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SEZIONE PRODUZIONI ANIMALI

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Doctoral Thesis

Alternative Feeding Resources in the Mediterranean areas to mitigate methane emissions from sheep

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To Peppino & Aisha

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Abstract

The objective of the present doctoral study is to evaluate the potential of using two locally byproducts namely carob pulp (*Ceratonia Siliqua)* and dehydrated citrus pulp (DCP) in view of reducing the carbon footprint, alleviating methane gas emission and reducing dependence on cereal feeding in fattening lambs. Two experimental trials were set up in parallel in an experimental farm in Villarosa (EN) both with Comisana lambs at 90 days of age. Two level of inclusion i.e. 24% and 35% fresh weight were investigated. Four types of parameters were monitored namely performance and productivity both in vivo and at slaughter, rumen pH and fatty acids, rumen microbial ecosystem and in vitro fermentation kinetics.

It was found that both carob pup and DCP ingesting animals had similar performance and productivity. The feed conversion ratio (FCR) was 0.25 for all the diets and the carcass weight was 43%.The rumen pH was similar across all the diets being around 6.6 which is within the range for optimal ruminal metabolism. For the rumen fatty acids profile, it was found that only vaccenic acid was significantly affected by ingestion of DCP, being at 4.99% in the 35% DCP containing diet (Cp35) compared to 1.5% in the control group. For the carob ingesting animals, the diet affected only the stearic acid which was lower in the 35% ingesting animals (Ca35) i.e. 26.4% compared to 38.3% in the control. These values demonstrate to some extent a change in the fatty acids metabolism due to ingestion of the two by products.

The rumen microbial ecosystem was not affected by inclusion of the byproducts remaining at a level of 11 (logcopies/gFM) for total bacteria. It was found also that the *in vitro* methane production was similar across all the diets demonstrating that inclusion of Carob pulp or DCP did not change methanogenesis.

The results of this study, demonstrated that Carob pulp and DCP can be used to substitute barley to some extent without any effect on performance and methane emission in lamb fattening. Rumen metabolism, in terms of ruminal fluid fatty acids, would seem affected only by dietary treatments based on the highest proportions (35%) of the two by products investigated.

Riassunto

L'obiettivo del presente studio è stato quello di valutare il potenziale di impiego di due sottoprodotti dell'industria agroalimentare siciliana : la polpa di carruba (CP) e la polpa disidratata di agrumi (DCP), allo scopo di diminuire l'impatto ambientale degli allevamenti ovini e di limitare l'impiego di cereali nelle diete.Due prove sperimentali sono state svolte in un' azienda sperimentale a Villarosa (EN), entrambe con agnelli di razza Comisana. Le diete sperimentali sono state formulate con due livelli dei sottoprodotti su citati (24% e 35% peso fresco) . Sono stati monitorati : le performances degli animali *in vivo* e alla macellazione, il pH e il profilo degli acidi grassi ruminali, la flora ruminale e i parametri della fermentazione *in vitro*.

È emerso che la produttività degli animali alimentati sia con la polpa di carruba sia con le polpe disidratate di agrumi è risultata simile a quella degli animali alimentati con la dieta a base di orzo. L'efficienza alimentare (kg ADG/kg SS intake) è risultata intorno al valore di 0,25 per tutte le diete e la resa al macello si è collocata su valori intorno al 43% . Nessuna differenza imputabile al trattamento alimentare ha riguardato il pH ruminale simile per tutte le diete, intorno al 6,6 ed indicatore di un metabolismo ruminale regolare. Per quanto riguarda il profilo degli acidi grassi del liquido ruminale solo l'acido vaccenico è stato significativamente influenzato dall'ingestione di DCP con valori del 4,99% del totale degli acidi grassi nel

gruppo alimentato con la dieta che includeva le polpe di agrumi in ragione del 35% rispetto all'1,5% presente nel fluido ruminale del gruppo di controllo. Per gli animali alimentati con la dieta che includeva la più alta proporzione di polpe di carrube (35%) l'acido stearico del liquido ruminale è risultato inferiore rispetto al controllo (26.4 vs 38,3% degli acidi grassi totali, rispettivamente). Questi valori dimostrano un cambiamento nel metabolismo degli acidi grassi causato dall' ingestione dei differenti sottoprodotti allo studio.

La flora ruminale non è stata influenzata dalle diverse diete sperimentali; il valore dei batteri totali è risultato pari a 11 logcopies / gFM. Inoltre si è visto che la produzione di metano, stimata in vitro, era simile per tutte le diete.

I risultati di questo studio, hanno dimostrato che sia le polpe di carruba (CP) che le polpe disidratate di agrumi (DCP) possono essere utilizzate per sostituire l'orzo in una certa misura senza avere un effetto negativo sulle performance degli agnelli da ingrasso e sull'emissione di metano. Il metabolismo ruminale in termini di acidi grassi del fluido ruminale sembrerebbe essere influenzato solo dalle diete che includevano la più alta percentuale (35%) dei sottoprodotti.

A- Bibliographic review

1-Introduction

There is growing concern around the world pertaining to effects of greenhouse gases on global warming. This is why there is a sense of urgency from both the scientific community and decision makers including politicians about the need to curb this trend exemplified by Kyoto protocol 1997, Climate Conference in Copenhagen 2009 and the Climate summit 2014. The main Green house gases (GHG) emitted by human activities are carbon dioxide, methane, nitrous oxide, and fluorinated gases. Moreover the 14% comes from agriculture sector (Figure1).

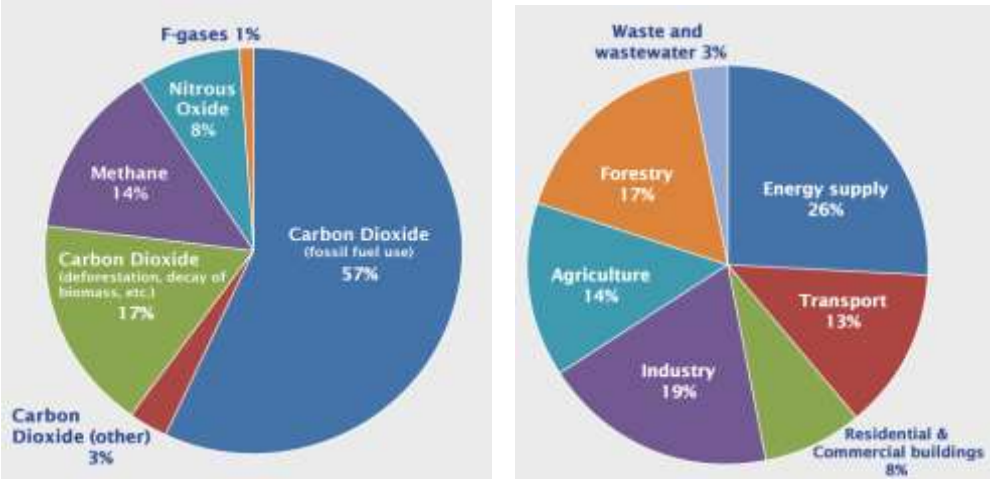


Figure 1 : Global GHG emissions by source (IPCC 2007)

In 2010, estimated worldwide emissions totaled nearly 46 billion metric tons of GHG, expressed as carbon dioxide equivalents. This represents a 35 percent increase from 1990 (Figures 2).

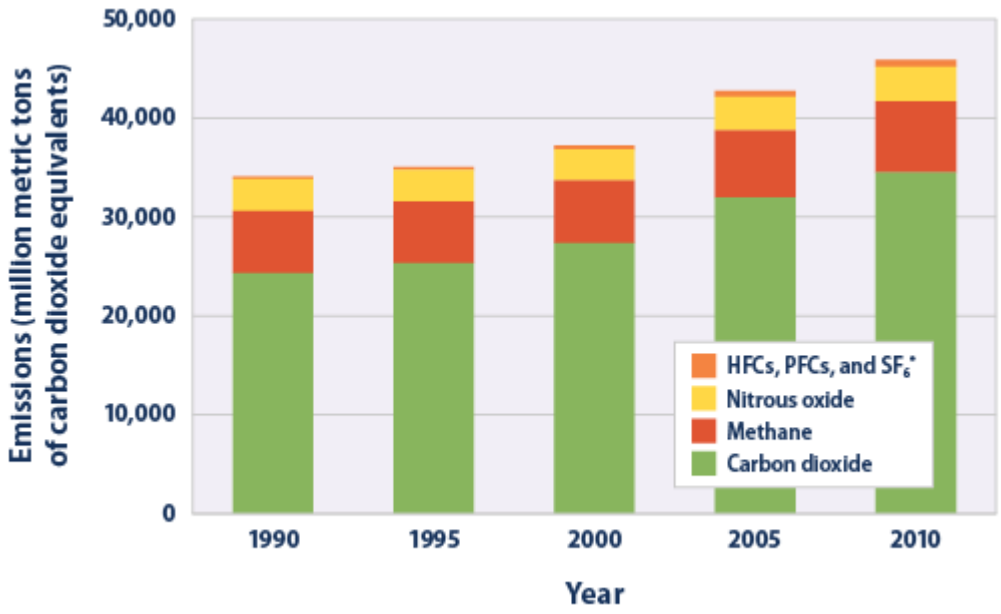


Figure 2 : Global Greenhouse Gas Emissions 1990-2010 (US-EPA)

Total GHG emissions from livestock supply chains are estimated at 7.1 gigatonnes CO_2-eq, they represent 14.5 percent of all human-induced emissions using the most recent IPCC estimates for total anthropogenic emissions. About 44 percent of the sector's emissions are in the form of methane (CH_4) (Gerber et al., 2013). The warming potency of methane is 21 times greater than carbon dioxide.

Ruminants emit CH_4 as part of their natural digestive processes. The microbial populations inhabiting the rumen are comprised of bacteria, protozoa, fungi and archaea. This enables the animal to digest and metabolize plant structural carbohydrates that otherwise could not do with only its digestive enzymes. In the process rumen microbes breakdown the nutrient into volatile fatty acids (VFAs) (acetate, propionate, butyrate), carbon dioxide and ammonia.

The VFAs are energy sources to the animal and the gases are removed by eructation (Fig 3). CH_4 is produced as it is the principal pathway of hydrogen elimination through the following reaction: $CO_2 + 4\ H_2 \rightarrow + 2\ H_2O$

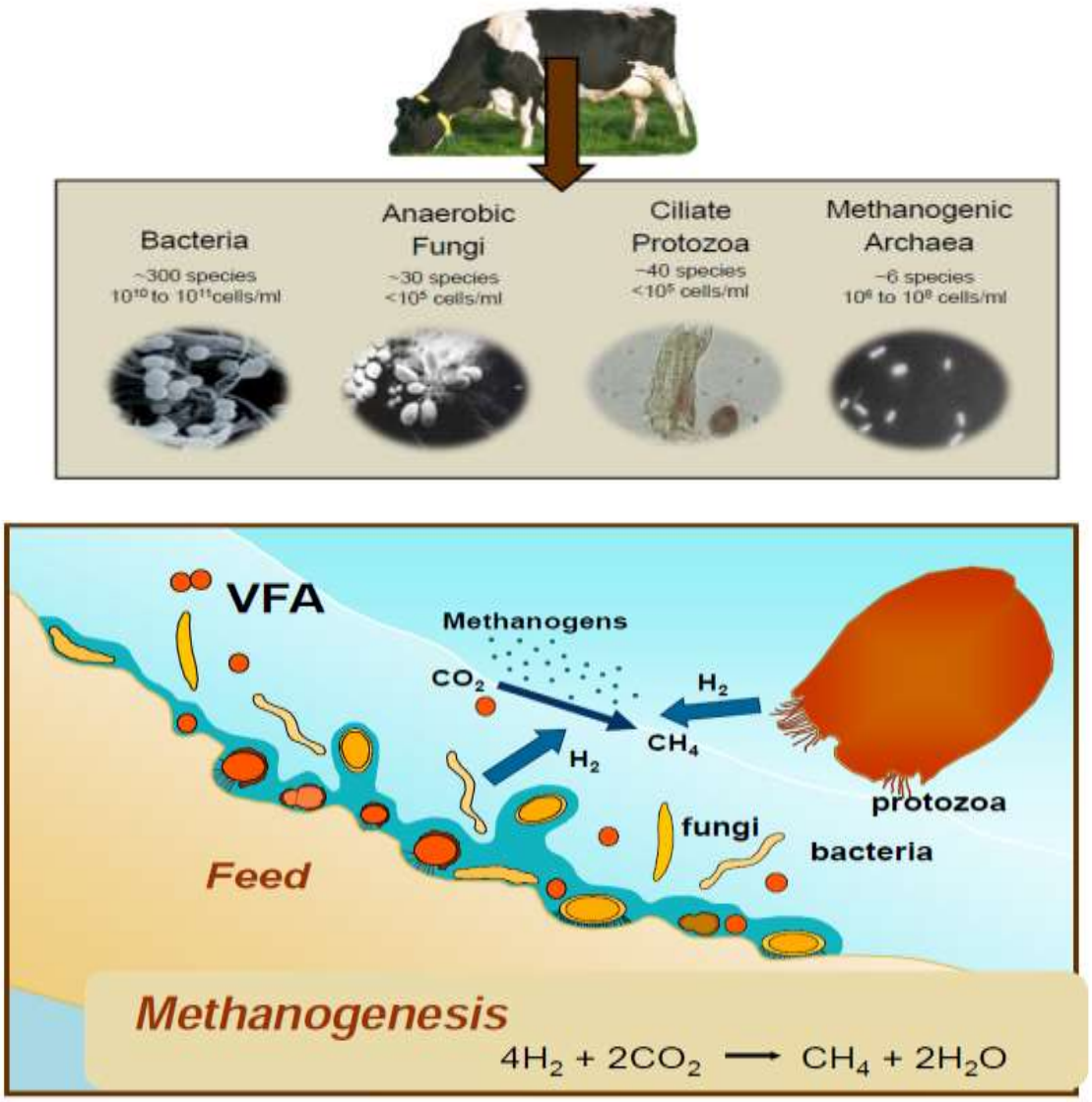


Figure 3: Rumen microbes and fermentation pathway which remove H_2 by conversion to CH_4 (Beauchemin, 2011)

While most of the CH_4 production occurs in the rumen, there is also some production in the lower digestive tract (hindgut), in fact Demeyer et al. (2000) reported 83-94% of the methane is produced in the rumen and the rest in the hindgut (6-13%).

The world demand for foods of animal origin is booming, and in order to meet this demand obviously there is a need to increase production which entails a further increase in methane emission. The demand for meat and milk is expected to be 58% and 70% higher in 2050 than their levels in 2010 and a large part of this increase will originate from developing countries (FAO, 2011).

Thus it is important for the sustainability of the livestock sector that innovations are brought to the conventional methods of farming. There are many studies that have been done to investigate how the level of CH_4 emitted by ruminants may be abated (Hristov et al., 2013; Steinfeld et al., 2006) and they are schematically depicted in figure 4.

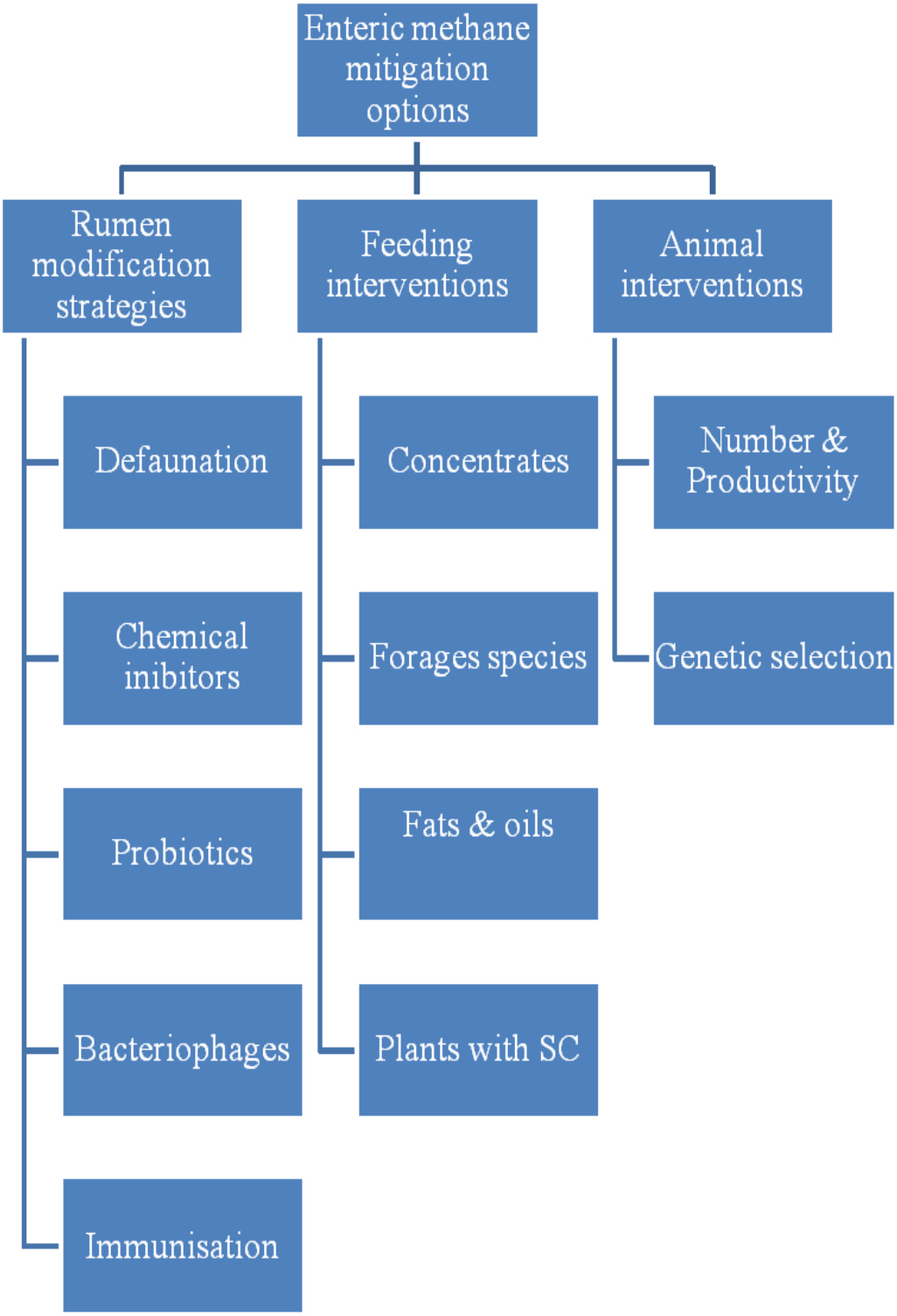

Enteric methane mitigation options
Rumen modification strategies
Feeding interventions
Animal interventions
Defaunation
Chemical inibitors
Probiotics
Bacteriophages
Immunisation
Concentrates
Forages species
Fats & oils
Plants with SC
Number & Productivity
Genetic selection

Figure 4 : Potential strategies for reducing methane production.

2- Methane mitigation strategies

There are a number of means that have been investigated and can be potentially implemented to abate enteric methane emissions. Hereunder, is an overview of some of the strategies that can be used at various levels of the production chain.

2.1- Rumen modification

2.1.1- Defaunation

The elimination of protozoa from the rumen is termed defaunation. As reported by Iqbal et al. (2008) defaunation techniques used have been reviewed by Hegarty (1999). These defaunation approaches include dietary manipulation; synthetic chemicals including copper sulfate, calcium peroxide, dioctylsodium sulfosuccinate and detergents; and natural compounds like vitamin A, non-protein amino acids. Defaunation has been shown to reduce CH_4 production by up to 50% depending on the diet. Mc Allister et al. (2008) showed a 26% reduction in CH_4 yield compared to faunated lambs, while another study reported no effect of defaunation on CH_4 production (Bird et al., 2008).

On the other hand, some reports indicate that the reduction of CH_4 production by defaunation is only temporary (Demeyer et al., 2000). This also limits the use of the above discussed option.

2.1.2- Halogenated compounds

Methanogenesis can be directly inhibited by halogenated methane analogues (Kumar et al., 2009). It is considered that the action of these compounds is due to an interaction with co-enzymes occurring in the methanogenesis process (Demeyer et al., 2000).

Bromoethanesulfonic acid (BES) is particularly effective at inhibiting CH_4 emissions. This analogue provokes a selective inhibition by interfering with the reductase of the methyl-co-enzyme M methanogenesis bacteria.

Recent research has shown that BES can reduce methane emissions from 3.9 to 0.6% of gross energy intake in feedlot steers (Mc Allister, 2008). But, unfortunately, other authors report that the inhibition was not durable suggesting the occurrence of adaptation in the methanogenic population (Iqbal et al., 2008).

2.1.3-Probiotics

The introduction of bacteria which are both active and capable of entering in competition with ruminal bacteria is an interesting approach (Martin et al., 2008).

Moss et al. (2000) reported that *Saccharomyces cerevisiae* (SC) and *Aspergillus oryzae* has been used, but the contrasted results suggest that their effects on CH_4 production remain inconclusive.

2.1.4- Bacteriophages and Bacteriocins

An advantage of bacteriophages is that they are naturally occurring, and have potential to survive in the rumen, and the capacity to lyse their hosts (bacteria and archaea) during the lytic phase of their development.

However, no phage specific to ruminal methanogens have been reported, and due to their host-specificity, this may pose a challenge due to the diversity of methanogens (Mc Allister et al.,2008).

Bacteriocins are bactericidal peptides that are produced by bacteria and may play a competitive role among microbial species for niches within the ruminal ecosystem. Nisin, a well known bacteriocin produced by *Lactococcus lactis* was used as an alternative to monensin reducing methane emission *in vitro* about 36% (Kumar et al.,2009).

2.1.5-Additives

*Ionophores : they are polyether antibiotics produced by soil microorganisms that modulate the movement of cations such as sodium, potassium and calcium across cell membranes. (Iqbal et al., 2008). The mechanism of action of Monensin, which is the mostly used ionophore, is extensively documented, and it affects methane production by four ways:

-Increasing feed conversion efficiency (Wallace et al.,1980) ;

-Shifting bacterial population from gram-positive to gram-negative organisms with a concurrent shift in the fermentation from acetate to propionate (Moss et al., 2000);

- Inhibiting the release of H2 from formate (Werner et al., 1984);

- Depressing ciliate protozoa population (Garcia et al., 2000).

However, the protozoan population can adapt to ionophores present in low or high concentrate diets. Kumar et al.,(2009) reported that monensin effect did not persist in the longer term. Also there is a risk that polyether ionophores may get absorbed from the rumen and reach meat or milk. These additives are now forbidden in the European Union as precautionary measure (Martin et al., 2008).

*Organic acids: dicarboxylic acids (malate, fumarate) are potential precursors of propionate which stimulate H2 utilisation for reduction of fumarate to succinate during propionate synthesis at the expense of enteric methane (Mc Allister et al., 2008).

An exceptional decrease in methane production by 76 % and by 62% has been shown by Wood et al. (2009) when encapsulated fumaric acid or fumaric acid were respectively used. However, supplementing diets with organic acids at the levels required to suppress methane emissions in vivo appears to be uneconomical (Martin, 1998). Nevertheless, some forages with a high malate content (like Lucerne) might serve as vehicles to increase dietary malate .

2.1.6- Immunisation

A new line of research has been developed in recent years, whereby the possibility of vaccinating ruminants livestock against methanogens is considered. The development of an anti-methanogenic vaccine is in progress. The aim would be to stimulate the animal to produce antibodies against known ruminal methanogens. It was found a non-significant reduction of 6% in the sheep after 4 weeks of vaccination with three methanogen mixtures and a significant 7.7% reduction in methane production per kg DM intake after revaccination (Wright et al., 2004).

However, Williams et al. (2009) have tested a vaccine based on a mixture of five methanogens in 36 two year old Merino wethers and did not observe a decrease in methane output in sheep that received the anti-methanogen vaccine in comparison to the level in the controls. Much more work is needed to make this technique effective, as there are multiple strains of Archaea in the rumen.

2.2- Feeding strategies

2.2.1-Forage to concentrate ratio

Many experiments showed that by increasing concentrate feeding there is reduction on CH_4 production (Yan et al.,2000; Lovett et al., 2003). A positive response to high levels of starch-based concentrate (grains) on methane reduction has also been reported by other authors (Beauchemin et al., 2005). Replacing dietary fibre with starch drives ruminal pH to decrease and modifies the microbial population. A shift of VFA production from acetate towards propionate occurs, thus resulting in less hydrogen production (Martin et al.,2008). Nevertheless, increased levels of concentrates may result in health problems (e.g. ruminal acidosis). Moreover the increased feeding costs related to high dietary levels of concentrates limit their use in some animal husbandry systems.

2.2.2-Forage type and quality

The use of more digestible fodder (less mature and processed forage) resulted in a reduction of methane emission (-15% and -21% respectively) (Benchaar et al., 2001 as cited by Iqbal et al., 2008). Similarly, CH_4 production was 28% lower for grass forage and 20% lower for good quality silage than for lower quality hay. Dietary characteristics affect rumen conditions and so alter the balance of methanogenic and other species, for example diets with high fermentable grains content, will be rapidly fermented. This will lower pH because of the rapid rate of VFA production and possibly of lactic acid; the latter acid itself, or lower pH, may then kill protozoa, removing one of the major habitats of methanogens (Martin et al., 2010).

2.2.3- Fat & oils

Dietary fat seems a promising nutritional alternative to depress ruminal methanogenesis without decreasing ruminal pH as opposed to concentrates. Fat inclusion in the diets causes a decrease in CH_4 production depending upon the levels of fat supplementation, fat sources, forms of fat supplementation, and types of diet. Beauchemin et al. (2008) created a dataset

based on 17 studies with beef. Over this broad range of conditions, CH_4 (g/kg DMI) was calculated to be reduced by 5.6% with each 1% addition of supplemental fat.

However, cost of fat supplementation with edible oils might not be economical for the livestock producers.

2.2.4- Plant secondary compounds

In the recent years, there is growing interest towards the use of plant secondary compounds (tannins, saponins) as a CH_4 mitigation strategy because of their natural origin in opposition to chemicals additives.

-*Tannins*: Tavendale et al. (2005) proposed two modes of action of tannins on methanogenesis: a direct effect on ruminal methanogens and an indirect effect on hydrogen production due to lower feed degradation. Moreover, tannins are known to decrease protozoal number (Goel et al., 2012).

Condensed tannins (CT) have been shown to reduce CH_4 production by 13 to 16% (DMI basis) (Carulla et al., 2005; Waghorn et al., 2006; Grainger et al., 2009). However, high CT concentrations may reduce voluntary feed intake and digestibility (Newbold et al., 2006).

- *Saponins*: The mode of action of saponins seems to be clearly related to their anti-protozoal effect (Newbold et al., 2006). The sensitivity of protozoa towards saponins may be explained by the presence of sterols in protozoa, but not in bacterial membranes (Williams and

Coleman, 1992 as cited by Hart et al., 2008). Thus, the sterol-binding capacity of saponins most probably causes the destruction of protozoal cell membranes.

Plant saponins also hold potential to reduce CH_4, being some saponins sources clearly more effective than others (Patra et al., 2009; Beauchemin et al., 2008).

In addition to saponin and tannin containing plants, a range of bioactive plant metabolites and plant extracts have or are being investigated in terms of their effects on rumen methanogenesis and several projects are currently underway in Europe such as"RUMENUP" which is involved in the screening of 450 plant species for anti methanogenic activity (http://www.abdn.ac.uk/research/rumen-up) or "SMEthane" in which different formulations and sources of plant extracts are tested as nutritional additives to reduce CH_4 and whose details are available on the web site : http://www.smethane.eu/en/index.html). Another example is the Australian project named "ENRICH" in which 128 native shrubs were evaluated as antimethanogenic feed additives (Bodas et al., 2008). But, still need for additional research *in vivo* to determine the optimal dose of the active compounds, to consider the potential adaptation of the microbes, the presence of residues in animal products as well as the potential anti-nutritional side-effects of such molecules.

2.3- Animal interventions

2.3.1- Number and productivity

CH_4 production is directly proportional to the number of animals. Removing nonproductive and low-producing animals from herds and maintaining just the high producing ones is often advocated in developed countries. In this way, although total production will be increased, CH_4 emissions per unit of product could be decreased. However, this is unlikely to be recommended due to socioeconomic and religious background in many developing countries.

Proper livestock management especially in developing countries such as reducing the incidence of disease and reproductive problems can decrease CH_4 emission in a herd for each unit of production (Eckard et al., 2010).

Increasing the productivity of animals could also lessen CH_4 emissions per unit of products.

In Australia, research has been undertaken to improve the feed efficiency of beef animals by selection for low residual feed intake. Animal production efficiency can be increased by selection of animals with improved animal performance. As a result of increased productivity, methane production per unit of milk or meat will decrease (Iqbal et al., 2008).

2.3.2-Genetic selection of animals

Ranking of animals based on CH_4 production is highly variable due to the high within-animals variability as demonstrated by several studies, when there are variations in diets and/or physiological stages or between successive measurements for a same diet.

This finding suggests the possibility of genetic differences between animals in CH_4 production, which could be utilized for genetic selection for low CH_4 production.

Hegarty et al. (2007) have demonstrated that ruminants with low residual feed intake (RFI; i.e. the difference between actual feed intake and the expected feed requirements for maintenance and production) emit less CH_4 than the animals with high RFI.

This may offer an opportunity for genetic selection for this trait and it can be selected without compromising the production traits (Patra, 2012).

Researches believes that likely one day breeders will be able to include CH_4 production as a selection and they are working on it by establishing the CH_4 measurement technique or associated traits suitable for genetic or genomic selection, considering that thousands of animals per species must be genotyped and/or sequenced in order estimate the heritability of traits CH_4 with the repeatability at different ages and on different diets in different countries this still part of a larger initiative to create selection lines for this trait so as to understand the anatomical, physiological and microbiological as well as productivity changes that accompany selection for this trait (Pinares, 2013).

3- Conclusion

There are an array of methods that can potentially be applied to mitigate CH_4 emission in livestock production. No single method by itself is likely to give tangible result at an economic cost (Cottle et al., 2011). These methods are relatively new and not easy to adopt in the present systems of production. Furthermore, the experiments done so far have very often resulted in inconclusive or even contrasting outcomes. So it is rather premature to devise at least one recipe. Consequently, for the time being it is pertinent to adopt several techniques which are synergistic among themselves resulting therefore in an overall reduction in methane emitted.

Figure 5 illustrate the findings by Beukes et al. (2010) who used a farm scale model based on farm grazing cows in New Zealand over different climate years, to evaluate the efficacy of selected mitigation strategies that improve efficiency without affecting production.

The simulation showed that the cumulative effect of the improved herd efficiencies and animal genetics in Farms C where improved animal efficiency methods have been adopted, and farm E which are home-grown maize silage (increased total metabolizable energy (ME)

yield and reduced nitrogen intake) resulted in a significant 15% reduction in CH_4/ha compared with Farm A, based on conventional (baseline) management.

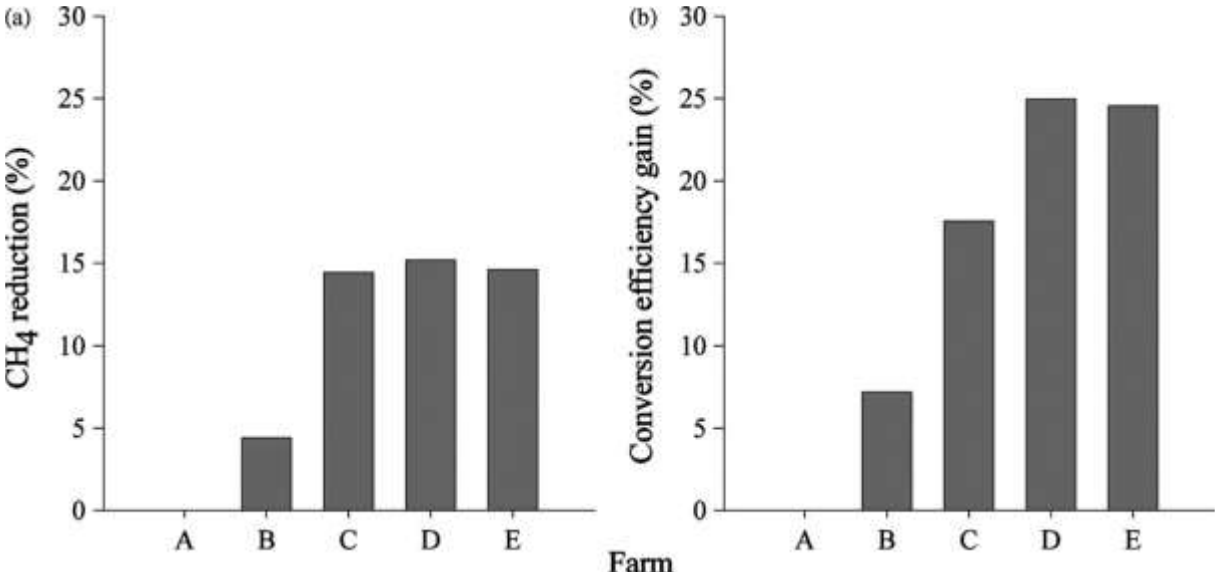


Figure 5 : Percent reduction in CH4 enteric emission per unit of area .

Farms A (baseline), B (improved herd efficiency), C (improved animal efficiency), D (improved pasture management) and E (home-grown maize silage).

B- Experiments

1- Context of the study

The Mediterranean region has a well-established livestock production sector with a wide variety of production systems ranging from pastoral based to stall-fed. Sicily has a relatively important livestock sector (Caracappa, 1999) and sheep production is estimated to represent

approximately 8,500 farms and 886,000 heads (ISTAT, 2010). It accounts 15 % of the national herd (Schilirò, 2006).

The sheep production is mainly directed to the breeding of breeds such as the Pinzirita, Valle del Belice and Comisana which are oriented to milk production and well adapted to the local contexts. Comisana is one of the most appreciated breed by farmers as it gives a good level of milk around 150-200kg/200 days of lactation (www.agraria.org) and has a good carcass yield and quality (Priolo et al., 1998; Priolo et al., 2005). Locally, ewe milk is mostly processed on farm site to produce a variety of traditional high quality homemade cheeses of which the most popular is Pecorino Siciliano, labeled as protected designation of origin (PDO). Generally, sheep feeding system is based on grazing on rangelands and pastures and concentrate supplementation to a certain extent often in milking parlor and hay supply in stall. The most common sources of concentrate supplementation are cereals and legumes. There is also some marginal utilization of agro-industrial byproducts especially supplemented with concentrates. The most commonly used byproducts derive from the olive oil, citrus and carob processing industries.

In line with the preoccupation of increasingly incorporate locally available resources so as to reduce the carbon footprint of the sector, studies are encouraged to devise means to achieve this in the various production systems of the region. The objective is to achieve at least comparable performances and at the same time to improve the sustainability of the sector through these innovations. Such strategy will not only reduce the dependencies of external inputs especially cereals and would be ecologically sound.

In the present study, the potential utilization of two alternative feeds namely citrus pulp (experiment 1) and carob pulp (experiment 2) as a substitutes for cereals in lamb fattening diet, were investigated and their potential in the abatement of methane emissions evaluated.

2-Experiment 1

In Italy, the citrus processing industry produces 1,000,000 tons of fruit per year and 600,000 tons of pulp and Sicily is one of the main contributors. About 30% of the production of citrus fruits (and 40% of orange production) in the world is processed (USDA-FAS, 2010) principally to make juice. The residue left after extraction of the juice is called citrus pulp (50−70 % of the fruit by weight) and contains the peel (60-65%), internal tissues (30-35%) and seeds (0-10%) (www.feedipedia.com).

Dehydrated citrus pulp (DCP) contains 5−10% of crude protein and 6.2% of ether extract , 10−40% of soluble fibre (pectins) and 54% of water soluble sugars. The composition of DCP is variable and depends mainly on the relative proportions of skins and seeds, which varies according to the citrus species, variety, harvesting season and processing undertook.

Citrus pulp is usually fed dehydrated and is introduced gradually into a ration to allow the animals to become accustomed to its distinctive smell and taste (Bampidis et al., 2006). In addition, the use of DCP is also determined by the profile of plant secondary compounds (PSCs) that is present. PSCs are a group of substances that are present in plants and whose metabolism was considered as secondary (Acamovic et al., 2005) compared to other substances like carbohydrates and proteins. Their roles are still not completely clear but they are basically toxic in nature and may have both positive and negative effects in animal nutrition (Waghorn e al., 2006). PSCs comprise a range of substances among which are polyphenols, saponins, essential oils and alkaloids just to name a few. DCP is known to have a range of PSCs (Ramful et al., 2010) and by virtue of these presence, their use in animal nutrition is limited. For instance, high tannins content would cause astringencies and may reduce substantially nutrients digestibility and assimilation (Frutos et al., 2004). However, the

presence of these PSCs is also what makes DCP interesting in methane abatement strategies. In fact, saponins and tannins are known to interfere with ruminal metabolism in particular either by modifying the ruminal flora (Goel et al., 2012) or interacting in the methanogenesis metabolism (Sliwinski et al., 2002; Kamra et al., 2006). Due to the wide variation in DCP composition with sites, processing types and variety and animal factors in terms of age, breed and sex, it is important to determine for each case, what would be the optimal rate of inclusion of DCP that would reduce methane without having deleterious effects on performance and animal well being.

The present study aims at investigating whether inclusion of locally available dried citrus pulp at two levels in concentrate mixtures as a substitute for barley, affects the productivity in terms of lamb growth performances and whether there are effects on rumen metabolism, rumen microbial ecosystem and the amount of methane emitted as assessed *in vitro*.

2.1- Materials and methods

2.1.1- Animals and diets

An experimental trial was set up in a sheep farm at Villarosa (523 m) in the centre of Sicily. There were used 29 male Comisana lambs all born on the same farm from the 10th to the 30th of November 2011. Animals were weaned at 2 months of age , dewormed and weighted. Based on the live weight recorded, the animals were randomly allocated to 3 groups with each group having a similar average liveweight (19,2± 4,468).Each group was assigned to a dietary treatment. In the experimental diets barley was the main ingredient of the commercial concentrates and was substituted with dried citrus pulp at two levels of inclusion (24%, Cp24

and 35%, Cp35). The ingredients and chemical composition of each diet are shown in Table 1.

Table 1: Ingredients and chemical composition of the diets (Control, Cp24 and Cp35 groups)

	Dietary treatments		
	C	**Cp24**	**Cp35**
Ingredients (g/100g as fed)			
Barley	60	35	23
Citrus pulp	0	24	35
Dehydrated lucerne	20	19	20
Soya bean meal	9	12	13
Wheat bran	11	10	9
Chemical composition			
Dry Matter (DM; %)	88,92	89,39	90,56
Crude Protein[1]	18,00	18,05	17,08
Neutral Detergent Fiber (NDF)[1]	34.6	31,7	33,10
Acid Detergent Fiber (ADF)[1]	13.7	15,9	18,03
Acid Detergent Lignin (ADL)[1]	8.3	8,21	11,5
Ether Exract[1]	2.23	1,38	2,25
Total Phenols[2]	4.0	6.7	7.9

[1] expressed as g/100g of DM,
[2] expressed as g of tannic acid equivalents/kg DM

After an initial period of 10 days for adaptation, the animals were kept on the respective diets for a period of 56 days. Animals were housed in individual pens (3m x 2m) and each pen was

equipped with a feeder and a drinker. Feed allocation was given on an *ad libitum* basis from 9 a.m. to 6 p.m. and water allocation was permanent.

The amounts of feed offered and refused were recorded daily in order to measure the daily voluntary feed intake. The animals were also weighed weekly before the feeds allocation. Feed samples were collected at four different times (days 9, 30, 44, 51) during the trial and stored at -30°C until analysis.

2.1.2- Slaughter sampling and measurements

The animals were slaughtered after a 24hr lairage, at a slaughter house (~100km) from the experimental farm. Slaughter was done by captive bolt followed by exsanguinations.

Within 15 min after slaughtering the rumen digesta were collected into plastic buckets and thoroughly mixed. Ruminal fluid pH was measured by a pH meter (Orion 9106;Orion Research Incorporated Boston,MA). Another aliquot of ruminal content (50ml) was stored at -80°C pending freeze drying. Another aliquot of ruminal content was filtered through two layers of cheesecloth and a portion of 100 ml was stored at -30°C until fatty acid analysis.

Carcasses were evaluated according to the EU score system (SEUROP classification system) and the carcass weight and yield recorded .

2.1.3 - Laboratory analysis

- Feed analysis

Pooled sample for each diet were ground to pass a 1mm sieve in a Wiley® mill and used for chemical analysis for neutral detergent and acid detergent fibre fractions (NDF and ADF, respectively) according to Van Soest et al. (1991). According to AOAC (1995) method,

feedstuffs were also analysed for crude protein (CP) and crude fat (CF) extracted with petroleum ether. Total phenolic compounds were extracted from the feeds as described by Makkar et al. (1993), using aqueous acetone (70% v/v), analysed by means of the Folin-Ciocalteau reagent and expressed as tannic acid equivalents.

- Rumen fatty acid analysis

Ten milliliters of rumen fluid was directly methylated using a combination of methods according to Kramer et al. (1997) modified by Park et al. (2001). The first step consisted of an alkaline methylation with sodium methylate/methanol (1 mL of 0.5 M-Sodium Methoxide) to esterify glycerides. The second step involved an acid methylation with HCl/methanol (1.5 mL of 5% methanolic HCl, 10 min at 50°C) as catalyst to esterify free fatty acids. Fatty acid methylesters (FAME) were extracted using n-hexane with C9:0 and C23:0methyl ester (Sigma Chemical Co., St. Louis, MO) as internal standards for quantification, and maintained in vials with hermetic closure to avoid the loss of volatile components. FAME were separated and identified by gas-chromatography on a GC equipped with a capillary column (CP-select CB for FAME Varian, Middelburg, The Nederlands: length, 100 m; i.d., 0.25 mm; film thickness, 0.20 μm), according to Buccioni et al. (2011). The injector and flame ionization detector temperatures were 270°C and 300°C, respectively. The programmed temperature was 40°C for 4 min, increased to 120°C at a rate of 10°C/min, maintained at 120°C for 1 min, increased to 180°C at a rate of 5°C/min, maintained at 180°C for 18 min, increased to 200°C at a rate of 2°C/min, maintained at 200°C for 1 min, increased to 230°C at a rate of 2°C/min and maintained at this last temperature for 19 min. The split ratio was 1:100 and helium was the carrier gas with a flux of 1 mL/min. Standard mix (47792 Supelco, Chemical Co., St. Louis, MO) and published isomeric profileswere used to identify the α-linolenic acid (ALA)

isomers. Two bacterial acid methyl ester mix (47080-U Supelco, Chemical Co., St. Louis, MO; GLC110, Matreya, Pleasant Gap, PA) and individual standard for methyl ester of iso 14:0, ante 14:0, iso 15:0 and ante17:0 (21-1211-11, 21-1210-11, 21-1312-11 and 21-1415-11, Larodan Malmo, SW) were used to identify branched fatty acid profile. Inter and intra-assay coefficients of variation were calculated by using a reference standard butter (CRM 164, Community Boureau of Reference, Bruxelles, Belgium) and detection threshold of fatty acids was 0.01g/100g of FA.

- q-PCR analysis of samples

Samples of rumen contents were freeze-dried and then mixed by physical disruption using a bead beater (Mini-Bead Beater 8, BioSpec Products, Bartlesville, OK). Extraction was then performed on 50-mg samples using the QIAamp DNA Stool Mini Kit (Qiagen Ltd., West Sussex, UK) with a modification: a higher temperature (95°C) was used for lysis incubation. The DNA samples were used as templates to quantify the copy numbers of 16S rRNA (for bacteria), methyl coenzyme M reductase A (mcrA) gene (for *methanogenic archaea*), and 18S rRNA (for protozoa) by real-time quantitative PCR (qPCR). The yield and purity of the extracted DNA were assessed using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Primer sets used were as follows: forward: 5c-GTG- STGCAYGGYTGTCGTCA-3c and reverse: 5c-ACGT- CRTCCMCACCTTCCTC-3c for total bacteria (Maeda et al., 2003) and forward:5c-GCTTTCGWTGGTAGT- GTATT-3c and reverse: 5c-CTTGCCCTCYAATCGT-WCT-3c for protozoa (Sylvester et al., 2004). The primer sets for detection and enumeration

of methanogenic archaea (mcrA) were forward: 5c-TTCGGTGGATCD- CARAGRGC-3c and reverse: 5c-GBARGTCGWAWC- CGTAGAATCC-3c (Denman et al., 2007).

Three replicates of each extract were used and a negative control was loaded on each plate run to screen for possible contamination or dimer formation and to set the back-ground fluorescence for plate normalisation. Real-time PCR analyses were performed on iQ5 multicolor Real-Time PCR Detection System (BioRad Laboratories Inc., Hercules, CA).

Two microlitre of DNA extract was added to amplification reactions (25 μL) containing 0.2 μL of each primer (10 μM) and 12.5 μL of iQ SYBR Green Supermix (BioRad Laboratories Inc.). Cycling conditions were 95°C for 5 min; 40 cycles of 95°C for 15s, 60°C for 30s, and 72°C for 55 s; and 72°C for 1 min. The threshold cycle (amplification cycle in which product formation exceeds background fluorescence) of each sample was determined during the exponential phase of amplification. The absolute amount for each microbial group, expressed as the number of DNA copies/g of fresh matter, was determined using standards. The qPCR standards consisted of the plasmid pCR 4-TOPO (Invitrogen, Carlsbad, CA) with an inserted 16S, mcrA, or 18S gene fragment corresponding to a conserved sequence of total bacteria, methanogenic archaea, or protozoa, respectively. The number of gene copies present in the plasmid extracts was calculated using the plasmid DNA concentration and the molecular mass of the vector with the insert. The concentrated plasmid was serially diluted (10-fold) to generate a standard curve.

- *In vitro* gas production test

To study the effect of using carob pulp on kinetics fermentation, Volatile Fatty acids (VFA's) profile and CH_4 production, an *in vitro* gas production assay was carried out according to Theodorou et al.(1994). A sample of 1 g was weighed in triplicate into 120 ml serum bottles.

Four 72 h runs were conducted using rumen fluid from sheep that have been already adapted to the control diet (alfalfa, barley, soya and wheat bran) for two weeks.

Rumen fluid was collected by a stomach tube before the morning meal from 3 fistulated sheep, and transferred into two pre-warmed thermos flasks, mixed and strained through two layers of cheesecloth.

Anaerobic buffer solution (containing micro- and macroelements, a reducing agent, and a reduction indicator of resazurin) was prepared and placed at 39◦C on magnetic stirrer under

continuous flushing with CO_2 until the color turns to pink (Menke et al., 1988). Then we poured the strained rumen liquid into the artificial saliva and continue stirring. About 60ml of buffered rumen fluid was dispensed into bottles containing the feeds.

Negative controls (blank) containing buffered rumen fluid but no substrate were also included in triplicate for correction of gas produced from small particles present in the ruminal fluid. All handling was under continuous flushing with CO_2. Bottles were sealed with rubber stoppers and aluminunim caps and inubated at 39°C. Cumulative gas production (ml/g DM) was recorded at 2, 4, 6, 8,12,24,48, 72 after incubation at 39°C. The pressure generated by the gas accumulated in the incubation bottles was measured through a pressure transducer connected to a digital reader .



After 24h of incubation about 0.8ml of bottle contents were sampled into a 2ml eppendorf containing 0.8ml of a solution (HCl , Crotonic and metaphosphoric acid) and stored at -20°c for subsequent VFA's determination. Then the samples centrifuged at 3000g (5300rpm) for 20 minutes ,and the supernatant transferred in vials, the individuals VFAs concentrations were analysed using gas chromatography technique.

To assess kinetics of fermentation, duplicate bottles were incubated, following the procedure described, but incubations were terminated at 72h. Rate and extent of gas production was

determined for each feed by fitting gas production data to the nonlinear equation Y = b(1 − e−ct) (Orskov and Mcdonald, 1979), where y is the volume of gas produced at time t, b the potential gas production (mlg−1 DM), and c the fractional rate of gas production.

At the end of the incubation periods the residues of fermentation were as stored at −20◦C for determination of neutral detergen fiber (aNDF) and digestibility. The NDF content were analysed following the technique described by Van Soest et al. (1991) using an ANKOM Model 220 fibre Analyzer (Macedon,NY). Fermentation residues were dried at 105ºC overnight and then incinerated in a muffle furnace at 550ºC for 12 h. Loss in weight after incineration was used as a measure of ash. The *in vitro* organic matter degradability (IVOMD) at 96 h of incubation was calculated as the difference between the OM content of the substrate and its undegradable OM.

2.1.4- Statistical analysis

Analysis of variance (ANOVA) was used to determine the effect of dietary treatment on performance indicators (feed intake, live weight gain, feed efficiency and carcass yield)., pH and rumen fatty acids profile, rumen microbial ecosystem and the in vitro parameters. Regarding intake and feed efficiency data, the individual average value for the whole experimental period has been included in the data base for ANOVA analysis. Data were analysed as a completely randomised design, with a model that included the diet as fixed effect. When the ANOVA was significant ($P < 0.05$), means were separated by pairwise comparison by means of the Tukey's method.

2.2- Results

2.2.1- Animal performances

The effects of the diets on dry matter intake, performance and nutrient intake are presented in table 2.

Table 2 : Performance and intake of lambs fed on Control (C) diet or two citrus pulp based diet (Cp24 and Cp35).

	C	Cp24	Cp 35	SEM	P
Performances					
DM Intake (g/d)	753.6	721.3	801.7	35.8	0.675
Average Daily Gain (ADG)	184.6	164.7	187.0	10.4	0.655
Initial weight	19.38	18.61	19.04	0.796	0.931
Final weight	29.91	28.00	29.69	1.30	0.822
Feed Efficiency (FE)	0.2580	0.2343	0.2429	0.00659	0.351
Carcass weight	12.99	12.55	12.96	0.657	0.959
Carcass yield	43.279	44.20	43.457	0.487	0.733
Nutrient Intake					
Crude protein[1]	135.91	133.4	142.3	6.40	0.856
NDF[1]	260.9	222.1	265.3	12.0	0.429

Hemicellulose[1]	157.5a	113.9b	120.8ab	7.25	0.021
Cellulose[1]	40.84	42.79	52.14	2.39	0.115
ADL[1]	62.54b	72.42ab	92.44a	4.55	0.015
Ether Extracts[1]	16.8a	11.25b	18.06a	0.958	0.003
Total Phenols[2]	30.14b	48.48a	63.06a	3.82	0.000

[1] expressed as g/100g of DM,
[2] expressed as g of (TA) equivalents / g of DM

From the Table 2, none of the parameters measured during the in vivo phase and at slaughtering were affected by the experimental diet. However, a different nutrient intake has been obtained for fibre fractions. The animals on Cp35 have ingested the highest level of lignin and the hemicellulose intake was highest in the control (P=0.0548 and P=0.0742, respectively) when compared to Cp24 and Cp35. Total phenols consumption was also affected by dietary treatment, as expected, showing the lowest values in Control lambs compared to Cp24 and Cp35 (P<0.05).

2.2.2- Ruminal pH & fatty acids profile

The effects of dietary treatment on ruminal pH and fatty acid profile are reported in Table 2. No significant difference among dietary treatments was reported on ruminal fluid pH. Pertaining to the fatty acid profile, the dietary treatment affected (P=0.03) vaccenic acid (VA) proportion with the highest level found in ruminal fluid from lambs fed the diet including the highest proportion of citrus pulp (Cp35) compared to Control. In ruminal fluid from Cp24 group the level of VA was comparable with its counterparts. Rumenic acid was not affected (P=0.388) by the dietary treatment, while the C18:2 trans 10, cis 12 CLA isomer increased (P=0.002) in ruminal fluid from Cp24 compared to Control.

Table 2 : pH and fatty acids (% total fatty acids) in the ruminal fluid of lambs fed on Control (C) or two citrus pulp based diets (Cp24 and Cp35).

	Diets

	C	Cp24	Cp35	SEM	P-value
pH	6.61	6.53	6.64	0.0473	0.616
C13:0	0.34	0.28	0.54	0.0601	0.193
C14:0	0.97	0.76	0.68	0.0812	0.328
C14:0 *iso*	0.36	0.67	0.76	0.063	0.014
C15:0	0.86	0.87	1.08	0.0874	0.511
C15:0 *iso*	0.29	0.26	0.28	0.0215	0.787
C15:0 *ante*	1.22	1.44	1.14	0.0844	0.346
C16:0	18.18	13.9	18.13	0.85	0.05
C16:0 *iso*	0.6	0.53	0.57	0.0793	0.941
C16:1	0.19	0.23	0.24	0.025	0.708
C17:0	0.55	0.62	1.94	0.486	0.441
C17:0 *iso*	0.87	0.79	1.13	0.106	0.408
C17:0 *ante*	0.65	0.36	0.64	0.0781	0.231
C18:0	38.28	34	30.45	2.02	0.299
Σ*iso* BCFA	2.14	2.26	2.75	0.167	0.296
trans-5 C18:1	0.043	0.062	0.067	0.0113	0.684
trans-6 to *trans*-8 C18:1	0.45	0.79	0.76	0.0986	0.295
trans-9 C18:1	0.24	0.38	0.36	0.0466	0.417
trans-10 C18:1	4.72	8.57	6.28	0.983	0.287
trans-11 C18:1	1.5a	1.86ab	4.99b	0.616	0.03
trans-12 C18:1	0.53	0.54	0.62	0.0357	0.579
cis-7 C18:1	0.89	1.23	0.88	0.0822	0.139
cis-9 C18:1	3.47	3.3	4.13	0.333	0.589
cis-11 C18:1	0.54	0.67	0.64	0.0341	0.281
cis-12 C18:1	0.57	0.39	0.34	0.0505	0.157
cis-15 C18:1	0.35	0.36	0.48	0.0347	0.201
cis-9, *trans*-11 C18:2 CLA	0.89	1.07	1.57	0.204	0.388
trans-10, *cis*-12 C18:2 CLA	0.24a	0.6ab	0.45b	0.0476	0.002
ΣCLA	1.14	1.68	2.03	0.206	0.213
cis-9, *cis*-12 C18:2 n-6	3.67	2.53	1.35	0.549	0.237
trans-11, *cis*-15 C18:2	0.06	0.13	0.11	0.0377	0.727
cis-9, *cis*-12, *cis*-15 C18:3	0.43	0.64	0.46	0.153	0.844
ΣSFA	59.18	52.3	50.4	2.09	0.215
ΣMUFA	13.32	18.19	19.56	01.40	0.164
ΣPUFA	4.16	3.31	1.93	0.553	0.262

[1]Σ*iso* BCFA = C14:0 *iso*+ C15:0 *iso*+C16:0 *iso* + C16:0 *iso*

[2]Σ SFA = C13:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0

[3]Σ MUFA = *trans*-5 C18:1+ *trans*-6 to *trans*-8 C18:1+ *trans*-9 C18:1+ *trans*-10 C18:1+ *trans*-11 C18:1+ *trans*-12 C18:1+ *cis*-7 C18:1+ *cis*-9 C18:1+ *cis*-11 C18:1+ *cis*-12 C18:1+ *cis*-15 C18:1

[4]Σ PUFA = *cis*-9 *cis*-12 C18:2 n-6+ *trans*-11. *cis*-15 C18:2+ *cis*-9. *cis*-12. *cis*-15 C18:3

2.2.3- Bacterial profile

The main microbial populations present in the rumen of lambs ingesting the control or the two citrus pulp based diets as identified by qPCR are shown in the Table 3.

The Archaea population was similar between the groups and was around 9 log10 copies/gFM. The Protozoa population was around 5 log10 copies/gFM and the total Bacteria around 11 log10 copies/gFM .

Table 3: Rumen microbial population of the animals ingesting the control and the diets with the different level of citrus pulp

	C	Cp24	Cp35	SEM	P value
Total Bacteria[1]	11.213	11.191	11.012	0.0472	0.157
Archaea	9.2388	9.081	9.065	0.0625	0.574
Protozoa	5.056	5.679	5.932	0.24	0.326

[1] log10copies/gFM

2.2.4-*In vitro* fermentation \

The *in vitro* ruminal fermentation parameters (A, c, CH_4, Total VFA, Ac/Pr and IVOMD) of the control and the experimental diets are shown in Table 4 .

Table 4 : *In vitro* fermentation characteristics of the Control and Cp diets

	C	Cp24	Cp35	SEM	P value
A	131.07	126.38	123.22	3.96	0.756
c	0.10432	0.11140	0.12850	0.00649	0.32
CH_4 (ml)	12.763	12.681	12.525	0.345	0.967
Total VFA	114.64	115.90	116.20	5.25	0.994
Ac/Pr	4.3059	4.244	4.173	0.112	0.907
IVOMD	61.74	63.230	62.062	0.647	0.66

A, potential gas production (ml/gDM)
c, fractional rate of gas production (h-1)
Total VFA, volatiles fatty acids
Ac/Pr : acetate to propionate ratio
IVOMD : *in vitro* organic matter digestibility

The diets did not differ in all of the parameters measured (P>0.05). However, numerically the diets with the highest level of citrus pulp produce the lowest level of CH_4 and had lowest potential of gas production, the opposite situation occurs for the fractional rate of gas production. These results seem to show that at the highest level of inclusion citrus pulp affected to some extent the *in vitro* fermentation.

Figure 2 depicts the *in vitro* gas production profile of the various diets over a period of 72hr of fermentation was similar.

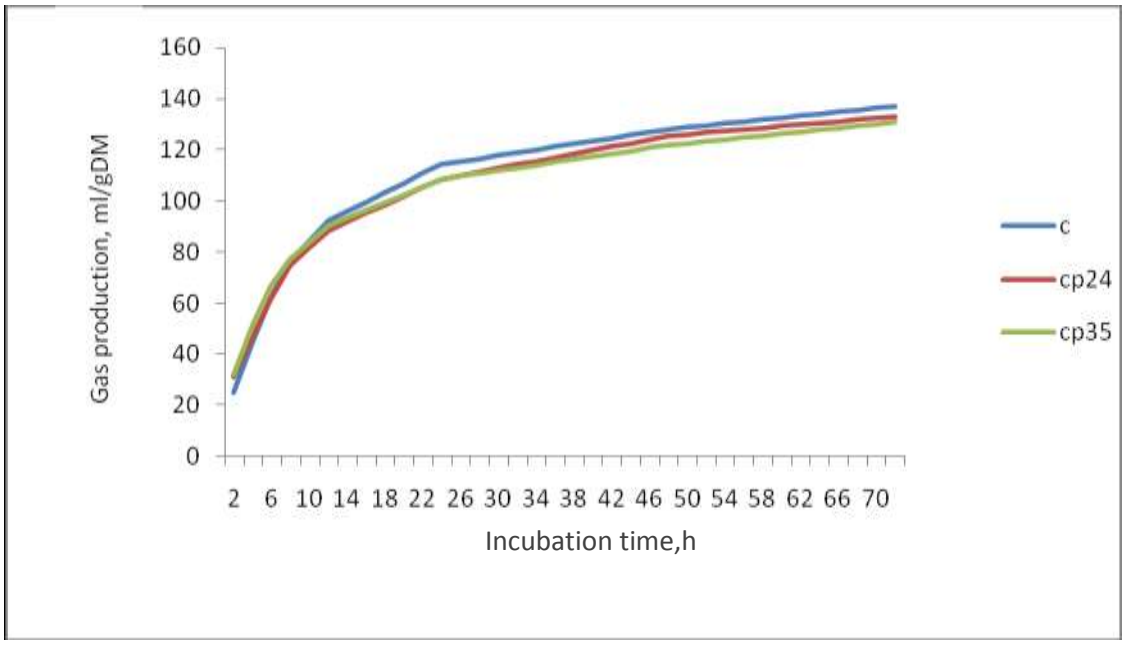


Figure 2: *In vitro* gas production profiles rations with the different levels of citrus pulp in sheep rumen inocula.

2.3- Discussion

2.3.1- Animal performances

Most of the *in vivo* parameters evaluated were similar across all the diets under study. These are indications that at the level of inclusion investigated, the animals fed the Cp24 and the Cp35 diets performed comparably to those fed on a conventional cereal-based diet.

This in concordance to a study of Caparra et al. (2007) that incorporate 30% of solar-dried citrus pulp in concentrate mixtures for fattening Merino lambs without adverse effects both in growth and slaughter performances as well as to Lanza et al. (2001) that replaced cereal grains with citrus pulp with no adverse effect of in vivo and at slaughter performance. Sparkes et al. (2010) carried out an in vivo study in sheep and outlined that the replacement of 30% of a lucerne diet with fresh citrus pulp decreased feed intake but did not affect performance in terms of ADG and wool growth in Merino ewes.

Macías-Cruz et al. (2010) indicated that replacing around 75% of buffel grass hay with fresh orange pulp in diets for fattening lambs resulted in the best growth rate and more efficient diet. Fonesca et al. (2001) reported that supplementation with citrus pulp up to 200f/kg of DM did not depress straw intake.

The DMI and the FE were similar across all diets ($P>0.05$) according to what reported by Pereira et al. (2008) who evaluated the substitution of corn silage by fresh pressed citrus pulp on nutrient intake and the performance of Santa Ines lambs and found that the feed conversion

remained unchanged (4.33 kg DM/kg of weight gain). This is also in agreement with Miron et al. (2002) but differs from the study done by Gilaverte et al. (2011) who reported a depression in FE in goats fed DCP.

The polyphenols in citrus diets were found to be equal to 6.7 and 7.9 mg GAE /100g DM at 24% and 35% level of inclusion, respectively, while in the Control diet polyphenols content was 4 mg TAE /100g DM. It is probable that the level of proteins and energy in the diets were more than sufficient such that the interaction of nutrients-toxins (polyphenols) was not detrimental in agreement with Priolo et al.(2000).

Generally, if ingested in relatively small quantity, polyphenols have a positive effect on nutrient use (Theodorou et al., 2006; Frutos et al., 2004) in ruminants. For example, tannins which form one of the main groups of polyphenols (Kumar et al., 1984; Devendra, 1988) are known to bind with proteins and carbohydrates (Mueller Harvey, 2006) protecting them to some extent from ruminal degradation. This eventually results in better performance in terms of weight gain and milk yield (Waghorn, 1998). However, beyond a certain level the inclusion of polyphenols may abate intake and reduce performance (Frutos et al., 2004) . In the present study, the fact that there were no differences in the performance indicators measured, lead to the inference that DCP ingestion did not disrupt the ruminal digestion either because of the low level of PSCs or due to the adequate level of nutrients in the diets. However, it would be adventurous to extrapolate these outcomes from the present study due to the very variable nature of DCP (Ramful et al.. 2010). The chemical composition of DCP may varies a lot especially in terms of bioactive compounds depending on season, varieties of citrus used and processing method. To our knowledge, considering only Sicily there are several citrus processing industries where DCP can be obtained (Regione Sicilia, 2012) which do not

operate at same scale, being their raw materials different from diverse areas and the processing method. All these, lead to the presumption that the DCP from these sources are likely to differ substantially among them in terms of chemical composition. So, ideally, for each source of DCP, the formulation of the diet should be adjusted.

Another possible hypothesis that can be put forward for the lack of any effect of DCP diets compared to the cereal based diet, is the difference in the fibre content. In a concentrate based diet as in the present trial, the role of fibre is important as such diets generally contains high level of fermentable sugars and highly digestible protein and lower levels of fibre if compared to a forage based diet. Fibre is important for ruminal function, it has been widely demonstrated that both the amount and physical form of dietary fibre are important in ruminant rations in order to maintain proper ruminal function, animal health status and product composition (Mirzaei-Aghsaghali et al., 2011)

The above situation may probably be accounted by the lignin and hemicellulose content which were both significantly higher in the DCP diets. Normally, a high fiber content is considered as being "poorer" compared to concentrate (Van Soest, 1982), but in ruminants feeding this argument is not completely true due the effect of the rumen flora which convert fibers into more digestible molecules. High lignin content in a diet is associated with low digestibility since lignin is highly resistant to microbial degradation. Since the DCP ingesting animals were also ingesting more lignin compared to the control a reduction in performances could have been expected

The carcass yield was comparable between all groups (P=0.733). This is in agreement with Rodrigues et al. (2008) who reported that replacement of corn by citrus pulp up to 68.4% of DM doesn't affect the carcass yield and meat characteristics of Santa Ines feedlot lambs.

2.3.2- Ruminal pH & fatty acids profile

Rumen pH did not differ (P>0.05) among the lambs fed on the 3 experimental diets being on average at 6.6. The values of ruminal fluid pH in the current study were optimal for normal rumen fermentation, in agreement with Mould et al. (1983) who suggested that a pH value of 6 to 7 as being optimal for cellulolysis, proteolysis and deamination.The inclusion of DCP was shown to have no effect on rumen pH (6.6) when beef cattle was fed a pelleted form (Villarreal et al. 2006).

In terms of fatty acid profile, an increase of DCP from 24% to 35% of the diet leads to a significant increase in the percentage of VA and C18:2 trans 10 cis 12. This may be attributed to the presence of polyphenols in the citrus pulp which affected to some extent the metabolism of ruminal fermentation and changed the rumen fatty acid profile. The fact that these fatty acids were higher in the rumen of animal ingesting citrus pulp compared to the control is an interesting finding although no changes occurred in rumenic acid and total CLAs but only in the CLA trans 10 cis 12 isomer. The CLAs represent cis and trans isomers of linoleic acid (C18 : 2) with conjugated double bonds, e.g. cis-9. trans-11 C18 : 2 (also called rumenic acid RA and a predominant isomer in meat and milk) and trans-10. cis-12 C18 : 2 .The CLA concentrations in animal products can be increased by nutritional practices that facilitate higher ruminal output of CLAs and vaccenic acid (trans-11 C18 : 1), being the latter

a precursor of cis 9, trans 10 C18:2 synthesis in animal tissues through the action of an enzyme named Δ 9 desaturase (Bauman, 2000). The CLAs have been shown to have beneficial effects especially in term on cancer prevention and reduction of cardiovascular complication (Park, 2009; Kazunori et al., 2013). That is the reason why there have been considerable interest to devise means to increase the levels of CLAs in milk (Nudda et al., 2014) and meat (Badee et al., 2014).

To our knowledge it is the first time that effect of including DCP in diets of lambs on rumen fatty acid metabolism has been investigated. Most studies have predominately rather focused on tannins (Vasta et al., 2009a;2009b; Vasta et al., 2010).

2.3.3- Ruminal microbial ecosystem

The average values of three of the main microbial population evaluated were comparable across all the diets investigated (Table 3). This is an indication that even at a relatively high level of ingestion of PSC's present in citrus diets, there were no effect on the ruminal bacterial population. PSCs have been reported in a wide source of literature as reviewed by Jayanegara et al. (2010) and Makkar et al. (2007) to have bactericidal effect in the rumen, in fact their use have been suggested as effective alternatives to antibiotics is suppressing rumen methanogenesis through their antimicrobial activity. Mathur et al. (2011) reported that citrus pulp contains various active compounds (saponins, alkaloids, flavonoids) and essential oils. In the present study we have found that the total phenolic content can reach up to 79 μg of tannic acid equivalents /g DM.

For example, Hernández et al. (2012) reported that DCP contains saponins at 5g/kg DM and there are several studies that correlated the inhibitor effect on methanogens in the rumen to saponins (Goel et al., 2012; Jayanegra et al., 2010).

For instance, methanogen populations decreased in the presence of *Sesbania sesban* saponins by 78% and fenugreek saponins by 22% in the *in vitro* fermentation media with the rumen liquor collected from cattle (Goel et al., 2008). One possible way in which saponins lead to the above situation has been reported to be through a reduction in the ruminal protozoal population. Rumen methanogens exist on the surface of ciliate protozoa and are responsible for 9- 37% of enteric methane production (Hegarty, 1999; McAllister et al., 2008).

A similar situation arises with flavonoids which is another group of polyphenols, known to have a similar effect on the bacterial population. Patra et al. (2010) reported that flavonoids have a direct effect on methanogens and they may decrease CH_4 production. Oskoueian et al. (2013) applied the *in vitro* technique to test the effect of 5 types of flavonoids in the pure forms at 4.5% of DM on rumen microbial activity and observed that total populations of protozoa and methanogens were significantly suppressed. However, Broudiscou et al. (2000) screened thirteen plant extracts, selected for their high flavonoid content on their action on fermentation and protozoa numbers in outflow fermenter and retained that just 2 among the 13 (*Equisetum arvense* and *Salvia offcinalis*) have an inhibitory action on methane production. This support that PSC's can have different result depending on the context, inclusion levels and the experimental conditions.

Limonene which is the main essential oil found in citrus, was evaluated to only at 48 mg/kg DM in the DCP used in this study. This concentration is at least 100000 times less than what could be expected normally in citrus pulp (Amparo et al., 1998). Therefore, this may explain to some extent the absence of any apparent of DCP ingestion on rumen bacterial population.

Another finding of the present study was that in all the diets, the proportion of the three groups of microorganisms evaluated was unchanged. This is rather unexpected, as DCP contains bioactives namely polyphenols and others which are known to have antimicrobial effects by acting against bacteria, protozoa and fungi (Burt, 2004). The antimicrobial mode of action, either microcidial or microstatic, is considered to come mainly from the potential of their intruding into the bacterial cell membrane to disintegrate membranes structures which causes ion leakage (Bodas et al., 2012). Therefore, by virtue of the different composition of the diets, a difference in the microbial population could have been expected. One possible reason could be the PSCs profile of the DCP used.

Our findings are similar to those of Vasta et al. (2010) but differ with those of Miron et al. (2002) who reported a decrease in ruminal bacterial population when animals ingested forage containing tannins. Inclusion of polyphenols in diets may have interfere with bacterial cell wall (Smith et al., 2004) leading ultimately to a reduction in the population. However, this did not happen in the present study which does imply that the distribution of the various bacterial species remained unaffected. This hypothesis can be illustrated by the study done by Broadway et al. (2012) who evaluated the ruminal bacterial diversity of cattle fed diets containing up to 20 % of citrus pulp pellets (CPP) and found that *Butyrivibrio* and *Carnobacterium* populations increased but in contrast, this was accompanied by linear decline in the population of *Dialister* and *Catonella* with increasing CPP concentrations resulting ultimately in a unchanged total bacterial population .

The sampling of ruminal fluid was done at the end of the trial after that the animals had ingested the same diet for more than 50 days. It may also be probable that, by that time, the ruminal bacteria had got acquainted, achieving an equilibrium whereby the overall bacterial

population returned to normal level but the proportion of the various species was altered but not detected. Makkar (2003) in his review explained the adaptive mechanisms through which the rumen microbes could tolerate and work efficiently in presence of high tannin levels.

Pertaining to the protozoal population, a similar situation as that for bacteria was obtained across all the diets which was not expected. Franzolin et al. (2010) compared the effect of ground grain corn vs. citrus pulp as energy source in diets administered to two different species. On one hand, in cattle, they reported that the diet did not change the total rumen ciliate protozoal population but affected the generic distribution of *Entodinium*, *Dasytricha* and *Charonia.*

On the other hand, in buffaloes the total ciliate population was increased by mixed feed of citrus pulp with urea but the generic distribution wasn't affected. Another recent study undertaken by Amira et al. (2014) showed that *Acacia saligna*, *Opuntia* and *Acacia nilotica* (some indigenous browse species present in large areas of Algeria and known to be rich in phenols) decreased the rumen protozoa by 3.68, 5.59 and 5.34 times respectively.

2.3.4- *In vitro* fermentation

The diets did not differ in all of the parameters measured ($P>0.05$) during the *in vitro* gas production assay. This is consistent with a study on 184 dairy cattle, where no differences were detected on VFA's or ruminal fluid between the animals fed 20.5% of dried citrus pulp or hominy diets (Leiva et al. 2000).

However, these results are in contrast to those reported by Hernández et al. (2012) in a previous trial , where DCP inclusion in goat diet increased asymptotic gas production and the IVOMD. The different results obtained could be explained by different amounts of PSC's in

the citrus pulp in fact in the current study the CP contain up to 79 mgTAE/kg DM while in the study of Hernández et al.(2012) the diet with lowest level of CP contained 36g of phenolics/kg DM of the total mixed rations.

Also Sparkes et al. (2010) conducted an in vitro study and demonstrated that the replacement of 30% of a lucerne diet with fresh citrus pulp improved total VFA yield, increased total gas production and improved IVDMD, while decreasing the production of ammonia, acetic acid and rumen pH. This is probably due to the use of fresh citrus pulp which contains a different amount and type of polyphenols than the DCP used in the present study.

DCP is known to be relatively rich in flavonoids (Levaj et al., 2009) and the former is known to reduce rumen degradability (Oskoueian et al., 2013) .Yaghoubi et al. (2010) reported that increasing doses of flavonoid extracts from propolis linearly reduced total gas production and protozoa numbers. These results confirmed the fact that effects of using a secondary compound as a stand-alone treatment or in natural substrate are not the same, which is mainly because in a natural substrate there are other compounds which may interact with the PSC's under study and thereby providing a diverse result.

2.4-Conclusion

The inclusion of citrus pulp into diets for lambs has been described as an efficient and cheap feeding strategy in the Mediterranean area. The results of this experiment demonstrated that substituting barley by DCP up to some level (35%of fresh weight) did not affect the performances and productivity of the animals. Also, it was found that lambs ingesting DCP had higher Vaccenic Acid in the rumen but the rumen pH was unchanged.

Furthermore, the microbial ecosystem in terms on profile and composition remained the same as compared to the animals ingesting a cereal based diet. This was accompanied by the absence of any adverse effects on rumen fermentation kinetics which could have been expected with high level of ingestion of PSCs.

However, further study is needed to clarify the mechanism by which DCP brought the changes that have been noted and a more comprehensive analysis of PSCs present in the DCP need to be carried out so as to better understand the reason for these changes.

3- Experiment 2

The current study investigated the use of carob pulp in the diet for growing lambs as relevant strategy in devising methane abatement.

Carob (*Ceratonia siliqua* L.) is a multipurpose tree common in Mediterranean areas for centuries, including Sicily. Carob contains high level of sugars (40–60%) but low protein (3–4%) and lipids (0.4–0.8%) (Marakis,1996). Carob pulp is a byproduct of carob pods processing for the production of carob gum, a galactomannan extracted from the seeds, which is a common food thickener and stabiliser (www.feedipedia.com).Carob pulp is relatively easy to find in the Mediterranean region and is used in animal feeding but, it is limited due mainly to the chemical composition (Calixto et al., 1982). Indeed, carob pulp contains high amount of soluble sugars (up to 44% of the DM; Calixto et al.,1982) which makes it relatively palatable but it also has PSCs in particular polyphenols (Silanikove et al., 2006; Priolo et al., 2000). Among the polyphenols class, it known in the literature that carob pods are rich in condensed tannins (Karabulut et al., 2006; Mohamed et al., 2008) which are polyphenolic

compounds of relatively high molecular weight having the ability to form insoluble complexes with proteins and digestive enzymes as well as carbohydrates (Biagi et al., 2010) resulting in the reduction of nutrients digestibility (Kotrotsios et al., 2011). Nevertheless, the presence of tannins in carob pods may have beneficial effects on human and animal health due to other properties, such as antidiarrheal, antibacterial, antioxidant and free-radical scavenging and antiproliferative activity in liver cells (Custodio et al.,2011).

Carob pods have been used in animal nutrition in diets of sheep (Karabulut et al., 2006), lambs (Priolo et al.,1998), rabbits (Gasmi-Boubaker et al.,2008), poultry (Sahle et al.,1992; Ortiz et al., 2004) and pigs (Andres-Elias et al., 2007; Biagi et al., 2010; Inserra et al., 2015).

In this study, the objective was to include a relatively cheap source of a locally available feed in the form of dehydrated carob pulp as a potential substitute to cereals (barley) in view to investigate whether there would be some modification of the ruminal flora and rumen fatty acid profile and evaluate by in vitro digestion the amount of methane produced.

3.1- Materials and methods

Twenty nine lambs were divided into three homogeneous groups, according to their weight (20.3kg ±4.4 kg (s.e.)) and randomly assigned to three experimental diets. The control group (C) was fed a total mixed concentrate diet consisting of barley, lucerne hay, wheat bran and soya bean meal that were coarsely ground. Two groups received a mixed concentrate with the same ingredients as in control group but with the addition of different proportions of carob pulp (24% and 35% on an as fed basis respectively for Ca24 and Ca35 groups). The diets

were formulated in order to supply an equivalent crude protein allowance. The ingredients and chemical composition of the diets are shown in Table 1.

Table 1: Ingredients and chemical composition of the diets (C,Ca24 and Ca35 groups)

	Dietary treatments		
	C	**Ca24**	**Ca35**
Ingredients (g/100g as fed)			
Barley	60	33	23
Carob pulp	0	24	35
Dehydrated lucerne	20	20	17
Soya bean meal	9	13	16
Wheat bran	11	10	9
Chemical composition			
Dry Matter (DM; %)	88.92	88.25	87.79
Crude Protein[1]	18.00	19.06	19.20
Neutral Detergent Fiber (NDF)[1]	34.6	34.4	34.6
Acid Detergent Fiber (ADF)[1]	13.7	18.0	22.7
Acid Detergent Lignin (ADL)[1]	8.3	10.9	11.4
Ether Exract[1]	2.23	2.46	2.52
Total Phenols [2]	8.6	14.2	16.6
Total Tannins[2]	1.6	3.4	4.5

[1]expressed as g/100g of DM

[2] expressed as g of tannic acid equivalents/kg DM

All the *in vivo* animals management, animal slaughter, sampling, laboratory analysis and *in vitro* gas production assay were performed as described in details in experiment 1. As both experiments were conducted in parallel for practical reason.

For the analysis of total phenols in the feed, samples were first treated as described by Makkar et al. (1993) with minor modifications. Briefly, 200 mg of finely ground feeds was extracted with 5 mL of diethyl ether containing 1% acetic acid to remove pigments and the supernatant was discarded. For extraction of total phenolic compounds, 10 mL of 70% (v/v) acetone was added and samples were subjected to ultrasonic treatment for 30 min in a cold water bath.

Samples were then extracted for 2 h using a rotating device and then centrifuged at 2500 x g for 10 min at 4°C. The supernatant was collected for subsequent analyses. The above extraction procedure was repeated and the supernatant collected. The residue from the acetone extraction was subjected to a further extraction using a modification of the method described by Silanikove et al. (2006). Briefly, 9 ml of citrate-phosphate buffer containing 0.5 mg/ml of urea (pH 4.7) was added to the residue and samples were incubated at 90°C for 2h. A clear supernatant was obtained by centrifugation at 2500 x g for 20 min.

In all the above extracts, total phenols were determined using the Folin-Ciocalteau reagent. The concentration of total phenols in feeds was calculated as the sum of the concentration measured in each extract. The assays were calibrated using standard solutions of tannic acid (TA) and results were expressed as mg of TA equivalents/g of feed (on a dry matter basis).

3.2- Results

3.2.1- Animal performances

The effects of the diets on total feed intake, performance and nutrient and ingredient intake date are presented in table 2.

Table 2 : Performance and intake of lambs fed on Control (C) diet or two carob pulp based diet (Ca24 and Ca35).

	C	Ca24	Ca 35	SEM	P
Performances					
DM Intake (g/d)	753.6	871.1	802.9	36.7	0.455
Average Daily Gain (ADG)	184.6	195.5	167.2	10.7	0.578
Initial weight	19.38	19.40	18.13	0.866	0.807
Final weight	29.91	30.54	27.66	1.34	0.676
Feed Efficiency (FE)	0.2580	0.2376	0.2154	0.0088	0.145
Carcass weight	12.99	12.98	12.07	0.628	0.807
Carcass yield	43.279	43.551	43.14	0.422	0.776
Nutrient Intake					
Crudeprotein[1]	135.91	170.9	154.4	7.43	0.159
NDF[1]	260.9	299.5	277.7	12.6	0.480
Hemicellulose[1]	157.5a	135.89a	95.32b	7.85	0.001
Cellulose[1]	40.84c	68.54b	91.00a	5,76	0.000
ADL[1]	62.54b	95.11a	91.34a	4.97	0.006
Ether Extracts[1]	16.8	21.40	20.24	0.964	0.126
Total Phenols2	30.14b	45.79a	45.86a	2.48	0.005

[1]expressed as g/100g of DM

[2] expressed as g of tannic acid equivalents/kg DM

No differences on dry matter ,intake, average daily gain, initial and final body weight, feed efficiency between animals fed the control diet and the other groups fed with the carob diets. Similarly, no differences ($P > 0.05$) were found among treatments for the performance parameters measured at slaughtering. Results of nutrient ingestion were similar in terms of protein and NDF but different for the fiber fraction, whereby lambs that ate carob pulp showed a lower hemicelluloses intake. The Control lambs had the lowest cellulose and ADL intake. Also there was a significant difference between the total phenols ingested by the control group compared to the Ca24 and Ca35 ($P= 0.0005$).

3.2.2-Rumen pH and fatty acid profile

Table 3 reports the pH and fatty acid profile of ruminal fluid of sheep that received the control and the carob containing diets. No dietary effect on pH or fatty acid profile except for the concentration of stearic acid ($P<0.05$), which was present at lower concentration in the ruminal fluid from lambs fed the Carob35% diet as compared to the Control treatment.

Table 3 : pH and fatty acids (% total fatty acids) in the ruminal fluid of lambs fed on Control (C) diet or two carob pulp based diet (Ca24 and Ca35).

	Diets				
	C	**Ca24**	**Ca35**	**SEM**	**P-value**
pH	6.61	6.73	6.53	0.0499	0.234
C13:0	0.34	0.61	0.632	0.0698	0.181
C14:0	0.97	0.95	0.63	0.09	0.389
C14:0 *iso*	0.36	0.57	0.63	0.0527	0.086
C15:0	0.86	0.94	0.93	0.0689	0.865
C15:0 *iso*	0.29	0.37	0.28	0.0275	0.311
C15:0 *ante*	1.22	1.08	0.91	0.0814	0.31
C16:0	18.18	17.94	17.28	1.03	0.939
C16:0 *iso*	0.606	0.85	0.79	0.0814	0.467
C16:1	0.19	0.22	0.36	0.0745	0.651
C17:0	0.55	0.44	0.5	0.0301	0.401
C17:0 *iso*	0.87	1.33	1.59	0.163	0.191
C17:0 *ante*	0.65	0.64	0.8	0.0874	0.735
C18:0	38.28a	29.68ab	26.39b	1.88	0.019
Σ*iso* BCFA	2.14	3.13	3.29	0.243	0.105
trans-5 C18:1	0.043	0.048	0.061	0.00892	0.723
trans-6 to *trans*-8 C18:1	0.45	0.654	0.653	0.0703	0.407
trans-9 C18:1	0.24	0.27	1.15	0.252	0.253
trans-10 C18:1	4.72	8.24	6.49	1.44	0.63
trans-11 C18:1	1.5	3.06	3.6	0.454	0.138
trans-12 C18:1	0.53	0.43	0.46	0.029	0.326
cis-7 C18:1	0.89	0.61	0.6	0.0732	0.18
cis-9 C18:1	3.47	3.47	4.51	0.337	0.366
cis-11 C18:1	0.54	0.49	0.54	0.0324	0.83
cis-12 C18:1	0.57	0.4	0.27	0.0582	0.095
cis-15 C18:1	0.35	0.33	0.35	0.0207	0.928
cis-9, *trans*-11 C18:2 CLA	0.89	1.05	1.03	0.164	0.926
trans-10, *cis*-12 C18:2 CLA	0.24	0.3	0.41	0.0848	0.708
ΣCLA	1.14	1.35	1.45	0.159	0.733
cis-9, *cis*-12 C18:2 n-6	3.67	1.86	1.7	0.53	0.254
trans-11, *cis*-15 C18:2	0.06	0.27	0.12	0.0649	0.418
cis-9, *cis*-12, *cis*-15 C18:3	0.43	0.18	0.41	0.086	0.424
ΣSFA	59.18	50.57	46.42	2.33	0.067

ΣMUFA	13.32	18.04	18.74	1.33	0.199
ΣPUFA	4.16	2.32	2.24	0.545	0.276

[1]Σ*iso* BCFA = C14:0 *iso*+ . C15:0 *iso*+C16:0 *iso* + C16:0 *iso*

[2]Σ SFA = C13:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0

[3]Σ MUFA = *trans*-5 C18:1+ *trans*-6 to *trans*-8 C18:1+ *trans*-9 C18:1+ *trans*-10 C18:1+ *trans*-11 C18:1+ *trans*-12 C18:1+ *cis*-7 C18:1+ *cis*-9 C18:1+ *cis*-11 C18:1+ *cis*-12 C18:1+ *cis*-15 C18:1

[4]Σ PUFA = *cis*-9. *cis*-12 C18:2 n-6+ *trans*-11. *cis*-15 C18:2+ *cis*-9. *cis*-12. *cis*-15 C18:3

3.2.3- Rumen microbial ecosystem

The main microbial population present in the rumen as identified by qPCR is shown in the table 4. The total Bacteria and Protozoa population were similar between the animals ingesting control or the two carob-supplemented diets. However the Archaeal population was numerically lower in the ruminal fluid from lambs in the Carob35% group when compared to the control, although the ANOVA did not show a statistically significant effect of the dietary treatment (P=0.139).

Table 4 : Rumen microbial population of the animals ingesting the control and the two carob diets

	C	**Ca24**	**Ca35**	**SEM**	**P value**
Total Bacteria[1]	11.213	11.179	11.052	0.0494	0.392
Archaea	9.2388	9.2978	8.919	0.0838	0.139
Protozoa	5.056	5.821	6.089	0.284	0.323

[1] log10copies/gFM

3.2.4-*In vitro* fermentation

The *in vitro* ruminal fermentation parameters (A, c,CH_4, Total VFA, Ac/Pr and IVOMD) of the control and carob based diets are shown in Table 5. The diets did not differ in all of the parameters measured (P>0.05).

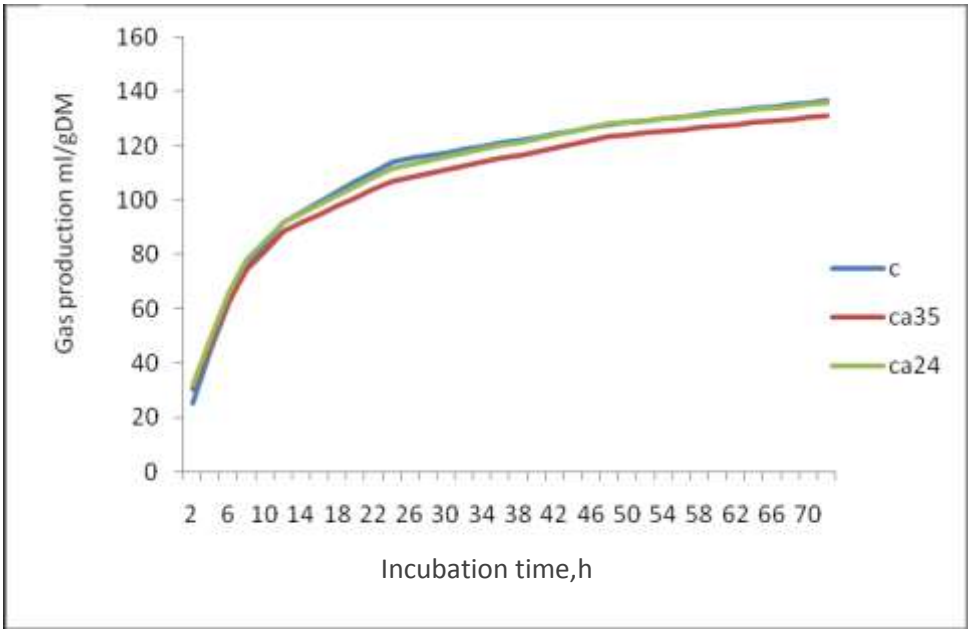
Table 5 : *In vitro* fermentation characteristics of the control and the diets with the different level of carob pulp

	C	Ca24	Ca35	SEM	P value
A	131.07	129.22	124.6	5.79	0.913
c	0.10432	0.11663	0.11783	0.000655	0.70
CH4 (ml)	12.763	13.028	12.358	0.573	0.909
Total VFA	114.64	113.58	113.75	4.74	0.996
Ac/Pr	4.3059	4.1232	3.9595	0.129	0.576
IVOMD	61.74	64.603	62.458	0.639	0.228

A,potential gas production (ml/gDM)
c, fractional rate of gas production (h-1)
Total VFA, volatiles fatty acids
Ac/Pr : acetate propionate ratio
IVOMD : *in vitro* organic matter digestibility

Figure 1 depicts that *in vitro* gas production profile of the various diets over a period of 72hr of fermentation was similar.

Figure 1 : *In vitro* gas production profiles rations with the different levels of carob pulp in sheep rumen inocula



3.3- Discussion

3.3.1- Animal performances

Dry matter intake (DMI) was not significantly affected by the dietary treatment as well as crude protein and NDF ingestion (Table 2). However, a different nutrient intake has been obtained for fibre fractions: the Ca35 group showed significantly ($P<0.05$) lower hemicellulose intake compared to Control and Ca24 groups. Lambs in Control group showed the lowest level of daily cellulose and ADL intake (Table 2). Total phenols consumption was also affected by the experimental diet, as expected, showing the lowest values in Control lambs compared to Ca24 and Ca35 ($P<0.05$).

Regarding animals' performance, none of the parameters measured during the *in vivo* phase and at slaughtering were affected by the experimental diets (Table 2). This implies that no

detrimental effect on animal performance indicators has been obtained even at the highest value of carob pulp inclusion. This is in line with the findings of Priolo et al. (1998) who observed that the inclusion of 20% of carob increased feed intake did not affect animals growth and worsened feed efficiency. Similarly, Obeidat et al. (2011) observed that replacement of barley grains by carob pods up to level 250 g/kg in fattening diets of Awassi lambs did not affect nutrient intakes, carcass characteristics and meat quality parameters. However, Silanikove et al. (2006) who used carob pods at level 520 g/kg cause the reduction in growth performance in Anglo-Nubian kids. The inconsistency of the different studies that contained carob pods could be related to differences in the diets, level of PSC's (especially tannins) and/or level of feeding. Waghorn (2008) explained that the impact of tannins upon ruminant performance depends on the amount and astringency in the diet, animal nutrient requirements and other dietary components. In this study, it has been observed that even at the level of 35% carob pulp allowed to maintain a level of growth and carcass weight and yield comparable to animals fed on conventional diet probably due to the high sugar content in carob which offsets the detrimental effect of tannins by allowing a sufficient amount of energy.

3.3.2. Ruminal pH & fatty acid profile

Feeding the highest level (35%) of carob pulp to lambs reduced the concentration of C18:0 in ruminal fluid ($P=0.019$) compared to Control and had no effect on the other rumen fatty acids or ruminal fluid pH.

A possible explanation for this effect is the highest content of polyphenols in the carob diets compared to the control one. A similar result was reported by Vasta et al. (2009a) who

reported that supplementation with tannins (4% of DM) did not affect rumen pH and significantly reduced the stearic acid concentrations (-49%). However in another study by Vasta et al. (2010), SA concentrations in the rumen was not affected by tannin supplementation. The same study of Vasta et al. (2009a) indicate that tannins affect biohydrogenation pathway by increasing rumenic acid and PUFA content and could be a useful strategy to reduce SFA in ruminants meat. This was not observed in the current study as the concentrations of these fatty acids were similar between the different diets. It probably due to the fact that carob pulp contains not just tannins but a mix of PSC's that affect in a different way the rumen metabolism and therefore the end products.

3.3.3- Rumen microbial ecosystem

The average value of three of the main microbial population evaluated were similar across all the diets investigated (Table 4). The level of the diverse microbial populations are similar to those as reported by Vasta et al. (2010) who reported a bacterial population of lambs are around 10 log 19 copies/g of FM and the protozoal was estimated to be 5 log 19 copies/g of FM. Pertaining to the protozoal population, a similar situation as that for bacteria was obtained across all the diets which was not expected.

So far there is no available literature about the effect of dietary carob pulp on the rumen metabolism and its microbial ecosystem. However, an early work by Tagari et al. (1965) demonstrated the inhibiting effect of carob pod extract in an artificial rumen on the cellulolytic and proteolytic activities of rumen microorganisms. Also, Henis et al. (1964) found an antimicrobial effect of carob pod tannin extract on *Cellvibrio fulvus* (a celluloytic bacterium) *in vitro*; that resulted in morphological changes indicating tannin effect on this bacterium. On the other side literature is rich in information on the effects of tannins on

ruminal microbial ecosystem. This group of polyphenols has been studied in different laboratories for the antimethanogenic effect both *in vivo* and *in vitro* conditions (Bhatta et al.,2013, 2009; Patra et al., 2009).

For example, alteration in gut microbial population was demonstrated in rats fed condensed tannins at 20 mg/kg diet and there was a shift in fecal microbial population favoring *Enterbacteriaceae* and the *Bacteroides* species (Smith et al., 2004). In ruminants, tannins inclusion in the diet is reported to either increase protozoal population in lambs (Vasta et al.,2010) or causes a depression (Miron et al., 2002). Tavendale et al. (2005) proposed two modes of action of tannins on methanogenesis: a direct effect on ruminal methanogens (methanogens who refer to the group of bacteria that involved in the pathway of the reduction of H_2 to CH_4) and an indirect effect on hydrogen production due to lower feed degradation. Abdalla et al.(2012) evaluated the effect of tannin-rich plants on *in vitro* CH_4 formation and attributed the effect to a decrease in fermentable substrate rather that a direct effect on methanogenesis. Tavendale et al. (2005) first demonstrated the inhibitory effect of tannins on the growth of a pure culture of rumen methanogen. This study suggested that low molecular weight tannins could be a more effective inhibitor of microbes including methanogens compared with high molecular weight tannins because low molecular weight tannins could form strong binding with microbial enzymes. and that the action of condensed tannins (CT) on methanogenesis may be attributed to the direct inhibitory effect on methanogens depending upon the chemical structure of CT and methanogen species. Also tannins have been shown to lower protozoal numbers (Patra et al.,2009) which may also decrease protozoal-associated methanogenesis. But still little information available showing the direct influence of tannins on rumen methanogens.

However in this study a natural product was used that is carob pulp instead of an only one secondary compound like tannins makes that interpretation of above observations more difficult to account for. Carob pulp and the other ingredients used in the formulation of the various diets.ie. soya bean, dehydrated Lucerne, wheat bran and barley, in the end makes a range of many diverse types of biochemicals namely carbohydrates, fats, proteins, PSCs, minerals (Rao et al., 2013) etc resulting in the fact that animals ingested a mix of substances that alone or together can have differential potential effects. The effects may be categorized into three main types, i.e. antagonistic, synergistic and complementary. For example, Makkar et al. (2005) revealed that the effects of both tannins and saponins on decrease in apparent and true digestibilities and gas production were additive.

Provenza et al. (1992) speculated that because of the very complex nature of the diverse metabolic processes that occur in particular in ruminants it is difficult to predict which exact effect a particular mixture would have in a given context. Thus, it is probable that in the present study although polyphenols was assayed, this fact alone is not sufficient to elucidate what has happened to the microbial population.

In fact, the nutrient level in terms of energy and protein can explain the above results. The measured performances indicators show that all the animals requirements in terms of growth and maintenance were satisfied and therefore it is highly probable that the superfluous amount of protein and energy interact with PSCs mechanisms leading to a compensation effect. So, on one hand polyphenols abate microbes population and on the other hand, nutrients availability especially sugars (Makkar,2003) favoured the microbial growth . This may account for the absence of any net effect on the microbial population .

3.3.4- *In vitro* gas production

The diets did not differ in all of the parameters measured (P>0.05) during the *in vitro* gas production assay (Table 5).

To our knowledge there are no studies that evaluated the rumen fermentation kinetics of a carob containing diet. Karabulut et al. (2006) applied the gas production assay to different fractions of carob pods (whole pods, seeds and carob kibbles i.e; the remaining pulp obtained after removal of the seed). It reported that the *in vitro* gas production of seed was lower than those of whole pods and kibbles and this is possibly due to high cell wall and low sugar contents of carob seed. Starch and sugar contained in carob byproducts are quickly available for microbial fermentation (higher production rate c).

More information is available about the effect of tannins on fermentation kinetics. Silanikove et al. (2006) evaluated the metabolites in rumen fluid on day 15 of the experiment in kids fed a control diet or diets containing carobs with or without PEG, and reported that no differences on rumen pH or total VFA's but a change in the VFA's profile by an increase in butyric and caproic acids concentrations.

Another interesting work has been done by Krueger et al. (2010) who examined the effects of source of tannin (condensed,CT vs. hydrolysable, HT) on ruminal fermentation parametersand outlined that there was no effect of tannin supplementation on rumen pH on the molar proportions of acetate and propionate as well as on the acetate:propionate ratio or the total concentration of VFA. Vasta et al. (2009b) incubated tannins extracted from carob, acacia leaves and quebracho in glass syringes and reported that VFA production was not affected by the different types of tannins used. This is not consistent with previous studies that have examined the effects of tannins on fermentation parameters in various species; Makkar et al.

(1995) found that both CT and HT tannins decreased VFA production *in vitro* when added at 0.8mg/mL of medium but that CT decreased VFA production to a greater extent than HT.

The different results obtained using different tannins could be attributed to their nature, structure or activity and to the concentrations at which they were used.

Under the conditions reported here, the lack of effect of dietary supplementation on the ruminal fermentation can be explained by the fact that PSC's could have diverse effect when is tested as sole substrate or a part of plant or more as an ingredient in a complex diet.

Nevertheless, the *in vitro* studies give insight into the mechanism of action of various compounds, their comparative effects and possible *in vivo* effects.

3.4- Conclusion

This research has demonstrated that feeding lambs up to 35% of carob pulp did not modify both the rumen microbial ecosystem and ruminal fermentations parameter and did not affect the performances and productivity of the animals.

Also, it was found that lambs ingesting the highest level (35%) of carob pulp had lower concentration of C18:0 in ruminal fluid, and no changes occurs on the other rumen fatty acids or ruminal fluid pH.

Overall, the level of CH_4 emitted was unchanged and to our knowledge this is the first time that the use of carob pulp in this respect has been carried out. Therefore, this study should be considered as a starting point to investigate in details the kinetic and mechanism in which carob pulp is involved in methanogenesis so that means that its use would be economically and ecologically sensible.

4- Consolidated conclusions

The results obtained in this thesis highlight interesting points that pave the way for further research. The use of locally available feed resources has great potential for improving animal production and reducing the carbon food print of livestock systems.

Dietary strategies are reliable CH_4 mitigation technologies that are feasible in real life situation and economically viable while improving ruminant production and thereby are the most recommended methods. It is a mean that can be relatively easily adopted by farmers and may be implemented on large scale (given that genetic selection/breeding program cannot yet be implemented at farm levels). Future research works shall focus on identifying plants that may have methane abatement potential with the objective of incorporating them in livestock diets. Besides these scientific considerations, the present doctoral study have enable me to hone my skills in scientific literature search, improve my aptitude to plan, organize and implement experimental trials and develop my abilities to work and integrate within a multidisciplinary group. I have also learnt through on-hand experimentation in lab works particularly in the fields of genetic screening and *in vitro* fermentation.

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