULTS AND DISCUSSION

3.1 Lactation trial

Data on feed intake, energy intake, body weight, and body condition score of sheep of the two experimental groups are reported in Table 3; data on milk yield and composition are reported in Table 4. Regarding the DMI, statistically significant differences were recorded ($P = 0.008$) between the two groups. In particular the L-NDF group had higher values of intake compared to H-NDF group (1.81 vs 1.46 kg/d respectively, covariates values in respect to the initial intake level) (Figure 4). Moreover, a significant difference ($P < 0.001$) on the interaction between the diet and the day of the measurement was observed between the two groups (Table 3). It has to be noticed that differences in DMI were not evident without the covariate analysis due to an higher DMI observed before treatments in animals included in the group H. Similar results were obtained in a paper by Obeidat *et al*., (2018) in dairy sheep. In this study, 3 types of diets containing forages with different NDF content were tested. These authors observed that DMI of diets containing good quality forage (alfalfa hay with lower NDF level) was greater than for those with lower quality forage (wheat straw with higher NDF level). According to these authors, the greater DMI was due to less time spent for rumination and more time spent for intake. These results are in line with those obtained by West et al.,

(1999), who noted that a low level of NDF in corn silage of dairy cow diets helps to increase DMI. Also, Criscioni *et al*., (2019) have obtained similar results in dairy goats. In this experiment animals fed a diet with lower NDF forage (NDF of the diet 52% DM basis) had higher DMI, about 12% higher than the animals fed a diet with higher NDF forage (NDF of the diet 61% DM basis). Ramos *et al.,* (2011) carried out a study to understand the effect of the forage to concentrate ratio (F:C) and two different forage species (alfalfa and ryegrass) on digestibility and rumen fermentations both in sheep than in goats. They showed that animals fed alfalfa (% NDF of the diet 37-42% DM basis) ingested 5 to 10 % more DMI than ryegrass-fed animals (NDF of the diet 40-50 % DM basis). However, in a recent work carried out on lambs by Gallo *et al.,* (2019) was observed that passing from a diet with an NDF content of 15% (% NDF on DM basis) to a diet with a content of 25%, the DMI increases by only 10%.

Data on milk production and composition are shown in Table 4. As for milk production, there was no significant effect of the diet between the two experimental groups. However, the interaction between the effect of the diet and the effect of the measurement day was significant (P = 0.02). In particular, as is possible see from Figure 5, which shows the trend of milk production of the two groups, milk yield was higher in H-NDF group (0.93 kg/d per head), compared to L-NDF group (0.83 kg/d per head), for almost the entire trial period (except for the last measurement carried out). Fernandez *et al*., (2019b) obtained similar results in a study carried out on dairy goats. In fact, even in their case no differences were observed regarding milk production despite the use of two diets with different NDF contents. Manousidis *et al*., (2018) recently performed a work on dairy goats evaluating the effect on milk production and digestibility of grass and brushes cut at different stages of growth. Animals fed with grass cut at an early stage, and therefore with a better quality (low NDF

level and high CP level) produced about 50% more milk than animals fed with grass cut in a more advanced stage of growth (0.60 vs 0.39 kg/d in 2010; 0.90 vs 0.40 kg/d in 2011 respectively). The results obtained are in contrast with those reported in a paper by Natel *et al*., (2013). Their study carried out on dairy sheep showed that increasing the NDF content of the diet (from 23% to 50% DM basis) milk production decreased by about 50 %. Results of our study are contrast with those obtained by West *et al*., (1999) which observed that diets containing a lower level of NDF favored the production in dairy cows. In addition L-NDF group had higher DMI than H-NDF group (Table 3). In fact, DMI is expected to be the most important factor influencing milk production (Rapetti *et al*., 1995). Oba and Allen (1999) and Waldo (1986) also underlined that DMI is influenced both by forage digestibility and quality.

Data on milk fat content did not show significant differences between the groups, even the milk the milk fat content of L-NDF group was more (6.37 %) than that of H-NDF group (6.07 %). Protein and lactose levels were not influenced by diet.

Milk urea level was statistically lower in animals of L-NDF group (26.06 mg/dl) compared to those of H-NDF group (31.43 mg/dl) (Figure 6; $P < 0.001$). It was attributed to the better quality of fiber of L-NDF forage, whose carbohydrate degradability could have been better synchronized with the proteins of the soybean meal. Similar results have been obtained recently by Maamouri *et al*., (2019). In their study carried out on dairy sheep, they noticed that by introducing feeds with a more digestible fiber into the diet, the milk urea concentration decreased by 15-20%. The importance of synchronization and energy and CP of diet to reduce milk urea levels was recently highlighted by Giovanetti *et al*. (2019). In this study, various diets with a similar CP content (range CP = 17.9–19.7 DM basis) but with different energy levels were tested on dairy sheep (range NE_L = 1.20–1.88 Mcal/kg DM). The results obtained showed that there is a close correlation between MUC (milk urea concentration) and the

energy level of the diet, and in particular with increasing energy the MUC decreases. According to the authors, the increase of the energy available for microorganisms in the rumen allows a greater use of nitrogen by the latter and a lower waste, therefore decreasing the milk urea concentration.

3.2 Digestibility trial

Data of DMI and nutrient intake of two experimental group are reported in Table 5. Data of digestibility of sheep from the two experimental groups are reported in Table 6. DM apparent digestibility was affected by dietary treatments (P = 0.028). As expected coefficient of DM digestibility was statistically higher in sheep of L-NDF group than sheep of H-NDF group (85.77 vs. 78.73 %) (Table 5). In a recent work by Rinne *et al*., (2019) the digestibility of different forages was evaluated. The results obtained have shown that forages with a high level of NDF and ADL present a minor DM digestibility, highlighting how the quality of the fiber is the main factor that influences the DM digestibility. In another work carried out by Rapetti *et al*., (1995) the digestibility of the forages used in diets for dairy goats was evaluated and forages with lower level of NDF showed higher digestibility. The results of the NDF digestibility are in line with those obtained for the DM digestibility, which showed only a tendency to significance (P = 0.057); from a numerical point of view, the NDF digestibility was greater in the L-NDF group than in the H-NDF group (80.72 vs 66.99 % respectively). Very similar results were obtained by Yang *et al*., (2018) in a work carried out on Tiberian sheep to evaluate the effect of forage quality on nutrient digestibility. The results obtained showed that the forages cut at vegetative stage (low NDF and high CP content) compared to forages cut in late maturity stage (high NDF and low CP content) are most digestible (DMD= 76.1 vs 52.2% respectively; P <0.001). Furthermore, NDF digestibility reflected values of

DM digestibility (NDFD= 71.5 vs 51.1% respectively P = 0.018). According to the authors there was a close correlation between the nutrient composition of forages and its digestibility with higher NDFD in forages with lower NDF level. It reflects to the greater amount of energy available for the ruminal microflora when good quality forages are used for ruminant feeding.

Also, Soto-Navarro *et al*., (2014) showed that with the same NDF intake the digestibility of the NDF depends on the quality of the fiber, and lower NDF content is associated with higher NDF digestibility. Similar results were obtained by Jalali *et al*., (2012), in an experiment carried out both on goats and on sheep to test the digestibility of two different forages with a different NDF content: the good quality forage had an NDF content of 58 % on DM basis, whereas the poor quality forage had of 81 % on DM basis. In sheep the NDF digestibility of low NDF forage was about 14 % higher compared to high NDF forage. In goats, on the other hand, the differences were minimal and not significant.

3.3 Gas measurement trial

Data of body weight, DMI intake, milk yield, digestibility coefficients and methane emissions of sheep of the two experimental groups are reported in Table 7. The BW of the animal of the two groups were similar because the 4 sheep were in order to have balanced BW. DMI intake was greater in the L-NDF group than in the H-NDF group (0.62 vs 0.59 kg/d respectively) although this difference was not significant. This result would seem to indicate how forage quality favors intake. Milk production was also higher in the L-NDF group compared to the H-NDF group (0.72 vs 0.50 kg/d respectively), but in this case the difference between the two groups was not significant. This result is in line with the observed DMI, indicating the close positive correlation between these two variables. NDFI was very similar in both groups

(Table 7). These results were determined by the fact that there were no high differences in the DMI and also in both groups diets with a similar content of NDF were used. The different composition of the forages used did not influence the apparent digestibility of DM and OM and neither the digestibility of other nutrients (CP and NDF). It should be emphasized, however, that although there were no significant differences, numerically the NDFD was greater in the H-NDF group than in the L-NDF group (72.71 vs 65.22 % respectively). This result was unexpected because the haylage fiber used to feed the L-NDF group was qualitatively better (< NDF < ADL) than the haylage fiber used to feed the animals of the H-NDF group (> NDF > ADL). This result is in contrast with data from Carulla *et al*., (2005) in a study carried out on sheep using forage with a different content of NDF. The results of their study showed that NDFD was influenced by the NDF level of the feed, and in particular forages that have a lower NDF content have a higher digestibility of NDF, as expected. On lambs, de Carvalho *et al.*, (2017) showed not differences in digestibility of forages with different content of NDF and ADL.

The methane emissions values observed in our study are show in Table 7. The use of forages with a different content of NDF did not significantly affect the ewe emissions of methane. However, although there were no significant differences, the L-NDF group emitted more methane (g/d) than the H-NDF group (32.16 vs 24.49 gCH_4/d respectively). This result was also unexpected. For example, recently Wang *et al*., (2019) have carried out an experiment very similar to the one presented in this chapter, with the aim of evaluating how different quality forages can affect methane emissions in sheep. The study involved the use of 3 different diets with different F:C ratio. The results obtained showed that animals fed a diet with a high F:C ratio (in their case 90:10) emit less methane than animals fed diets with a lower F:C ratio (60:40) (15.2 vs 17.1 g CH_4/d respectively). The explanation given by the

authors was that increasing the amount of forage in the diet there was a decrease in the digestibility of NDF, and since the fiber was the main substrate used by methanogenic bacteria, lower digestibility caused lower methane production. This is in contrast to what we obtained in our study, as the L-NDF group had a lower NDFD and higher methane emissions. However, expressing the methane emissions on g NDF digested, the differences between the two groups become very small, and in any case not significant (Table 7). A similar result was obtained by Criscioni *et al*., (2016) on dairy goats. In their case the forage with a higher NDF level was more digestible (in terms of NDFD) than forage with a lower NDF level (60.9 vs 58.1% respectively); also, in this case as in our study, the difference in digestibility of the NDF did not affect methane emissions, which on the contrary were in a small extent greater in forage with a lower NDF content than forage with the higher NDF content (28.5 vs 25.9 g CH_4/d respectively). Also, Vargas *et al*., (2018) carried out an *in vitro* study to understand variation of methane emission from different forage species and forages cut at different stages of maturity. They showed that i) the forages that are cut at a young maturity stage produces less methane than the forages cut at an advanced maturity stage; ii) the results are different between the various species and in particular clover and ryegrass have two opposite emission trends during the various phases of maturation. To explain the results the authors proposed that in plants such as ryegrass, which during the growth phase tend to significantly increase their NDF content, the methane production is mainly due to the fiber degradation and in particular to the fact that there is a large production of acetate, which is the main precursor of the hydrogen which will then be transformed into methane. However, there are different cases such as clover, that tend to produce less methane as growth progresses probably due to an effect of the CP level on substrate degradation, but the mechanism is not yet fully accepted.

Methane production was also expressed on milk production (gCH_4/kg milk). In this case the L-NDF group obtained lower values than the H-NDF group (38.6 vs 66.8 gCH_4/kg milk). The results in this case were influenced by the higher milk production recorded in the L-NDF group, fed the best quality haylage (Table 7). Similar work has been done by Fernandez *et al*., (2019a) on dairy goats to evaluate the effect on productive performance and methane emissions using alfalfa hay and silage. The emissions of the group fed silage were lower than the group fed hay (21.5 vs 26.6 g CH_4/d), while the production performances were in favor of the group fed hay. Methane emissions expressed per kg of produced milk resulted 14.3 vs. 15.9 g CH_4/kg milk, for the groups fed haylage and hay, respectively. Therefore, the emission intensity relates the animal performance to the environmental impact of the milk production.

Figures 7 and 8 show the hourly pattern of methane emissions recorded during the experimental period. It seems that the pattern of emissions is not particularly linked to the type of diet, but rather the trend of emissions seems to be determined by the animal itself and the time of feeding. In fact, the two-day patterns different for the same animals are very similar to each other (for example Figure 8A and 8B), and this indicates a certain trend linked to the animal. Figure 7D shows the typical trend of methane emissions during the day and in particular the emission peaks are linked to the moment in which the animal ate. This is not evident in all animals, probably related to their feeding habits. This typical trend is very similar to those already described previously in dairy cows (Crompton *et al*., 2011), on which the administration of the ration in one or more meals, equally divided and distributed in the day, produces as many peaks as there are offered, with the reduction of methane production between one meal and another. These patterns were also confirmed in sheep (Zhang *et al*., 2007). In fact, even in their work the peaks of methane emissions were recorded immediately after the administration of the diet, which in their study was divided into two daily meals.

Very similar trends in methane emissions were also seen on goats (Martínez-Fernández *et al.*, 2013), with emissions peaks recorded after the intake of the main meal.

4. CONCLUSIONS

In this study, no significant differences emerged between the two groups regarding milk production, despite the use of different forage quality. However, forage quality also influenced the level of MUN, which was lower in the L-NDF group. This was attributed to a better use of nutrients by microorganisms at rumen level in diets with high quality fiber. On the other hand, forage quality did not increase significantly the total digestibility of nutrients. This result was unexpected and highlight the need to improve studies on haylages in dairy sheep feeding. The daily amount of methane (g CH_4/d) was no significantly different among treatments, but when expressed per kg of produced milk, ewes from the L-NDF group produced about half of the methane compared to the ewes fed H-NDF. However, the present study has shown that haylage can be a good forage source for the feeding of dairy sheep, and that its quality might help to reduce methane emission intensity in sheep. Further studies should be conducted to understand the dynamics of methane emission.in sheep fed diets with similar level of NDF of diet and different proportion of fiber fractions.

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6. TABLES

Table 1. Chemical composition of haylages used in sheep diet

	Haylage	
Nutrients[1]	H-NDF	L-NDF
DM, %	39.60	31.60
CP, % DM	12.15	16.66
NDFom, % DM	48.73	37.12
ADF, % DM	33.66	25.98
ADL, % DM	5.20	2.69
Ash, % SS	10.79	12.87

[1] DM: dry matter; CP, crud protein; NDF, neutral detergent fiber determined on an organic matter basis; ADF, acid detergent fiber; ADL, acid detergent lignin.

Table 2. Chemical composition of diet used in the adaptation phase and in lactation trial

Nutrients	Adaptation phase	Lactation trial[1]	
		H-NDF	L-NDF
DM, %	41.10	50.88	34.08
CP, % DM	17.11	17.09	17.22
NDFom, % DM	35.41	32.58	33.20
ADL, % DM	8.86	9.50	7.14
Ash, % DM	5.23	5.27	5.31
NE_L, Mcal/kg DM	1.67	1.70	1.72

[1] H-NDF, sheep fed diet containing forage with high level of NDF; L-NDF sheep fed diet containing forage with low level of NDF.

Table 3. Effect of diet, time and their interaction on intake, body weight (BW) and body condition score (BCS) of dairy sheep (n=16) during the lactation trial.

	Diet[1]		SEM[2]	P			
	H-NDF	L-NDF		Diet	Time	Diet x Time	COV
DMI, kg/d	1.46	1.81	0.03	0.008	< 0.001	< 0.001	< 0.001
NE_L, Mcal/d	2.75	2.77	0.05	NS	<0.001	<0.001	-
BW, kg	45.90	46.94	0.79	NS	NS	NS	-
BCS	2.70	2.74	0.02	NS	NS	NS	-

[1] H-NDF, sheep fed diet containing forage with high level of NDF; L-NDF sheep fed diet containing forage with low level of NDF.

[2] SEM, standard error of the mean.

Table 4. Effect of diet, time and their interaction on milk yield and composition of dairy sheep (n=16)

	Diet[1]		SEM[2]	P		
	H-NDF	L-NDF		Diet	Time	Diet x Time
Milk yield, kg/d	0.93	0.83	0.03	NS	< 0.001	0.02
FPCM, kg/d[3]	0.89	0.82	0.03	NS	<0.001	0.08
Fat, %	6.07	6.37	0.09	NS	< 0.001	NS
Protein, %	5.74	5.82	0.06	NS	NS	NS
Lactose, %	4.72	4.53	0.03	NS	NS	NS
Urea, ml/dl	31.43	26.06	0.71	< 0.01	< 0.001	NS

[1] H-NDF, sheep fed diet containing forage with high level of NDF; L-NDF sheep fed diet containing forage with low level of NDF.

[2] SEM, standard error of the mean.

[3] FPCM, fat and protein corrected milk with 6.5% of fat and 5.8% of protein calculated as FPCM kg/d = milk yield * (0.25 + 0.085G + 0.035SAT) (Pulina and Nudda, 2001).

Table 5. DMI and nutrient intake (expressed in kg on DM) of the two experimental groups (L-NDF group and H-NDF group), of the digestibility trial.

	Diet[1]		SEM[2]	P		
	H-NDF	L-NDF		Diet	Time	Diet x Time
DMI, kg/d	1.06	1.05	0.06	NS	<0.001	NS
OMI, kg/d	0.96	0.92	0.05	NS	<0.001	NS
CPI, kg/d	0.15	0.16	0.009	NS	<0.001	NS
NDF, kg/d	0.41	0.38	0.02	NS	<0.001	NS
NDF, % DM	38.05	35.51	0.57	0.09	0.04	NS

[1] H-NDF, sheep fed diet containing forage with high level of NDF; L-NDF sheep fed diet containing forage with low level of NDF.

[2] SEM, standard error of the mean.

Table 6. *In vivo* digestibility coefficient (expressed in % on DM), milk yield and body weight of the two experimental groups (L-NDF group and H-NDF group).

	Diet[1]		SEM[2]	P
	H-NDF	L-NDF		
BW, kg	46.1	43.63	1.58	NS
Milk yield, kg/d	0.93	0.67	0.12	NS
DM apparent digestibility	78.73	85.77	1.74	0.028
OM apparent digestibility	79.64	86.23	1.73	0.044
CP apparent digestibility	72.72	78.70	1.87	NS
NDF true digestibility	66.99	80.72	3.75	0.057

[1] H-NDF, sheep fed diet containing forage with high level of NDF; L-NDF sheep fed diet containing forage with low level of NDF.

[2] SEM, standard error of the mean.

Table 7. Dry matter intake, body weight, milk yield, digestibility coefficients and methane production of the two experimental group in the gas measurement trial.

	Diet[1]			
	H-NDF	L-NDF	SEM[2]	P
DMI, kg/d	0.59	0.62	0.07	NS
BW, kg	50.3	44.5	2.53	NS
Milk yield, kg/d	0.50	0.72	0.18	NS
NDFI, kg/d	0.24	0.23	0.04	NS
DMD, % DM	75.6	77.1	0.49	0.084
OMD, % DM	77.2	78.3	0.42	NS
CPD, % DM	56.1	72.3	6.54	NS
NDFD, % DM	72.7	65.2	3.05	NS
DMdig, g	445	478	53.8	NS
NDFdig, g	172	146	26.5	NS
Methane emissions				
L/d	24.5	32.2	4.25	NS
g/d	17.5	23.0	3.05	NS
g/kg milk	66.8	38.6	22.4	NS
g/kg BW	0.35	0.54	0.09	NS
g/g DMI	0.03	0.04	0.002	NS
g/gNDFI	0.04	0.05	0.003	NS
g/g DMdig	0.09	0.1	0.02	NS
g/g NDFdig	0.12	0.15	0.02	NS

[1] H-NDF, sheep fed diet containing forage with high level of NDF; L-NDF sheep fed diet containing forage with low level of NDF.

[2] SEM, standard error of the mean.

7. FIGURES

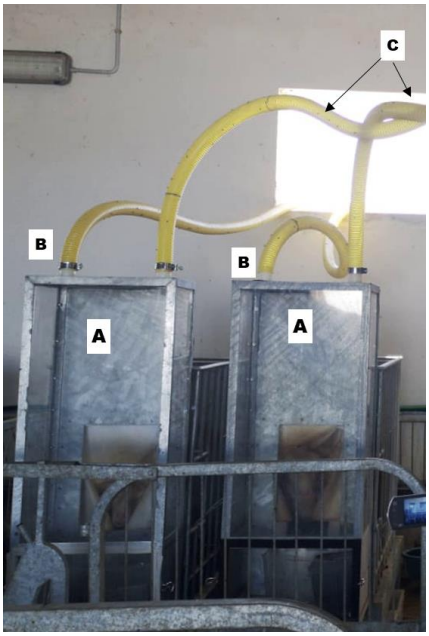

Figure 1. Ventilated hood heads system (**A**). Pipe line of ambient air entering the hood head (**B**); Pipe line from the two head hoods to the air drawing/analyzer equipment (**C**).

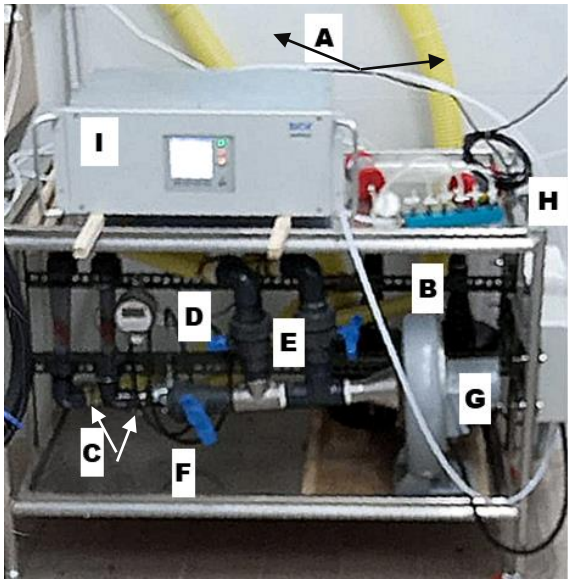

Figure 2. Air drawing/ analyzer equipment. Pipe line (yellow) from the two head hoods (**A**); dust filters **(B**); flowmeters (**C**); pipe lines for connections (**D**); regulation valves (**E, F**); centrifugal fun (**G**); manual micro valves switch system and pipe line connection (**H**) to the gas analyzer (**I**).

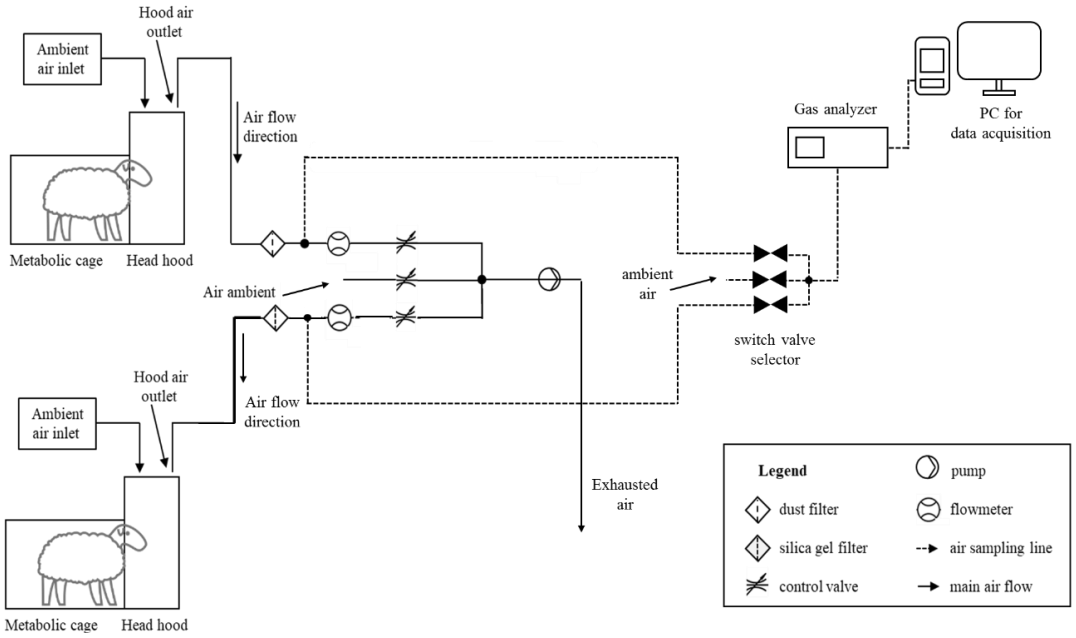

Figure 3. Schematic describing an overview of the updated ventilated hood system, air sampling and gas analyzer.

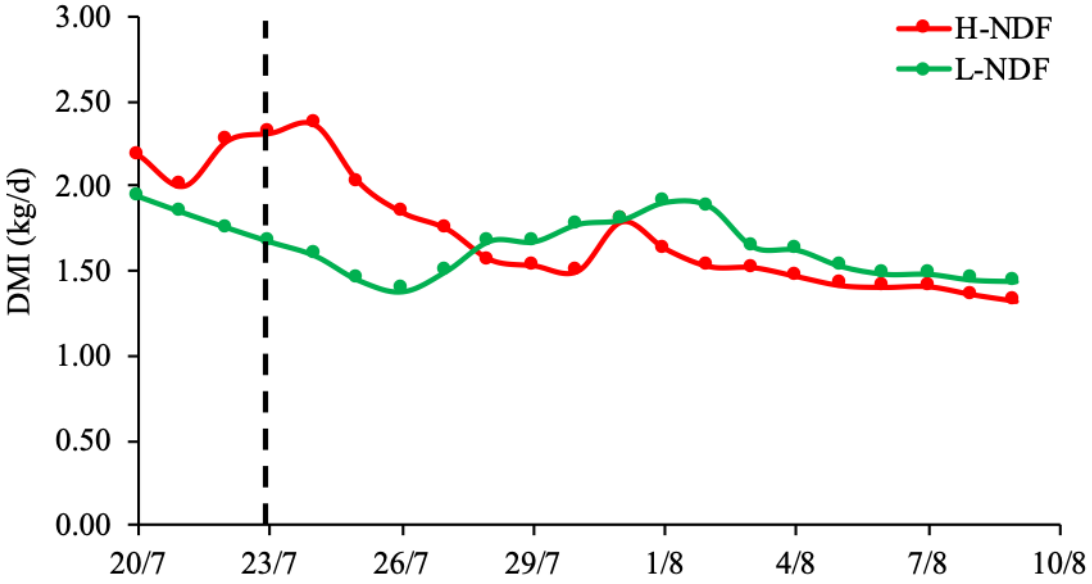

Figure 4. Evolution of DMI of sheep (n=16) of the two experimental groups (red line= H-NDF group: sheep fed diet containing forage with high level of NDF; green line = L-NDF group: sheep fed diet containing forage with low level of NDF). Dashed line = trial start date.

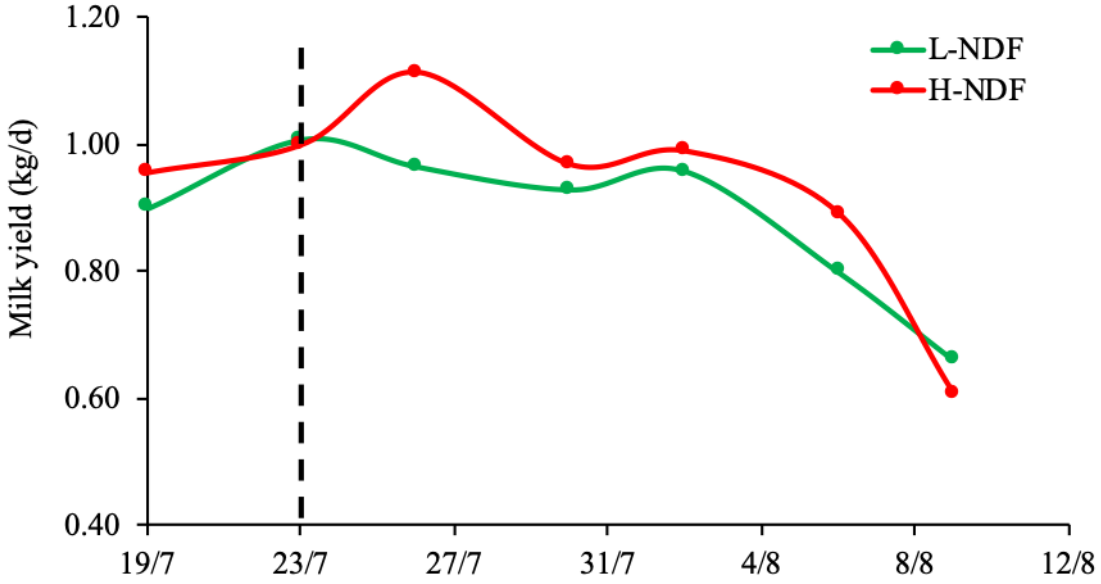

Figure 5. Evolution of milk yield of sheep (n=16) of the two experimental groups (red line= H-NDF group: sheep fed diet containing forage with high level of NDF; green line = L-NDF group: sheep fed diet containing forage with low level of NDF). Dashed line = trial start date.

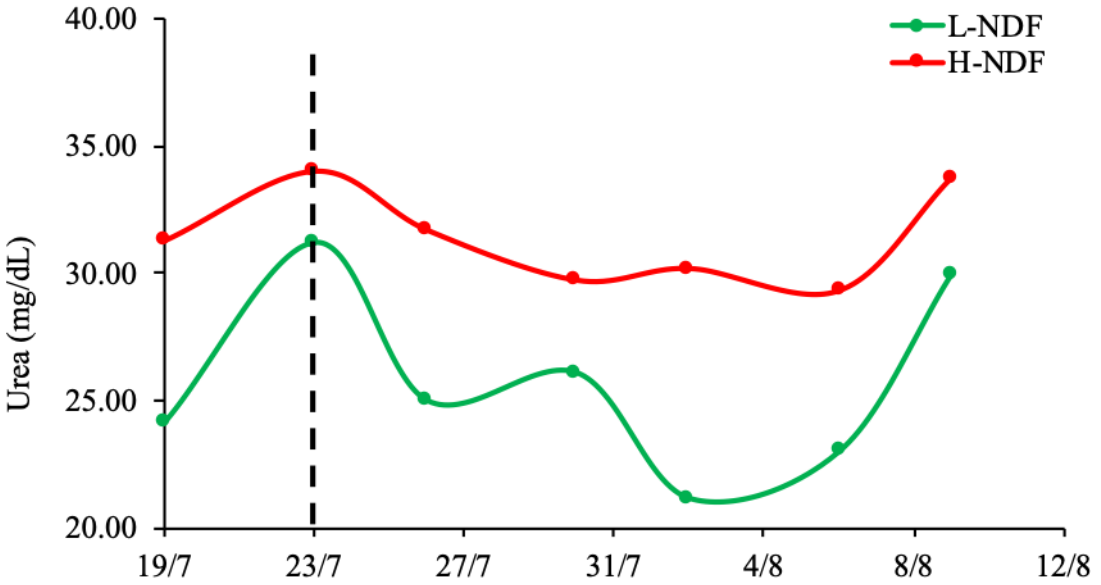

Figure 6. Evolution of milk urea of sheep (n=16) of the two experimental groups (red line= H-NDF group: sheep fed diet containing forage with high level of NDF; green line = L-NDF group: sheep fed diet containing forage with low level of NDF). Dashed line = trial start date.

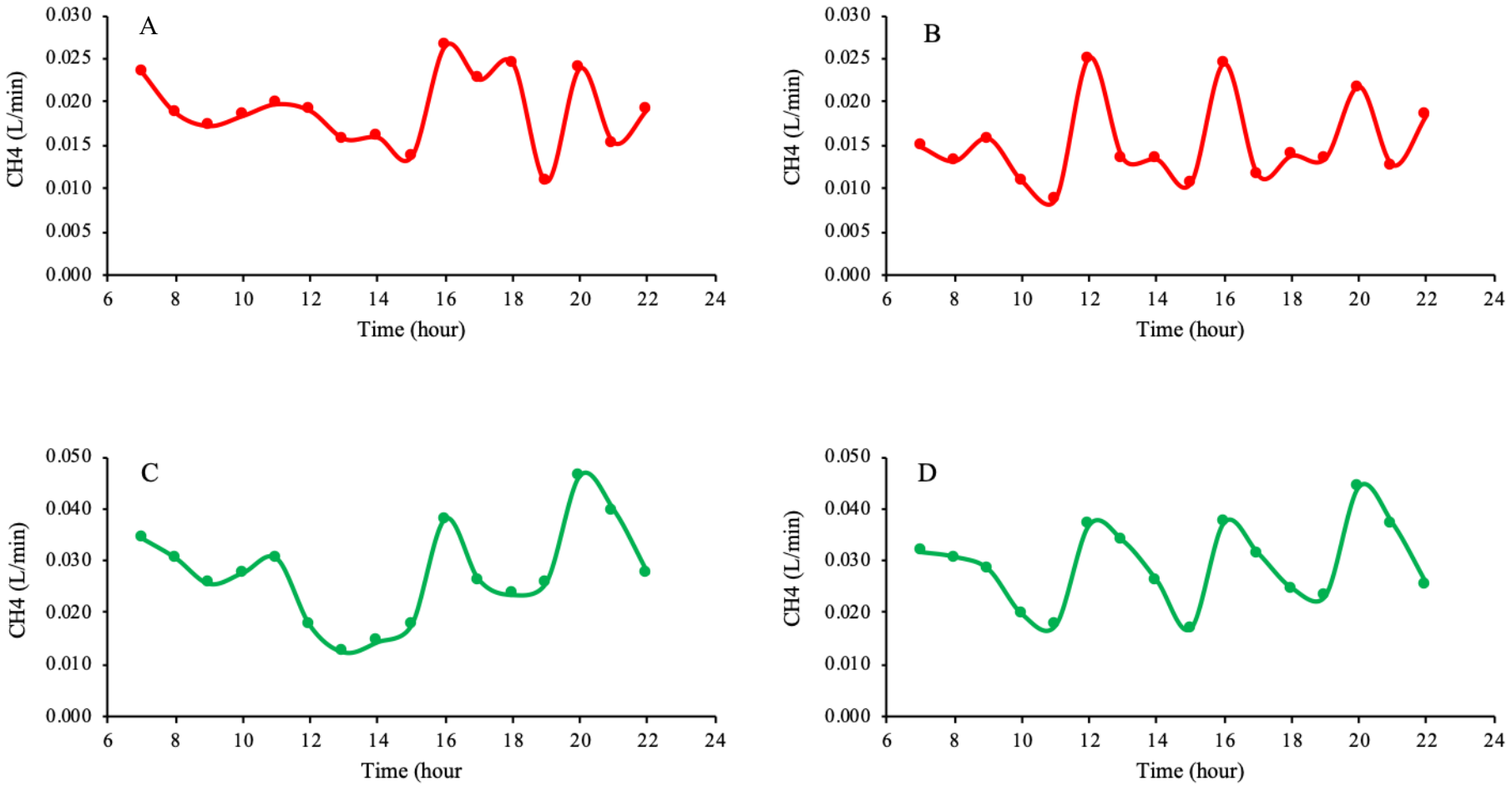

Figure 7. Emission patterns of animals used in the methane measurement test. The figures on the left show the trend of methane emissions of animals during the first day (figure A sheep of the H-NDF group, figure C sheep of the L-NDF group). The figures on the right (B and D) show the trend of methane emissions always of the same animals during the third day of trial.

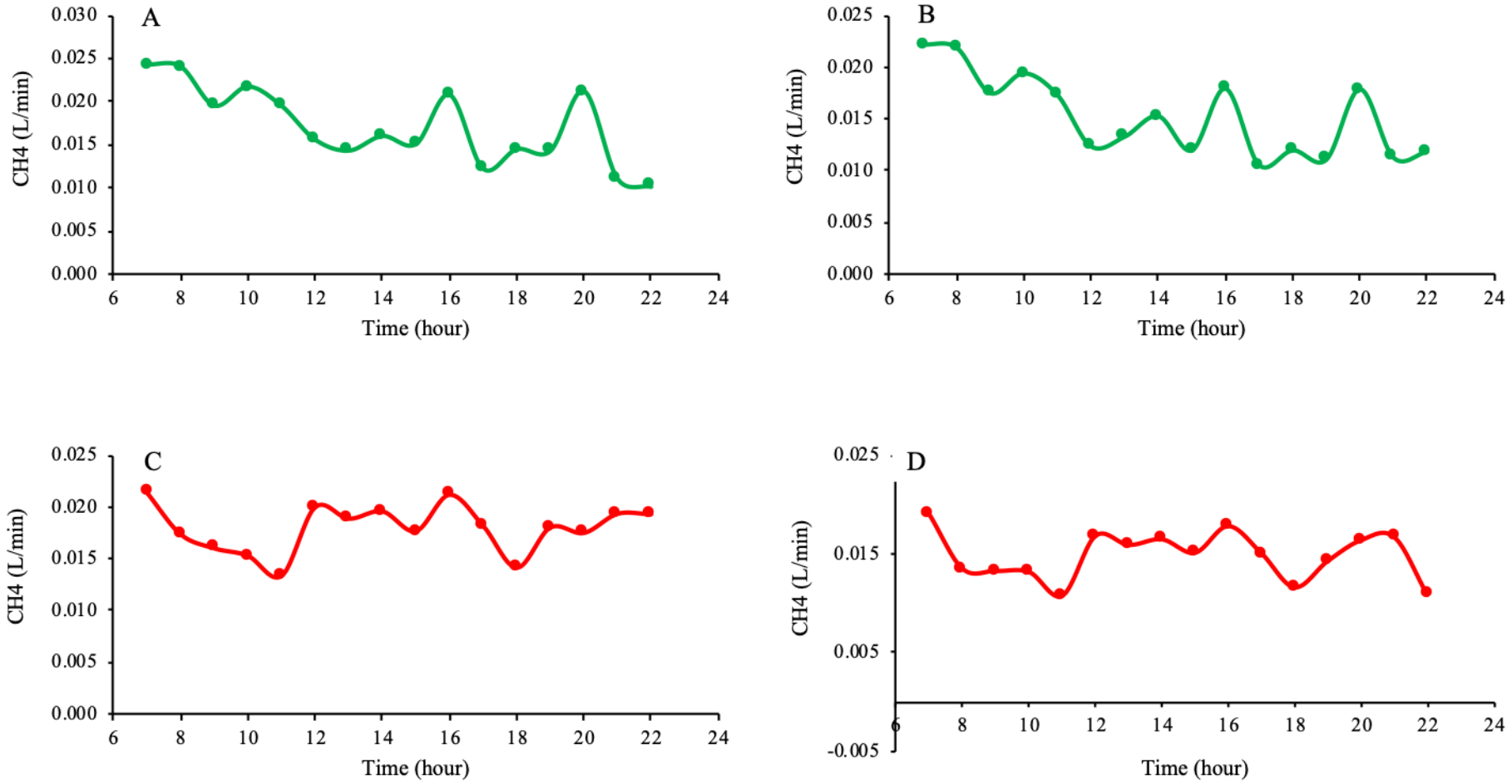

Figure 8. Emission patterns of animals used in the methane measurement test. The figures on the left show the trend of methane emissions of animals during the second day (figure A sheep of the H-NDF group, figure C sheep of the L-NDF group). The figures on the right (B and D) show the trend of methane emissions always of the same animals during the fourth day of trial.

CHAPTER IV

In vitro test of nitrate and acrylate additives, alone or combined with nanocarriers to reduce methane emissions of ruminant diets

ABSTRACT

In vitro methods represent an important technique to simulate rumen fermentations, to estimate nutritional values of the feeds and to measure methane emissions. The aim of this study was to evaluate, with *in vitro* tests, the effects of nanoparticles carriers associated in different combinations to nitrate based compounds and other additives to reduce the methane emission of feeds. The experimental hypothesis assumed that nanocarriers might enhance additive activity in the rumen. A randomized factorial design was applied in two runs of gas production. The treatment included the i) Control (CTR) consisting of TMR and other 7 treatments on which the TMR was incubated with different additives (dosis as % of DM) and nanocarriers of doubled layered hydroxides (Nano): ii) Aluminum nitrate (NAl) 2 %; iii) Acrylate (A) 2 %; iv) Aluminum nitrate 1 % + Acrylate 1 % (NAl+A); v) Nano NAl 1 % of nitrate; Nano A 1 % of acrylate; vi) 2 Nano 0.5% of nitrate + 0.5 % of acrylate; vii) Calcium nitrate (NCa) + 2 %. Six analytical replicates for each TMR were simultaneously incubated in diluted rumen fluid. At each time of incubation (i.e., 2, 5, 9, 24, 36 and 48 hours) one replicate was used to determine the gas volume and composition, as well as rumen VFA. In particular, two ml of gas were sampled from the headspace of these bottles and analyzed by gas-chromatography to determine gas composition (CH_4, O_2, H_2). 10 ml of fermentation fluid were used to determine VFA, and residues for DM and NDF digestibility at 24 h and 48 h.

The treatments poorly affected the cumulative gas production per g of DM at 48 h from incubation. Cumulative methane emissions at 48h (ml of CH_4 per g of DM) were significantly affected by treatments ($P < 0.001$). Highest values were observed for CTR and NCa (60.9 and 59.3 ml) whereas the lowest values were observed for Nano NAl, NAl and NAl+A (46.2, 50.5 and 51.8 ml) confirming the efficacy of NAl to reduce methane emissions and the potential role of nanocarriers to reduce emissions with lower doses of nitrates. When methane emissions were analyzed in respect to their control the effect was even more evident. NAl and Nano NAl showed the lowest emissions per g of DM, 86 % and 85 % of CTR respectively ($P < 0.05$). Emissions per g of NDF showed that Nano A, NAl+A, 2 Nano, NAl and Nano NAl leaded to a reduction of 86, 85, 85, 83 and 76 % of methane in respect to CTR ($P < 0.05$).

This work confirm the opportunity to continue research on nano materials as possible coadjutant in enhancing the effect of bioactive compounds in the rumen and to quantify the dose-response effects in ruminant diets.

1. INTRODUCTION

About 90 % of methane produced in the gastrointestinal tract of a ruminant occurs at ruminal level and is expelled with eructation, whereas the remaining part is produced in the gross intestine (Murray *et al.*, 1976). Through the fermentation of feed substrate (organic matter, OM), a large amount of hydrogen and electrons are formed inside the rumen and are used by the *Archea* to reduce CO_2, resulting from the hydrolysis of the OM, in methane (Hook *et al.*, 2010; Nolan *et al.*, 2010). Methane production is a physiological need of ruminant to maintain the reductive potential ruminal environment (Moss, 2002). On the other hand, reduction of enteric emissions from ruminants represent a nutritional challenge to face global warming (van Zijderveld *et al.*, 2010). In addition, methane production represent a quite relevant energy loss (from 6 to 10% of gross energy; Johnson and Johnson, 1995) and the reduction of methane emissions causes increases in feed efficiency and energy harvesting that might significantly reduce production costs at farm level (Hristov *et al*., 2013).

Direct techniques to measure dry matter digestibility and methane emission are expensive, time consuming and require complex labor and equipment whereas *In vitro* methods represent a consolidated important technique to simulate rumen fermentations, to estimate nutritional values of the feeds and to measure methane emissions (Pirondini *et al*., 2012). A large number of *in vitro* trials have been executed to quantify and measure the methane emission potential of feeds and the effects of bioactive compunds on ruminal fermentation and gas production (Asanuma *et al*., 1999; Eckard *et al*., 2010; Bodas *et al*., 2012; Lee and Beuchemin, 2014). *In vitro* fermentations also allows different diets to be tested simultaneously, alone or in presence of additives and inhibitors, for their effect on methanogenesis (Pirondini *et al*., 2012).

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Many additives have been tested to reduce methane production in the rumen using different compound and substances such as polyphenols (Vasta *et al*., 2019), and other bioactive substances. Substances can act directly as competitor of hydrogen acceptors (i.e: polyunsaturated fatty acids) or also acting on microbial groups of methanogens (Kumar *et al*., 2009). Recently, Patra *et al*., (2017) reviewed the effects of a large number of substrates and compunds with antimethanogenic effect. Other molecules such as acrylate have been used with idea to increase the efficiency of energy harvesting (Newbold *et al*., 2005)

One of the strategies known since many decades to be used in anaerobic system to limit methanogenesis is the use of nitrogen-based compounds (i.e.: nitrates), which act as hydrogen and electron acceptors (Allison and Reddy, 1984). Nitrate effects on ruminants have been well established and quantified and have a recognized and significant effect on methane reduction in rumen (Latham *et al*., 2016). Nolan *et al.*, (2010) carried out a study on sheep concerning the effects of adding nitrate in the diet on fermentation, methane production and digestibility. In this work they could see how the addition of urea and 4 % of potassium nitrate (KNO_3) in the diet led to decrease in methane production (L/kg DM) od around 24 % compared to a diet containing exclusively urea. Van Zijderveld *et al.*, (2010) have experimented four different dietary treatments (control, nitrate, sulfate, nitrate + sulfate) on sheep; the results showed that the diet containing nitrate lowered the production of methane compared to the other treatments. Other nitro-compounds, like nitropropanol, have been successfully tested to reduce methane emissions (Martinez Fernandez *et al*., 2018). Otherwise nitrate compounds are dangerous for oxygen balance in the organisms causing hypoxia and

high toxicity for nitrate poisoning (Eckard *et al*., 2010), triggered by higher blood methemoglobin levels with increasing nitrate consumption of ruminants (Lee and Baucheamin, 2014).

Recently the use of nanotechnologies to mitigate methane emissions is of particular attention. In a recent study carried out by Sarker *et al.*, (2018) which evaluated the effect on methane emissions of increasing levels of nano zinc oxide (nZnO) added to various types of feed, it has been show that nanoparticles have a positive effect in lowering methane emissions by ruminants. Despite this, the use of nanomaterials as a tool to mitigate methane emissions is still very limited (Sarker *et al.*, 2018).

The efficacy of nanoparticles relies in their ability to increase the diffusion of the additives in the medium and improve their effect on the microbial community (Bugatti *et al.*, 2019) in this sense the use on nanoparticles could be useful to decrease the amount of nitrates to be included in the animal diets and reduce the risks of toxicity. Nanoparticles of double layered hydroxides have been successfully developed and used in the last years with many applications (Gorrasi, 2015; Mishra *et al.*, 2018). Recently Double Layered Hydroxides have demonstrated significant effects on modulating microbial populations (Bugatti *et al*., 2019). Other nanocarriers based on Zinc molecules have also demonstrated their efficacy on reducing methane emissions (Sarker *et al*., 2018).

However, it should not be forgotten that various compounds used to mitigate methane emissions intervene by decreasing the digestibility of the organic matter and thus making the nutrients less available to the animal, decreasing feed efficiency (McGinn *et al.*, 2004). Thus, the most recent studies have focused on researching additives that do not affect digestibility but act as hydrogen acceptors instead of methanogenic bacteria (Asanuma *et al.*, 1999).

Possible ways include the improvement of enhancers of propionate pathways in order to improve the H_2 utilization and increase ruminal propionate formation which is stoichiometrically associated with a reduction in methane production (Lan and Yiang, 2019). The acrylate path is also an important propionate stimulating pathway in the rumen, it involves the *Megasphaera elsdenii* as lactate-utilizer (Russell and Wallace, 1997; Lan and Yiang, 2019). Possible use of acrylate acting as an alternative H_2 sink and reduce CH_4 production (McAllister and Newbold, 2008; Bodas *et al.*, 2012).

The objective of this study was to evaluate, with *in vitro* tests, the effects of nanoparticles carriers associated to nitrate based compounds and other additives in different combinations to reduce the emission potential of a generic animal diet. The experimental hypothesis assumed that additives associated with nanocarriers might have similar effects than additives alone by using half concentration of the active molecule.

2. MATERIALS AND METHODS

The experiment was conducted at the experimental facilities of the Università Cattolica del Sacro Cuore, located in Piacenza, in the north-west of Emilia Romagna, Italy, and was divided into 2 different parts: i) *in vitro* rumen gas production experiments; ii) *in vitro* digestibility experiments.

2.1 Experimental design

A randomized factorial design was applied to test the effects of different additive combinations against a control diet without additives in an *in vitro* gas production trial.

The treatments were designed considering to test the additives alone with in inclusion of 2 % of DM in the fed, the additives with nanocarriers in half concentration of the additives alone and the mixes of additives in half concentration in respect to single inclusion. The final dosis were defined as follow: 1) Control (CTR) consisting of TMR; 2) Aluminum nitrate (NAl) consisting of TMR + 2 % of aluminum nitrate on DM basis; 3) Acrylate (A) consisting of TMR + 2 % of acrylate on DM basis; 4) Aluminum nitrate + Acrylate (NAl+A) consisting of TMR + 1 % of aluminum nitrate + 1 % of acrylate on DM basis; 5) Nitrate in nanocarrier (NAl Nano) consisting of TMR + 1 % of nitrate mounted in a double layered hydroxide nanocarrier (aluminum nitrate/nanocarrier = 1:5; 5 % total additive) on DM basis; Acrylate in nanocarrier (A Nano) consisting of TMR + 1% of acrylate nanocarrier (acrylate/nanocarrier = 1:3.85; 3.85 % of total additive) on DM basis; Nitrate in nanocarrier + Acrylate in nanocarrier (2 Nano) consisting of TMR + 0.5% of nitrate in nanocarrier + 0.5 % of acrylate in nanocarrier on DM basis, 4.4 % of additive; Calcium nitrate (NCa) consisting of TMR + 2 % of calcium nitrate on DM basis. Each treatment consisted of 8 replications. For this experiment three syringes with amylose maize starch (220 ± 0.002 mg) were used as internal standard.

For this experiment was used the rumen fluids of two fistulated dairy cows (Holstein). Cows were raised at the experimental farm of the University, CeRZOO (Centro per la Ricerca e la Sperimentazione in Agricoltura, San Bonico, Piacenza, Italy). Two distinct fermentation runs were executed on different days with the same TMR and additives mixtures. Each fermentation was carried out by sampling the rumen fluid from the same two dry fistulated cows the day of the trial. The ruminal fluid cow donors were fed a diet formulated in agreement with dairy cow requirements (NRC, 2001) and based on grass hay (700 g/kg), corn silage (200 g/kg) and concentrate (100 g/kg) on a dry matter basis, and was characterized by a

level of 15 % CP and 34 % NDF (% on DM basis). Characteristics of TMR are show in Table 1. The procedure of the chemical analysis on TMR was previously described by Gallo *et al.*, (2015).

For each tested TMR, height analytical replicates were simultaneously incubated in diluted rumen fluid to measure rumen ferment ability according to Menke and Steingass (1988) in a single run. The gas production and its composition, as well as VFA, ammonia nitrogen and soluble sugars were determined. In particular, about 220 mg of each sample (about 200 mg of DM) were weighed into graduated 100-mL glass bottles, then 30 mL of diluted rumen fluid (buffer to rumen ratio of 2:1, vol/vol) collected from the rumen of 2 fistulated dry cows were added. Rumen liquor was maintained in a warm insulated flask, filtered through 2 layers of cheesecloth, and used within 20 min from collection. Before injection into bottles, the medium was saturated with CO_2. Bottles were then placed in a water bath at 39 °C. Six blanks samples (diluted rumen fluid only) and two internal standard samples (Gelose 80 maize starch, Penford Food Ingredients Co., Centennial, CO) were incubated in each run.

Gas measurements. Gas volume was measured at 2, 5, 9, 24, 36, and 48 h of incubation. At each incubation time, bottles were gently shaken. After gas volume recording, on each replication at each time point the headspace was degassed with a needle and recovered to atmospheric pressure in order to avoid CO_2 saturation and inhibition of microbial growth (Tagliapietra *et al.*, 2010). the bottle assigned to the specific incubation time was rapidly cooled with ice (2-4 °C) to stop fermentation. Two milliliters of gas were sampled from the head space of bottle and immediately analyzed by gas-chromatography (7820A GC System, Agilent Technology) to determine gas composition (CH). Calibration was done with a mixture of gases having a Methane of 2, 5 and 25 %. The bottles were then emptied and three aliquots of fermentation fluid (10 mL) were kept at −20 °C until the following analysis. In

particular, NH_3-N was analyzed by using the kit Ammoniak Ammonia UV methos (r-Biopharm, Germany). The VFA content was analyzed by a GC FID Shimadzu 2025.

Measurements at 24 and 48 h were made on two flasks for each treatment. One flasks was also saved for microbiological determination on the liquid phase.

Volatile fatty acids analysis. The analysis of fatty acids contained in the rumen liquid was carried out following the method previously described by Gallo *et al*., (2015). 2 ml of rumen supernatant were taken and 1 ml aqueous solution containing pivalic (internal standard) and formic acids (1 g/l and 50 ml/l, respectively) was added. Samples were analyzed by gas chromatography (Varian 3350 system; Varian Inc., CA) equipped with a flame ionization detector and a split injector. A silica capillary column was used to performed the analysis (DB-5, Agilent Technologies, USA) injecting 1 µl/sample (volume). The carrier gas was high-purity helium with a flow rate of 1·3 ml/min. The injection temperature was 200 °C while the detector temperature was 220 °C. The peak identification was performed using an external standard (WSFA-2, cat. no. 47 056, Supelco/Sigma Aldrich, Milan, Italy).

Digestibility of DM. Two samples of each treatments incubated at 24 and 48 hours were used to estimate digestibility of DM and NDF. At the end of the gas production measurements, the content of each syringes was filter using a ceramic filter with the addition of filter paper (Whatman 40) and weighted (filter paper + residual content). Filter with residual content were placed in a forced-air over for 24 h. Dry matter digestibility was calculated with the following equation:

$$IVDMD\ (\%) = (FW-IW)/IW*100$$

where: IVDMD is the *in vitro* dry matter digestibility, FW is the final weight of the DM (after the fermentation) and IW is the initial weight of the DM (before the fermentation);

The same procedure described for the DM was used to determine the digestibility of NDF (NDFD).

2.2 Model and curve fitting

Exponential model with a discrete Lag (EXP_{Lag}) was used to fit gas production data and obtain curve parameters, such as final volume (Vf, mL/g of DM), fractional rate of fermentation of the substrate (kd, h^{-1}) and the discrete Lag phase time (Lag, h). The equation used was the following:

$$V = Vf\,(1 - exp^{(-kd\,(t - Lag))})$$

2.3 Statistical analysis

For each treatment, gas production, CH_4 production, DMD and NDFD were evaluated in a complete randomized design by using the MIXED procedure of SAS (Version 9.0, SAS Institute Inc., Cary, NC) with the following model:

$$Y_i = \mu + \alpha_i + bj + \varepsilon_{ij}$$

where Y_i is the dependent variable, μ is the general mean, α_i is the effect of treatment (i=8); b_j is the random effect of the fermentation and ε_{ij} is the residual error.

Orthogonal polynomial contrasts were used to examine treatment effects on response variables. Significant effect of diet was declared at $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1 *In vitro* gas production and degradability

Model and curve fitting. Fermentation patterns of each treatment as obtained from the average values of all replicates were reported in figure 1. They were expressed as ml of gas per g of DM and showed the expected trend of *vitro* gas production (GP). Gas production values from all replicates were also analyzed looking at fitted kinetic parameters of gas production detailed in Table 2. The Vf and Lag values were not influenced by the treatments, with Vf and Lag ranging from 203 to 236 mL/g of DM and from 0.14 to 0.38 h, respectively. kd values were influenced by treatment (P = 0.017) ranging from 4.5 to 6.5 %/hr. The fermentation run significantly affected Vf and kd, probably due to the differences in rumen liquid quality collected in the two different days. Due to this difference the comparisons among treatments were performed per gram of fermented substrate, as percent of produced gas or as difference in respect to the control values obtained in each run. In particular, significant differences emerged between the NAl treatment, which showed the lowest value, and the control treatment (CTR), which showed the highest value. It indicated the degradability rate of the DM. Average production of gas per g of DM were included within the range observed in literature for fermentation of dairy cow diets (Pirondini *et al*., 2012; Tagliapietra *et al*., 2012; Gallo *et al*., 2015). On the other hand the total gas production indicated by Vf observed for the CTR was slightly lower than that observed for NCa which is in contrast to what observed by Lund *et al.*, (2014) who found a lesser final volume and less DM digestibility when nitrates were used as additives to the diet. Even Wang *et al.* (2016) with increasing amount of sodium nitrate as an additive to mitigate the methane production, found lower values of Vf compared to the CTR treatment where no additives were added.

Proportionally, the calculated kd were also different from those observed in these studies even if different methods were used to model the gas production kinetics in (Wang *et al.*, 2016) and (Lund *et al.*, 2014).

Methane emissions when expressed per g of DM (Figure 2) and as % of produced gas (Figure 3) showed a similar trend than observed for GP, whereas the increasing slope from 24 to 48 hours highlights the higher emission rate of the low digestible carbohydrate fractions, that are fermented later, after sugars and starch (Patra *et al*., 2017). Observed emission values of methane, per g of DM and as % of GP at 48 h, stayed within the range of values reported by Maccarana *et al.* (2016) which reviewed literature from 30 studies and 339 *in vitro* observations on methane emissions from different substrates with and without additives (from 7.3 to 77.5 ml of CH_4 per g of DM; from 6.4 to 40.6 ml of CH_4 per ml of GP).

A deeper focus on fermentations and treatment effects can be performed looking at data of *in vitro* gas production at 24 h from incubation are reported in Table 3 and 48 h reported in table Table 3.

Effects on digestibility. Treatments had a significant effect on both DMD and NDFD ($P < 0.05$). The DM digested in the CTR with slightly higher than DMD of the TMR with additional NAl (53.4 vs. 46.6 % of DM respectively), whereas other values were not statistically different among them. Numerical values revealed the proportion among DMD and GP already discussed. DMD and kd of NAl treatment were in fact lower than CTR. No significant differences were observed for NDFD despite large numerical differences among treatments. Whereas only numerical differences were noticed for DMD and NDFD at 48 h (Table 4). The results are agreement with Wu *et al*., (2019) that evaluated in vitro the effects of various additives on gas and methane production and observed not significant differences

for DM and NDF digestibility among treatments even with slightly higher values in the treatments with nitrate than in the control.

Gas production and methane at 24. When cumulative values from 0 to 24h of gas production were analyzed only considering the two replicates were methane emission measures were performed, significant differences were observed among treatments with the same proportions detected for DMD values (Table 3). In particular, the inclusion of NAl and Nano NAl were associated to a reduction of 13 % and 15 % in gas production compared to CTR (199 vs 173 and 199 vs. 169 mL of GP, respectively; $P < 0.023$). Gas production per g of NDF tended to be higher in CTR then in other treatments ($P < 0.06$; Table 3). Whereas no significant differences were detected when gas production at 24 h was expressed per g of digested DM or digested NDF.

Therefore, the results show that there is less gas production when aluminum nitrate is used, both in pure form and in combination with the nanocarriers which is also proportional to a decrease in DMD. As well known with increased DM digestibility the gas production also increases from the greater fermentation of nutrients (Gasmi-Boubaker *et al.*, 2005) being DMD one of the most important factor affecting *in vitro* GP (Kamalak *et al.*, 2005).

No significant differences were observed between TMR treated with aluminum nitrate alone or with nanocarriers (Table 3), even though it has to be noticed that Nano Al includes half doses of nitrate in respect to NAl and causes a similar effect on fermentation. Similar trends for GP were observed at 48 h (Table 4).

Gas production at 48 h. Data of *in vitro* gas production from 0 to 48 h from incubation reported in Table 4 indicated that the treatments were very similar in terms of gas production expressed in ml of GP per g of DM, with lowest values for Nano NAl (208 ml per g of DM) but without significant differences in respect to the control, except for NCa which showed the

highest value (245 ml per g of DM; $P < 0.02$). Similar differences and proportions were observed when values were expressed per g of NDF. Inthapanya *et al.*, (2011) recorded lower gas production with the use of calcium nitrate. On the other the effect of calcium nitrate on gas production is more pronounced in the first hours of fermentation, while as the process continues the effects are made less visible (Inthapanya *et al.*, 2011) which is in line with our results. Capelari and Powers (2017) evaluating the effect of nitrates on ruminal fermentations observed no significant differences on GP between the CTR and the two treatments with nitrates (1.25 % and 2.5 % on DM basis) among 12-24 hours of incubation. However, between 12-48 hours the total of CTR GP was significantly higher of about 5 % and 10 % ,respectively, than treatments with nitrates, even if no differences were observed for DMD. Total methane emission was lower in the two nitrate treatments compared to CTR (57 % and 67 % respectively). The authors explained that lower methane production in both nitrate treatments, would lead to an effective action of hydrogen acceptor action with both positive effect on GP and methane reduction They also showed that both total GP and total methane production decreased with increasing nitrate doses.

Methane emissions at 24 h. Methane emissions were largely influenced by the treatments (Table 3 and 4). Already at 24 h from incubation ml of CH_4 per g of DM resulted significantly higher for CTR (23.5 ml per g of DM) than for the all treatments ($P < 0.0001$), except than 2 Nano and NCa (Table 3). The lowest values were observed for NAl (16.1 ml/g of DM). Similar trends were observed for ml of CH_4 expressed per g of digested DM, per g of NDF, digested NDF and per ml of GP (Table 3). Contrast of treatments categories (CTR vs. additives vs. additives with nanocarriers) were not significantly different, except that ml of CH_4 per g of digested DM tended to be higher in CTR vs. additives without nanocarriers ($P < 0.10$). The results observed with these contrasts evidenced that the observed effects were

quantitatively dependent of the single additive and mixes more than to the presence of the nanocarriers. It has to be noticed that Nano A, Nano Al and 2 Nano, were associated to a reduced dose of the bioactive compounds in respect to A, NAl, and NAl+A, thus similar methane reduction per unit of substrate of these treatments in respect to control should be considered a positive outcome of the trial.

Methane emissions at 48 h. Data of *in vitro* gas production from 0 to 48 h from incubation reported in Table 4 indicated that the treatments were very similar in terms of gas production expressed in ml of GP per g of DM, with lowest values for Nano NAl (208 ml per g of DM) but without significant differences in respect to the control, except for NCa which showed the highest value (245 ml per g of DM; $P < 0.02$). Similar differences and proportions were observed when values were expressed per g of NDF. Inthapanya *et al.*, (2011) recorded lower gas production with the use of calcium nitrate. On the other the effect of calcium nitrate on gas production is more pronounced in the first hours of fermentation, while as the process continues the effects are made less visible (Inthapanya *et al.*, 2011) which is in line with our results.

Methane emissions at 48 h, expressed as ml of CH_4 per g of DM, were significantly affected by treatments ($P< 0.001$). Highest values were observed for CTR and NCa (60.9 and 59.3 ml) whereas the lowest values were observed for Nano NAl, NAl and NAl+A (46.2, 50.5 and 51.8 ml) confirming the efficacy of NAl to reduce methane emissions and the potential role of the nanocarriers in cause an important reduction of emissions with a lower dose of nitrates (Table 4). Significant differences were also observed when emissions were expressed per g of NDF ($P < 0.001$). The lowest values were observed in NAl and Nano NAl (145.1 and 132.9 ml of CH4/g of NDF) in respect to the highest values observed in the CTR (175.0 ml/g of NDF).

A study carried out by Zhang *et al*., (2018) evaluated the effect of the use of nitrogen compounds (urea, nitrates and the mix between the two products) on the GP and methane emissions in *in vitro* ruminal culture. The results showed that the exclusive use of urea did not decrease the methane production after 72 hours of incubation (mL/g DM), while if the urea is used in combination with nitrate there is a decrease of about 10% of methane production. These differences were very pronounced on rice straw compared to wheat straw. Probably the lower NDF digestibility recorded in the urea+nitrate treatment led to a lower production of VFA and therefore a lower methane production.

Zhao *et al*., (2018) also showed how the use of nitrate-based compounds could be explained through 3 different mechanism: i) nitrate have a negative effect on the methanogens growth; ii) nitrate activity act also as hydrogen acceptor and in competition with other electron acceptors; iii) nitrates have an inhibitory activity on different enzymes that play a strategic role in methanogenesis.

When methane emissions measured in each fermentation run were analyzed as difference in respect to their control the effect of treatments was more evident. In term of methane emitted per ml of GP, CTR showed the highest emissions whereas. Oppositely, NAl and Nano NAl showed the lowest emissions 86 % and 85 % of control respectively ($P < 0.05$). Similarly, emission expressed per g of DM showed that Nano NAl caused the highest emission reduction (89 % in respect to CTR). These evidences were even more clear when emission were expressed per g of NDF showing that Nano A, NAl+A, 2 Nano, NAl and Nano NAl leaded to a reduction of 86, 85, 85, 83 and 76 % of methane in respect to control ($P < 0.05$). It might indicate that effects of NAl and Nano additives are related with pathways of fiber fermentation.

VFA production. results of VGA production are detailed in Table 5. Acetate production was not significantly influenced by the diet, although the results of the statistical analysis show a tendency towards significance (P = 0.06). The lowest acetate production was recorded for Nano NAl and Nano A treatments (46.7 and 47.1 mmol/l respectively), while the CRT and NCa treatment led to a greater production of acetate (53.6 and 53.8 mmol/l respectively). Acetate is highly related with methane emissions thus higher proportions of acetate were expected to be associated to emitted methane (Hook *et al.,* 2010). As already seen from Iwamoto *et al.*, (1999) the use of nitrate-based compounds should lead to a lower acetate production. However, Zhou *et al.*, (2012) showed that the use of sodium nitrate might increase the acetate production when added to alfalfa hays. Significant differences were observed among treatments for propionic (P = 0.03) and butyric (P < 0.02) which can be possibly related to DMD of each treatment. Newbold *et al.*, (2005), tested different propionate precursors as electron acceptors with the aim of mitigate the production of methane, have shown how the use of acrylate increases the amount of propionate produced. In our work we observed similar production of propionic acid among CTR and A and NAl+A, whereas a reduction of propionic acid was observed with Nano A (Table 5).

Differently than what observed for GP and CH_4, contrast among treatment categories were significant (Table 5). Especially the ratio acetic/propionic acids resulted higher for treatments with nanocarriers than for others (P = 0.01) whereas presence of nanocarriers was associated to lower butyric acid (P < 0.001). It indicates that nanoparticles modifies the fermentation patterns in the rumen, probably involving microbial communities, as already observed in literature (Patra *et al*., 2017; Sarker *et al.*, 2018). Similarly, Riazi *et al.*, (2019) did not observed *in vitro* significant differences in acetate production whit zinc nanoparticles addition to the substrate even detecting reduction in methane.

4. CONCLUSIONS

The results obtained in this work provide a preliminary test on the use of different additives to reduce methane emissions in ruminant diets. From an applicative point of view the use of nitrates, in association or not to nanocarriers, did not gave an unequivocal response of methane mitigation that allow to choose the best additive and quantify its possible use at field level. Even though the Nitrate aluminum, alone and in combination with nanocarriers, showed the highest reduction potential of methane emission *in vitro* in respect to other additives tested.

Cumulative methane at 48 h from incubation, expressed as ml of CH_4 per g of DM, were significantly affected by treatments being the highest emissions observed for CTR (60.9 ml) and the lowest values of emissions observed for Nano NAl, NAl (46.2 and 50.5 ml, respectively). It confirmed the efficacy of NAl to reduce methane emissions (-18 % of methane per g of DM). In addition, the observed effects of the nanocarriers was that caused a similar reduction of methane using only half dose of nitrates (-25 % of methane per g of DM). Effects of aluminum nitrate and nanocarriers were even more evident when emissions were expressed per g of NDF. Microbiological determination would help to verify if nanocarriers influences the microbial population.

It can be speculated that, if these evidences would be confirmed by future studies, the use of nanocarriers will allow to improve the efficacy of nitrates in ruminant nutrition by reducing their inclusion in ruminant diets but without reduce their mitigation potential on methane emissions. From a scientific point of view this work confirm the opportunity to continue research on nanomaterials as possible coadjutant in enhancing the effect of bioactive compounds, specifically to explain the biological mechanism that modulate their action in the rumen and to quantify the dose-response effects in ruminant diets.

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6. TABLES

Table 1. Ingredients and chemical composition of experimental total mixed ration (TMR)

Ingredients	DM, %
Corn meal	18.2
Barley meal	1.7
Soybean, solvent meal 44%	8.6
Grass hay	1.9
Alfalfa hay	2.6
Mineral-vitamin supplement	1.2
Fat (palm oil)	0.8
Corn silage	35.0
Alfalfa silage	30.0
Chemical Composition[1] (% DM)	
DM, (% as fed)	44.6
CP	15.2
NDF_{om}	33.6
ADF_{om}	24.6
ADL	4.3
EE	3.4
Starch	20.8
Sugar	4.1
Ashes	8.6
N_{EL}, Mcal/kg DM	1.59

[1] DM: dry matter; CP, crud protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; EE, ether extract.

[2] NE_L evaluation was calculated by using equations of (NRC, 2001)

Table 2. Fitted kinetic parameters of gas production as estimated average of the two fermentation runs.

		Treatments									P		Contrasts		
Parameters[1]		CTR	NAl	A	NAl+A	Nano NAl	Nano A	2 Nano	Nca	SEM	Treat	Run	Nano vs non nano	CTR vs non nano	CTR vs nano
Vf	mL	218.9	226.5	218.6	211.4	203.2	216.9	210.9	236.3	8.09	0.313	<0.001	0.157	0.176	0.964
kd	h^{-1}	0.065^{a}	0.045^{b}	0.055^{ab}	0.055^{ab}	0.050^{ab}	0.055^{ab}	0.060^{ab}	0.050^{ab}	0.00	0.017	0.011	0.456	0.067	0.060
Lag	h	0.28	0.30	0.14	0.37	0.38	0.20	0.31	0.27	0.05	0.968	0.153	0.610	0.740	0.994

[1] Vf = final volume of gas production (mL/g OM); kd = rate of gas production (h^{-1}); Lag = Lag time (h).

CTR: Control TMR; Nano: Nanocarriers of double layered hydroxides (Nano): NAl Aluminum nitrate 2%; A: Acrylate 2 %; NAl+A: Aluminum nitrate 1 % + Acrylate 1%; Nano NAl : nano +1 % of nitrate; Nano A: nano+1% of acrylate; iv) 2 Nano: Nano+ 0.5% of nitrate + 0.5% of acrylate; NCa: Calcium nitrate + 2 %.

Within rows, different letters indicates significant differences of treatment effect for $P < 0.05$.

Table 3. Effect of treatments on *in vitro* DMD, NDFD, gas production and CH_4 production, 24 h after incubation.

		Treatments								P		Contrasts		
Parameters[1]		CTR	NAl	A	NAl+A	Nano NAl	Nano A	2 Nano	NCa	SEM	Treat	Run	Nano vs non nano	CTR vs non nano	CTR vs nano
DMD	% DM	53.4^{a}	46.7^{b}	48.2^{ab}	49.5^{ab}	48.9^{ab}	48.6^{ab}	48.6^{ab}	51.1^{ab}	1.25	0.048	<0.001	0.777	0.631	0.453
NDFD	% NDF	68.5	60.3	68.0	59.3	66.5	61.5	72.4	59.2	1.80	0.493	0.429	0.196	0.269	0.049
Gas Production															
	mL GP/g DM	198.8^{a}	172.5^{b}	178.2^{ab}	176.6^{ab}	169.2^{b}	179.3^{ab}	184.8^{ab}	188.1^{ab}	4.97	0.023	<0.001	0.688	0.410	0.588
	mL GP/g dig. DM	386.1	384.2	382.7	368.3	358.2	382.0	392.1	381.0	18.06	0.526	<0.001	0.449	0.276	0.269
	mL GP/g NDF	487.9	423.2	436.8	426.5	411.8	439.1	452.7	452.4	12.38	0.059	<0.001	0.437	0.466	0.460
	mL GP/g dig. NDF	717.1	752.9	646.4	719.8	620.4	721.3	639.9	771.6	26.85	0.633	0.001	0.549	0.545	0.203
CH_4 emission															
	mL CH_4/g DM	23.52^{a}	16.09^{c}	17.75^{bc}	16.75^{bc}	15.93^{c}	18.16^{bc}	19.62^{abc}	20.55^{ab}	0.84	<.0001	<.0001	0.411	0.223	0.301
	mL CH_4/g dig. DM	56.79^{a}	44.64^{b}	46.78^{ab}	43.20^{b}	41.99^{b}	48.42^{ab}	52.31^{ab}	52.01^{ab}	2.98	0.004	<0.001	0.457	0.098	0.133
	mL CH_4/g NDF	71.10^{a}	48.85^{b}	53.32^{b}	49.88^{b}	47.96^{b}	55.21^{b}	59.52^{ab}	61.00^{ab}	2.55	0.0001	<.0001	0.327	0.212	0.232
	mL CH_4/g dig. NDF	104.21	87.26	78.61	84.20	72.06	90.39	85.18	104.42	4.53	0.216	<.0001	0.697	0.973	0.472
	mL CH_4/mL GP	11.67^{a}	9.27^{c}	9.94^{bc}	9.46^{bc}	9.39^{bc}	10.05^{bc}	10.47^{abc}	10.75^{ab}	0.20	0.0001	<.0001	0.405	0.240	0.277

1 DMD = dry matter digestibility (% DM); NDFD = NDF digestibility (% NDF); GP = gas production (mL); CH4 = methane production (mL).

CTR: Control TMR; Nano: Nanocarriers of double layered hydroxides (Nano): NAl Aluminum nitrate 2%; A: Acrylate 2 %; NAl+A: Aluminum nitrate 1% + Acrylate 1%; Nano NAl : nano +1 % of nitrate; Nano A: nano+1% of acrylate; iv) 2 Nano: Nano+ 0.5 % of nitrate + 0.5 % of acrylate; NCa: Calcium nitrate + 2 %.

Within rows, different letters indicates significant differences of treatment effect for $P < 0.05$.

Table 4. Effect of treatments on *in vitro* DMD, NDFD, gas production and CH_4 production, 48 h after incubation.

		Treatments									P		Contrasts		
Parameters[1]		CTR	NAl	A	NAl+A	Nano NAl	Nano A	2 Nano	NCa	SEM	Treat	Run	Nano vs non nano	CTR vs non nano	CTR vs nano
DMD	% DM	50.9	50.0	49.7	51.8	49.5	48.27	48.1	52.4	1.22	0.732	<0.001	0.680	0.244	0.404
NDFD	% NDF	66.8	68.1	64.9	67.91	70.2	69.08	72.7	73.9	1.36	0.796	0.176	0.266	0.295	0.673
GP															
	mL GP/g DM	231.2^{ab}	225.2^{ab}	223.9^{ab}	217.3^{b}	208.2^{b}	223.4^{ab}	223.6^{ab}	244.8^{a}	6.52	0.015	<0.001	0.720	0.828	0.471
	mL GP/g DMD	459.5	465.0	468.6	435.7	438.1	483.0	486.2	482.6	22.96	0.488	<0.001	0.869	0.220	0.420
	mL GP/g NDF	538.0^{ab}	524.4^{ab}	521.2^{ab}	506.0^{ab}	484.7^{b}	505.4^{ab}	494.9^{ab}	567.2^{a}	15.36	0.034	<0.001	0.900	0.228	0.104
	mL GP/g NDFD	805.2	767.5	812.2	747.0	687.5	741.4	681.2	806.7	26.82	0.652	<0.001	0.553	0.215	0.246
CH_4															
	mL CH_4/g DM	60.9^{a}	50.5^{cd}	53.5^{bc}	51.8^{cd}	46.2^{d}	53.7^{bc}	54.7^{abc}	59.3^{ab}	1.92	<0.001	<0.001	0.980	0.238	0.896
	mL CH_4/g DMD	149.5	128.8	138.5	128.8	120.1	144.5	148.0	145.1	7.61	0.045	<0.001	0.933	0.084	0.288
	mL CH_4/g NDF	175.0^{a}	145.1^{c}	153.7^{abc}	148.9^{bc}	132.9^{c}	150.0^{bc}	149.5^{bc}	169.7^{ab}	5.51	<.0001	<.0001	0.654	0.692	0.242
	mL CH_4/g NDFD	261.8	212.3	239.5	219.8	188.7	220.1	204.8	241.5	9.16	0.1823	<.0001	0.479	0.479	0.299
	mL CH_4/mL GP	26.3^{a}	22.4^{cd}	23.8^{bcd}	23.8^{bcd}	22.3^{d}	23.9^{bcd}	24.3^{b}	24.0^{bc}	0.26	<0.001	<0.001	0.379	0.170	0.387

[1] DMD = dry matter digestibility (% DM); NDFD = NDF digestibility (% NDF); GP = gas production (mL); CH4 = methane production (mL).

CTR: Control TMR; Nano: Nanocarriers of double layered hydroxides (Nano): NAl Aluminum nitrate 2%; A: Acrylate 2%; NAl+A: Aluminum nitrate 1% + Acrylate 1 %; Nano NAl : nano +1 % of nitrate; Nano A: nano+1% of acrylate; iv) 2 Nano: Nano+ 0.5 % of nitrate + 0.5 % of acrylate; NCa: Calcium nitrate + 2%.

Within rows, different letters indicates significant differences of treatment effect for $P < 0.05$.

Table 5. Molar ratios of single VFA measured after 48 h incubation as average of two fermentation runs.

		Treatments									P		Contrasts		
Parameters[1]		CTR	NAl	A	NAl+A	Nano NAl	Nano A	2 Nano	Nca	SEM	Treat	Run	Nano vs non nano	CTR vs non nano	CTR vs nano
Acetic	mmol/l	53.6	48.6	50.4	49.0	46.7	47.1	52.5	53.8	2.23	0.058	<0.001	0.556	0.219	0.258
Propionic	mmol/l	18.0^{a}	16.3^{ab}	17.3^{ab}	16.7^{ab}	15.5^{b}	15.5^{b}	16.6^{ab}	17.0^{ab}	1.30	0.033	<0.001	0.068	0.733	0.752
Butyric	mmol/l	14.9	13.6	14.9	13.8	12.5	12.3	12.7	13.2	1.03	0.017	<0.001	0.007	0.056	0.550
A/P		3.2^{b}	3.3^{ab}	3.2^{b}	3.3^{b}	3.4^{ab}	3.4^{ab}	3.4^{ab}	3.5^{a}	0.14	0.008	<0.001	0.011	0.034	0.515
(A + B)/P		4.1	4.2	4.1	4.1	4.2	4.2	4.2	4.3	0.14	0.077	<0.001	0.045	0.254	0.949

[1] A/P = acetic/propionic ratio; (A + B)/P = (acetic + butyric)/propionic ratio.

CTR: Control TMR; Nano: Nanocarriers of double layered hydroxides (Nano): NAl Aluminum nitrate 2 %; A: Acrylate 2 %; NAl+A: Aluminum nitrate 1 % + Acrylate 1 %; Nano NAl : nano +1 % of nitrate; Nano A: nano+1 % of acrylate; iv) 2 Nano: Nano+ 0.5 % of nitrate + 0.5 % of acrylate; NCa: Calcium nitrate + 2 %.

Within rows, different letters indicates significant differences of treatment effect for $P < 0.05$.

7. FIGURES

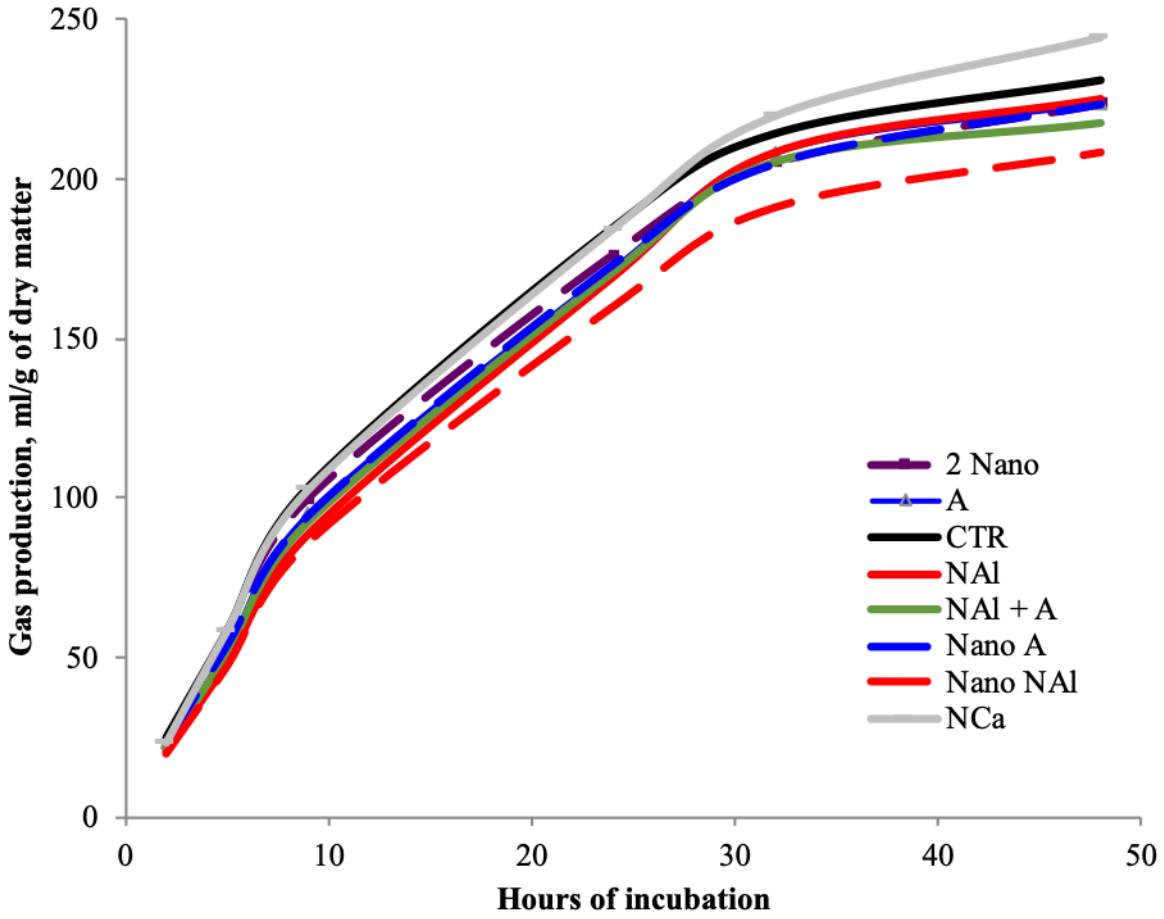

Figure 1. Gas production (ml/g of dry matter) in the batch fermentation of the 8 treatments.

CTR = Control without additives; Nano: Nanocarriers of double layered hydroxides (Nano): NAl Aluminum nitrate 2%; A: Acrylate 2 %; NAl+A: Aluminum nitrate 1% + Acrylate 1%; Nano NAl : nano +1 % of nitrate; Nano A: nano+1% of acrylate; iv) 2 Nano: Nano+ 0.5% of nitrate + 0.5% of acrylate; NCa: Calcium nitrate + 2 %.

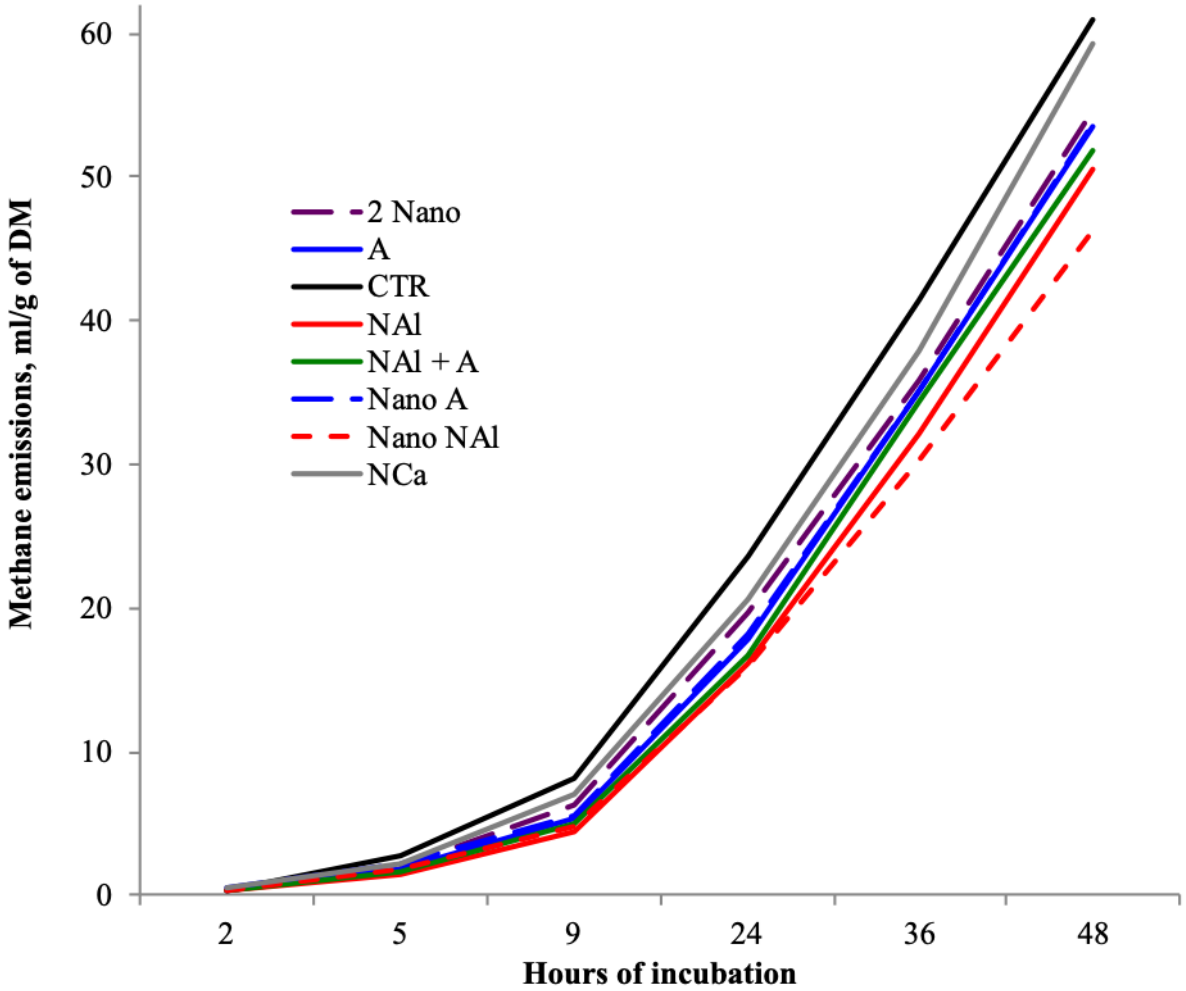

Figure 2. Methane emission (ml of CH_4/g of dry matter) in the batch fermentation of the 8 treatments. CTR = Control without additives; Nano: Nanocarriers of double layered hydroxides (Nano): NAl Aluminum nitrate 2 %; A: Acrylate 2%; NAl+A: Aluminum nitrate 1% + Acrylate 1%; Nano NAl : nano +1 % of nitrate; Nano A: nano+1% of acrylate; iv) 2 Nano: Nano+ 0.5% of nitrate + 0.5 % of acrylate; NCa: Calcium nitrate + 2 %.

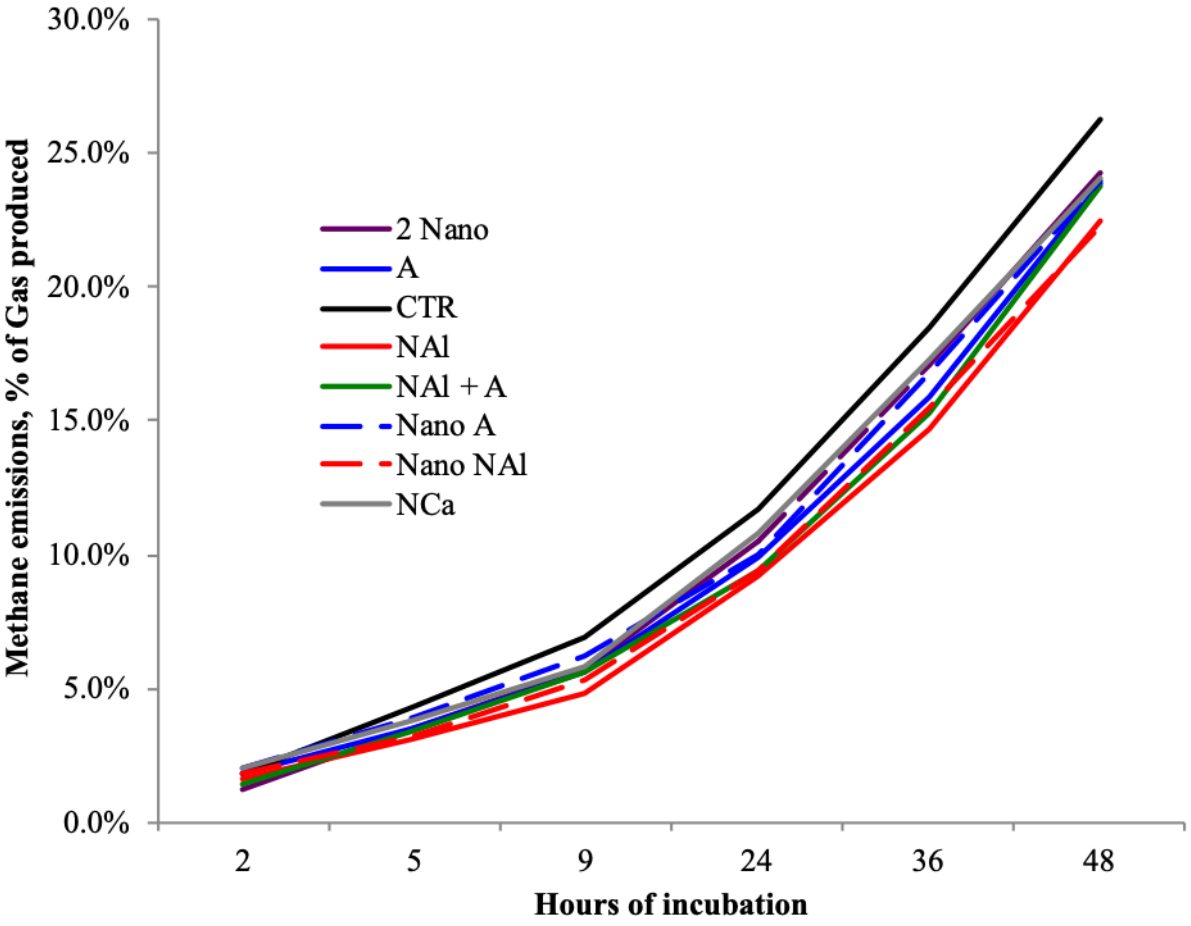

Figure 3. Methane emission (methane as % of gas produced) in the batch fermentation of the 8 treatments. CTR = Control without additives; Nano: Nanocarriers of double layered hydroxides (Nano): NAl Aluminum nitrate 2%; A: Acrylate 2%; NAl+A: Aluminum nitrate 1 % + Acrylate 1%; Nano NAl : nano +1% of nitrate; Nano A: nano+1% of acrylate; iv) 2 Nano: Nano+ 0.5 % of nitrate + 0.5 % of acrylate; NCa: Calcium nitrate + 2 %.

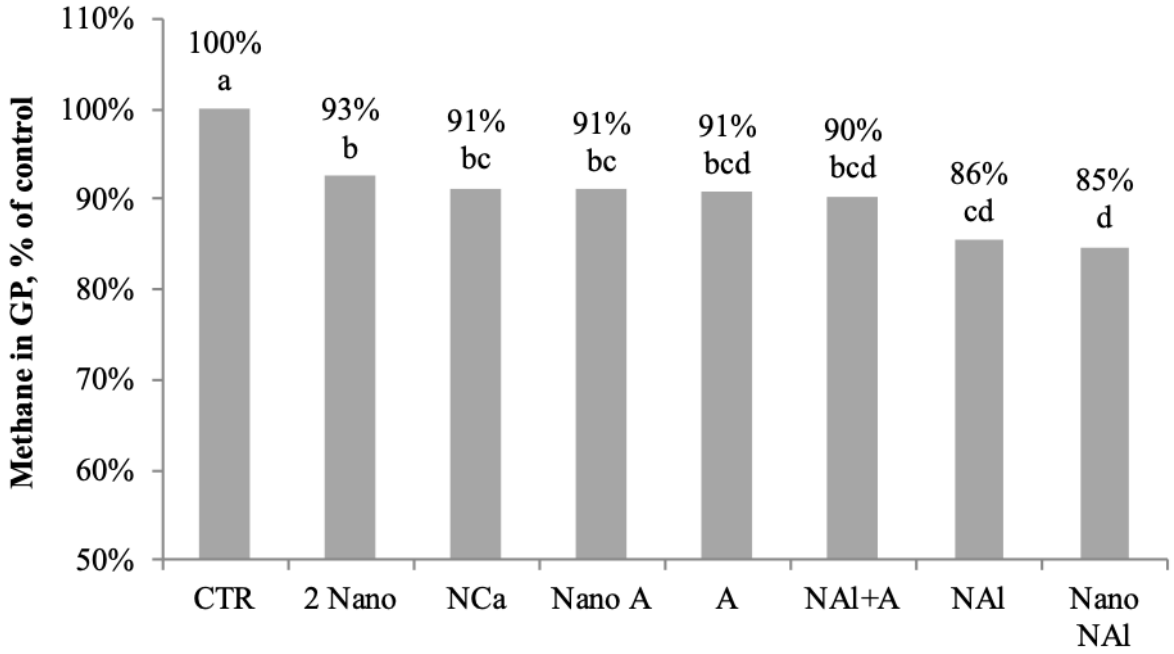

Figure 4. Relative methane emission as proportion of produced gas (control=100 %) in the fermentation of the 8 treatments. CTR = Control without additives; Nano: Nanocarriers of double layered hydroxides (Nano): NAl Aluminum nitrate 2%; A: Acrylate 2%; NAl+A: Aluminum nitrate 1 % + Acrylate 1%; Nano NAl : nano +1% of nitrate; Nano A: nano+1% of acrylate; iv) 2 Nano: Nano+ 0.5 % of nitrate + 0.5 % of acrylate; NCa: Calcium nitrate + 2 %.

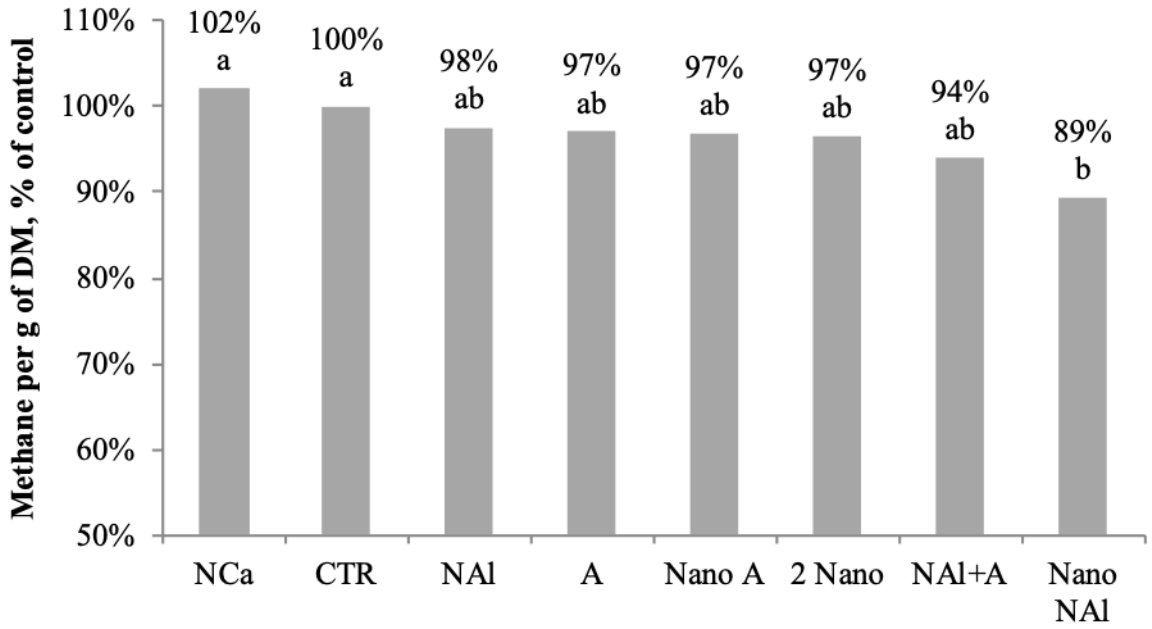

Figure 5. Relative methane emission per g of DM (control=100 %) in the batch fermentation of the 8 treatments. CTR = Control without additives; Nano: Nanocarriers of double layered hydroxides (Nano): NAl Aluminum nitrate 2 %; A: Acrylate 2%; NAl+A: Aluminum nitrate 1% + Acrylate 1%; Nano NAl : nano +1 % of nitrate; Nano A: nano+1 % of acrylate; iv) 2 Nano: Nano+ 0.5% of nitrate + 0.5 % of acrylate; NCa: Calcium nitrate + 2 %.

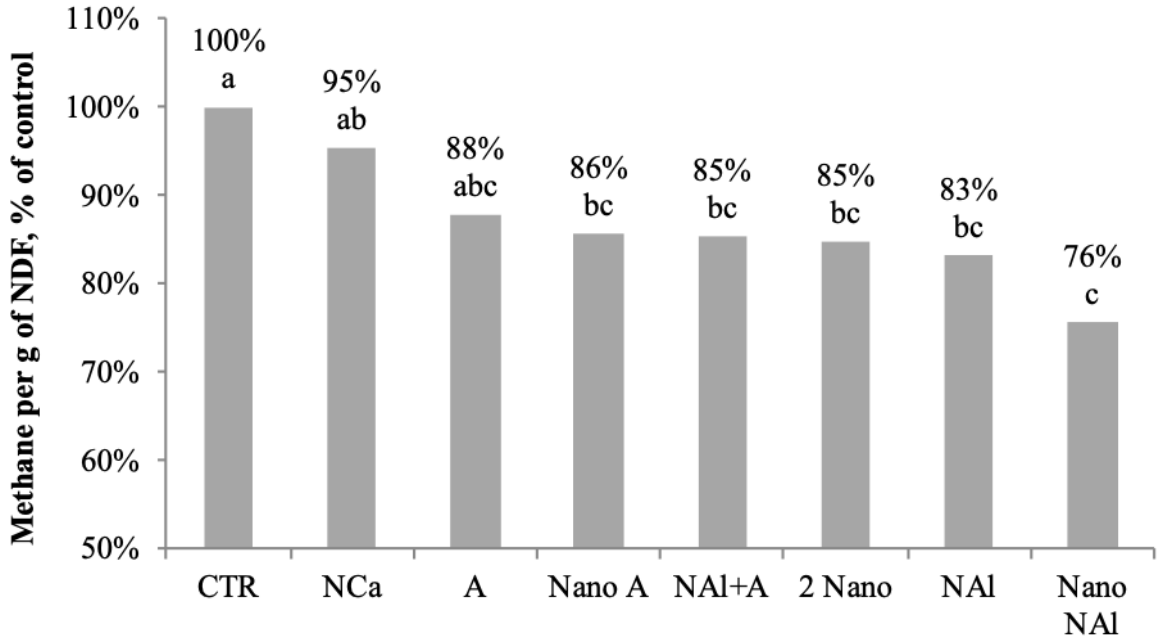

Figure 6. Relative methane emission per g of NDF (control=100 %) in the batch fermentation of the 8 treatments. CTR = Control without additives; Nano: Nanocarriers of double layered hydroxides (Nano): NAl Aluminum nitrate 2%; A: Acrylate 2 %; NAl+A: Aluminum nitrate 1 % + Acrylate 1 %; Nano NAl : nano +1% of nitrate; Nano A: nano+1% of acrylate; iv) 2 Nano: Nano+ 0.5 % of nitrate + 0.5% of acrylate; NCa: Calcium nitrate + 2 %.

CHAPTER V

GENERAL CONCLUSIONS, SCIENTIFIC AND PRACTICAL IMPLICATIONS

The work of this thesis focused direct methane measurements in *in vivo* and *in vitro* trials to test possible nutritional strategies aimed to reduce methane emissions from dairy sheep, specifically working on their diets in term of forage quality and additive inclusion.

The work of this thesis included also the development of a ventilated hood system for direct measurements of methane in vivo on dairy sheep. This equipment was firstly developed with a single hood and a sampling method consisting on gas accumulation bags that allowed to analyze emissions in intervals of one hour. The equipment was utilized in the first trial. In a second step the equipment was improved with two ventilated hoods that allowed to measure emissions from two animals of different groups at time and the methane determination method was improved including an methane analyzer that allowed to have record of methane within short intervals. The improved equipment was used in the second trial. These equipments represent a significant advance in the experiments of the animal nutrition research group of the University of Sassari.

The utilization of the hoods allowed running two experiments focusing the effects of forage quality on animal performance, diet digestibility and methane emissions of dairy sheep.

The first study focused the use of hays with different level of NDF (54 vs. 66 % of NDF on DM) for L-NDF and H-NDF group of sheep, respectively. The forages were offered ad libitum and integrated with a fixed amount of concentrates. The main findings highlighted that lowering NDF content of forages of diets allows to increase DMI and improve productive performance in dairy sheep. In particular, dairy sheep fed with high quality hay and lower NDF (L-NDF) produced more milk (about + 35%) and had longer milk persistency and lower level of milk urea than those fed with poor quality hay. The daily amount of methane (g

CH_4/d) was no significantly different among treatments, but if methane emission was expressed per unit of DMI it resulted 20% lower in animals fed L-NDF vs. H-NDF forage.

In the second trial the use haylages in dairy sheep diets was pointed out by feeding two groups of dairy sheep in late lactation with two different silages having 37 and 49% of NDF for the L-NDF and H-NDF groups, respectively. The forages were included in diets with different supply of concentrates in order to reach an average NDF of the diet in both groups. No significant differences emerged between the two groups regarding milk production, and methane emissions per g of DMI. The DMI was statistically higher in the L-NDF vs. the H-NDF group. Milk urea was significantly lower in the L-NDF group probably for a better synchronization of nutrient fermentations in the rumen. The H-NDF vs. L-NDF group produced more methane per unit of milk (66.8 vs 38.6 gCH_4/kg milk) without significant differences among treatments.

In general the two trails indicated that good quality forages with low NDF are better sources for sheep feeding allowing higher nutrient supply, higher milk yield in late lactation and at least in the case of hays lower methane emissions per kg of DMI. In addition it is confirmed that haylages are an excellent source for the feeding of dairy sheep and below 49% of NDF on DM basis we observed comparable animal performances.

These findings confirmed previous evidences already observed in cattle and slightly contributed to knowledge in dairy sheep. Even though considering that measurements were carried out in low producing animals with low levels of energy requirements and DMI further studies should be focused on sheep with higher milk yield and intake level both to study effects of forage quality on performances and methane emissions. Further studies should be also conducted to understand the patterns and dynamics of methane emission in small dairy ruminants considering the individual variation in respect to their feeding behavior. In

applicative terms good practices of dairy sheep feeding should promote for sure the use of low NDF hays and haylages in order to improve production performances and environmental benefits of dairy sheep.

The third study focused a preliminary test on the use of different additives to reduce methane emissions in ruminant diets. In particular effects of Aluminum nitrate and acrylate, alone or in combination with nanocarriers of double layered hydroxides on methane emission potential of a generic diet. From an applicative point of it was a preliminary test and the results does not allow to directly get recommendation from the field application. Even though it is possible to state that the Nitrate aluminum (NAl), alone and/or in combination with mentioned nanocarriers (Nano), showed the highest reduction potential of methane emission *in vitro* in respect to other additives tested.

Cumulative methane at 48h from incubation, expressed as ml of CH_4 per g of DM, were significantly affected by treatments and the lowest values of emissions were observed for Nano NAl, NAl which resulted lower than control for 15% and 14% as proportion of methane in the produced fermentation gas. In these terms nanocarriers caused a similar reduction of methane using only half dose of nitrates (from 2% in NAl to 1% in Nano NAl). Microbiological determination would help to verify if nanocarriers influenced the microbial population and to explain little better the mechanism involved in methane production pathways. From a scientific point of view this work confirm the opportunity to continue research on nanomaterials as possible coadjutant in enhancing the effect of bioactive compounds. Specifically, further research should be useful to explain the biological mechanism that modulate their action in the rumen and to quantify the dose-response effects in ruminant diets to promote reduction of methane emissions.