A Valeria,
"Ti daranno la possibilità di scegliere
tra affondare e galleggiare.
E tu scegli di volare"

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Forage Provided By Mediterranean Area And Preserved As Hay: Assessment Of Nutritional Quality And Eco-Sustainability

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Abbreviations

a.s.l.	at sea level
ADF	acid detergent fiber
ADL	acid detergent lignin
CF	crude fiber
CH_4	methane
CO_2	carbon dioxide
CP	crude protein
dCH_4	methane production related to degraded organic matter
DM	dry matter
DMVPA	Department of Veterinary Medicine and Animal Production
dOM	organic matter degradability
EE	ether extract
FAO	Food and Agriculture Organization of the United Nations
GC	gas chromatography
GHG	greenhouse gases
GP24	gas production registered after 24 hours of incubation
H_2	hydrogen
iCH_4	methane production related to incubated organic matter
IVGPT	*in vitro* gas production technique
N_2O	nitrous oxide
NDF	neutral detergent fiber
NSC	non-structural carbohydrates
OM	organic matter
OMCV	cumulative volume of gas related to incubated OM
pCH_4	methane production as percentage of total gas
PUFA	polyunsaturated fatty acids
MUFA	monounsaturated fatty acids
CLA	conjugated linoleic acid
SF6	sulphur hexafluoride
SFA	saturated fatty acids
UFL	Unité Fourragère du Lait
VFA	volatile fatty acids
Yield	cumulative volume of gas related to degraded OM

Summary

The thesis is organized in six chapters: a general introduction, four experimental contributions and a main conclusion. The general purpose was to characterize the forages preserved as hay made in some regions of the Mediterranean area (Southern of Italy) and utilized as forage basis in the feeding plan of dairy cow bred to produce dairy products of high dietetic and nutritional quality. In particular, sensory evaluation, chemical composition, *in vitro* fermentation characteristics and kinetics, and methane production were determined.

In the general introduction (Chapter 1.), the importance to evaluate the quality of hay, the principal fibre source in ruminant diets of temperate climate area is described; this aspect is more relevant when hay represents a significant fraction of the diet. The hay quality is very variable, mainly due to the characteristics of the starting forage and the growth stage at cutting, but also climate condition at harvesting and storage phases influence it consistently. The most reliable approach for evaluating the quality in hay is a combination of sensory, chemical and biological examination. The high quality of hay in ruminants' nutrition guarantee high quality of meat and milk resulting; moreover, the use of forage elevated in value can limit the environmental impact of livestock, mainly due to the lower methane production, because the larger digestibility of the fiber fraction.

In the first contribution (Chapter 2.), a database containing information on mixed hays (No. 56) produced in different agricultural farms of Southern of Italy and utilized in the diets administered to dairy cow is

reported. The general propose was to characterize the local resources and, if possible, to identify some critical points in the production steps in order to improve their quality. A new method to evaluate the sensory characteristics of hay is proposed. A cluster analysis was used to assess whether distinct groups could be created on the basis of chemical constituents. The sensory evaluation system proposed has demonstrated to be a useful and practical tool to judge the hays; data obtained are often in line with the analytical characteristics determined in the laboratory. All the hays sampled were also tested with the *in vitro* fermentation, including methane production. Overall, the hay produced in the farms of the Mediterranean Area considered are characterized by a medium quality due to the low crude protein level, the high structural carbohydrates content, the low leafiness, but the good softness. The sensory evaluation system proposed has demonstrated to be a useful tool for assigning a first quality judgment at the hay, which is often in line with the analytical characteristics determined in the laboratory.

In the second study (Chapter 3.), eight hays produced in four farms located in four Provinces of Southern of Italy, different for environmental condition, were characterized for sensory evaluation, nutritive value and *in vitro* fermentation characteristics and kinetics, including methane production. The differences observed between the farms evidenced that the forage produced in Avellino area is the most interesting in terms of chemical composition, nutritive value, *in vitro* characteristics and environmental impact. Data obtained allows having more information

about forages produced in the study area, useful for farmers to make balanced rations to maintain animal health and guarantee high level of production.

The objective of the investigation, reported in the third contribution (Chapter 4.), was to evaluate the environmental impact, in terms of *in vitro* methane production, of forages sampled over a 3-year period of collection provided by a farm located in Southern of Italy and preserved by haymaking. The substrates to test were incubated *in vitro* for 24 h with cow rumen fluid under anaerobic condition. The methane was measured by gas-chromatography and estimated with stoichiometric calculation. The results evidenced that the climatic condition can influence forages quality.

In the fourth contribution (Chapter 5.), the effect of haymaking in three mixed forages, on the *in vitro* fermentation characteristics, including methane production was studied. The hypothesis was that the *in vitro* gas production technique could point out the changes due to the preservation method. The fermentation characteristics and kinetics were studied with the *in vitro* gas production technique utilizing a manual system and cow rumen fluid as *inoculum* source. Methane production was measured after 24 h of incubation by gas-chromatography. As results, a worsening of the *in vitro* fermentation characteristics emerged as direct consequences of haymaking process. The methane production also increased moving from fresh forage to hay.

Summary

In the last chapter, some final considerations derived from the results obtained in the four experimental contributions are reported.

Chapter 1.

General Introduction

The importance of hay quality in ruminants' nutrition to guarantee high quality of animal products and low environmental impact

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ABSTRACT

The evaluation of hay quality, the principal fiber source in ruminant diets of temperate climate area, is very important, mainly when it represents a significant fraction of the diet. The hay quality is very variable, mainly due to the characteristics of starting forage and growth stage at cutting, but also climate condition at harvesting, soil fertilization and storage phases influence it consistently. The most reliable approach for evaluating the quality in hay is a combination of sensory, chemical and biological examination. The high quality of hay in ruminants' nutrition guarantee high quality of meat and milk resulting. The use of high quality forage in ruminant diet can limit the environmental impact of livestock, mainly due to the lower methane production, because the most digestible fiber.

Keywords: *milk and meat quality, fatty acid profile, sensory evaluation, methane.*

INTRODUCTION

Choosing or producing high quality forage to utilize in ruminant diets means obtain animal products of high nutritional quality, respect animal physiology (i.e., rumen functionality) preserving their welfare and limit environmental impact mainly in terms of greenhouse gas (GHG) emission. Moreover, the utilization of high quality forage is also one of the most important economic aspects for livestock managers: any nutrient not given by forage has to be complemented by concentrates, which increase

the whole cost of the ratio. These considerations are even more important if, as in the case of the Latte Nobile™ (Rubino, 2014) the amount of forage included in the ratio must represents a substantial fraction (at least 70%) as reported by the production disciplinary (www.lattenobile.it).

In temperate areas, in order to guarantee a forage basis constant ingredient in the diet during the year, most of the forage portion in the dairy cow diets is represented by dried forage, administered to the animal as hay. Hay is the oldest, and still the most prevalent, conserved fodder, despite its dependence on suitable weather at harvest time. Hay is a grass crop, which is cut, harvested, and stored. Using the proper equipment and accurate storage techniques, these methods keep the nutritive value of the starting forage and limit the losses for the crop. Haymaking involves reducing the moisture content of cut herbage in the field from 70–90% to 15–20%; in theory it is very easy, but very difficult in practice depending on weather condition, and demanding skill and judgment from the farmer (Suttie, 2000; www.fao.org). However, the quality of obtained product can be of very variable, due to the characteristics of the starting forage, or to the storage phases not properly carried out.

The present review is focused on the importance of hay quality. After mentioning the role of the fiber in ruminants' nutrition, mainly bring by hay, the methods to evaluate the quality of hay, in terms of sensory aspects, nutrient availability and environmental impact, are reported. Subsequently, the more recent studies regarding the influence of hay

quality administered to ruminants on the quality of their products (i.e., milk and meat) is mentioned. Finally, some arguments are presented concerning the hay quality and GHG emission, with particular regards to methane.

ROLE OF FIBER IN RUMINANT NUTRITION

Ruminants, due to the enzymatic activity of the microbial population colonizing the rumen, are able to hydrolyse the structural carbohydrates (mainly cellulose and hemicellulose) contained in the forage (commonly defined fiber); from this fermentation mainly three volatile fatty acids derived (acetic, propionic and butyric acids) that, pass through the wall of the rumen, through the blood coming to the liver, where they are metabolized and represent the main energy source for the host animal. The presence of fiber in the diet of ruminants, especially if structured and coming from forage, also has an important role in stimulating chewing, which guarantees an copious saliva production, whose buffer substances (i.e. sodium bicarbonate) regulate rumen pH and motility. The ideal value of the forage:concentrate ratio (F:C) is 70:30 in medium-low milk production cows. This condition is optimal for this type of dairy cow, rumen pH is established between 6.8 and 6.2 and the production of the volatile fatty acids (VFA) is in perfect balance (acetate: 60–70%; propionate: 15–25%; butyrate: 10–20%). Propionic acid allows the necessary production of glucose, which guarantees the amount of milk production, while acetic acid promotes the milk fat synthesis and

positively influences the health general state of animal. Therefore, the percentage of fat in milk is strongly influenced by F:C (McDonald et al.; 2010). When a diet is low in forage, in favor of a high proportion of concentrates the conditions can be unfavorable for growing and development of cellulolytic bacteria, necessary for structural carbohydrates digestion, for the benefit of other amilolytic bacteria, which fermenting the starch contained in concentrates.

In intensive livestock production system, ruminant diets are characterized by high proportion of concentrate (also 50%), mainly represented by cereals. The increasing starch amount ingested by the animal affects consistently rumen pH (on average < 6.0) and maximum is the propionic acid production (25% of total VFA). The high propionic acid production outcomes in a elevate glucose production and subsequent in the deposition of adipose tissue, a positive phenomenon for meat production, but not in dairy cows, where missing the acetic acid, precursor of the short chain fatty acids, a decrease fat content of milk is observed. If the amount of concentrates administered further increases, the rumen pH is further lowered, reaching values comprised between 5.0 and 5.5, due to the rise of lactic acid. In this condition, pathological consequences are generated: rumen, and then metabolic, acidosis, excess of hematic lactic acid, adverse liver and kidney effects, negative impact on the general health state even at the expense of the productions quality. However, such circumstances if properly controlled favor the increase of milk

production and weight gain, reason why the breeders adopt F:C ratio in favor of concentrates.

WHAT IS THE MEANING OF QUALITY IN HAY?

The most reliable approach for evaluating the quality in hay is a combination of sensory, chemical and biological examination (Grazioli et al., 2016). Using these different systems, it is possible to characterize hay for sensory quality (i.e., color, odor, presence of powder, texture, etc.), nutrient contents (i.e., dry matter, fiber, protein, lipids content and quality, mineral and vitamins) and nutritive value (energy content, digestibility, palatability, intake). However, for the sensory evaluation, it is necessary to have sensory skills to make sure the forage evaluation reflects the actual quality of the hay. The chemical analysis is not always feasible or cost effective. The biological analysis, including *in vitro* or *in vivo* approach that use animal or material came from animal (i.e., rumen fluid), are not always feasible in terms of laboratory work, financial commitment and ethical issue.

Sensory evaluation

This kind of assessment is useful because perform directly in the field, but it is a subjective analysis and needs of some experience for a correct evaluation. Factors related with sensory evaluation include stage of maturity, leafiness, color, odor and dustiness (Taylor, 1998).

Stage of maturity refers to the growth stage of a plant at harvest. Both in grasses and legumes it is easy to detect in the field, but in the hay its detection becomes more complicated especially if certain weather conditions delay normal development of plant parts. This aspect will largely discuss later.

Leafiness is important since the majority of digestible nutritive characteristics (i.e., energy, protein, minerals and vitamins) are placed in the leaves of the plants. Leafiness is influenced by harvest and handling methods and stage of maturity. The leafiness changes also depending botanical family, *Graminae* or *Leguminosae*: grass plant has a single long and lanceolate leaf, well anchored to the stem, on the other hand legume plant has many more leaves attached to the stem by a small petiole. In this case, during haymaking, especially due to an excessive drying is easy to miss leaves with consequential lost of nutritional value. Depending on the care of handling and how the hay is administered, detached leaves may or may not be utilized by the animal eating the hay. If the hay is ground and administered in a total mixed ration or fed in a manger, most all of the leaves could be utilized. However, if the hay is fed on the ground, most of the detached leaves will drop and be wasted.

Also color and odor are indicators of hay quality. A bright green color usually suggests that the plant was cut at a relatively early stage of maturity, rapidly and properly cured, with no damage from rain or overheating during storage. There are different reasons why hay loses its green color, some more detrimental to quality than others. Over mature

grasses often have stems and seed heads that are golden yellow to yellow. A pleasant odor indicates hay is been stored properly, while musty odors may occur when the hay is stored at moisture contents above 16 to 18%. Hay that is been damaged by rain will change color in dark brown and promotes the growth of mold on leaves and stems resulting in a significant reduction in quality. The exposure to sunlight will also bleach hay. A fresh aroma, free of mold, is often associated with green, well-cured hay, which is generally more palatable.

Regarding texture, softness usually results from early cutting, high leaf content, and a suitable moisture level at baling. When hay is “very soft” and pliable, it is difficult to distinguish between stems and leaves; “soft” hay is downy to the touch, but stems can be simply identified; “slightly harsh” hay has stems that are a little rough. “Harsh” hay is dry and unpleasant to the touch; “extremely harsh” hay can harm an animal’s mouth and decrease intake (Bal et al., 2001).

At visual inspection, extraneous substance can be detected (i.e. dust, sticks, rocks, wire, etc.), which represents something obviously undesirable and sometimes even harmful to the animal. Dustiness can reduce palatability and intake indicating quality problems often associated with rain damage or poor storage conditions.

Nutrient determination

A typical forage laboratory analysis includes measurements of dry matter, ash, crude protein and structural carbohydrates (i.e. cellulose,

hemicellulose) according to official protocol by Association of Official Analytical Chemists (www.aoac.org) and Van Soest et al. (1991). In particular, dry matter (DM), the portion of forage without the water, is determinate as the loss in weight that results from drying at 100 ± 5 °C of a known weight of feed. Typically, nutritive value is reported on a DM basis to eliminate the dilution effect of moisture, allow direct comparison of feeds, and facilitate the diets formulation of and the prices comparison. Ash represent the inorganic constituent of the feed (mainly minerals) and its content is determinate by ignition of the feed at 550°C; the analytical determination of mineral elements in foods can be also qualitative, evaluating the individual mineral elements in atomic absorption spectroscopy. Minerals are inorganic constituents and the composition of plants will influence the animal's mineral intake. The mineral content is influenced by species and stage of maturity of the plant, type of soil, climate and seasonal conditions. Legumes tend to be richer in the major minerals and certain trace elements than grasses. The minerals are divided into macro elements (Ca, P, K, Na, Cl, S, Mg) heavily represented in the diet and expressed in g/kg and microelements (Co, Cu, Fe, Zn, Mn, I, F, Mo, Se) present in small amounts (expressed in mg/kg) but essential for life; rarely there may be a deficit of microelements (McDonald et al., 2010).

The carbohydrates, which include the low molecular weight sugars, starch and various cell wall and storage non-starch polysaccharides (NSP) are the most important energy sources for livestock animals. The NSP and lignin

are the principal components of cell walls (Theander and Westerlund, 1993; Theander et al., 1993). The non-structural carbohydrates (sugars, starches, organic acids, and other reserve carbohydrates such as fructans) are less represented in dried forage because they get lost during the haymaking process. The structural carbohydrates (crude fiber, acid and neutral detergent fiber) are the most common measures of fiber used for routine feed analysis. The fiber is the organic residual feed extracted by successive treatments with boiling acid and alkali of defined concentration and includes only part of cellulose, hemicellulose and lignin. Neutral detergent fiber (NDF) is the method that best separates structural from non-structural carbohydrates in plants. NDF measures most of the structural components in plant cell wall (i.e. cellulose, hemicellulose, lignin and pectin); whereas acid detergent fiber (ADF) primarily represents cellulose and lignin and does not include hemicellulose. ADF is often used to calculate digestibility, as NDF is used to predict potential DM intake; as the fiber increases, forage quality declines (Van Soest et al., 1991).

Protein is one of major component of all ruminant products. It is commonly measured by Kjeldahl's technique in which the feed is digested with sulphuric acid getting crude protein (CP), which is 6.25 times the nitrogen content of forage. Rumen microbes can convert non-protein nitrogen into microbial protein; in this way, the animal can use them. High-performing animals need larger amounts of protein to be absorbed

from the intestines than rumen microbes produced. Therefore, they need a certain amount of by-pass protein in the ration (Bittante et al., 2012).

Ruminants are able also to use non-protein nitrogen sources (NPN) for the synthesis of new proteins. The sources of NPN are essential for the development of the microbial flora and the fermentation activity. In grass, the NPN constitutes 5–15% of the total crude protein. Ruminants require a share of degradable and soluble proteins for the proper functioning of the microbial flora, but a fraction of non-degradable protein (by-pass protein), which is not digested in the rumen and pass in the small intestine, is also present in the diet (McDonald et al., 2010)

Lipids are a group of substances, insoluble in water but soluble in common organic solvents; in animals, they are the major form of energy storage. Plant storage lipids occur in fruits and seeds and are predominantly esters of fatty acids with glycerol (triglycerides). Their physical and chemical nature is determined by their fatty acid composition; high-molecular weight saturated acids (SFA) confer chemical stability and physical hardness, whereas unsaturated acids confer chemical reactivity and physical softness. The unsaturated acids possess different physical and chemical properties from the saturated acids: they have lower melting points and are more chemically reactive. Unlike SFA, the unsaturated acids contain one (MUFA), two, three or more double bonds; fatty acids with more than one double bond are frequently referred to as polyunsaturated fatty acids (PUFA). In function of the number of carbon atoms the fatty acids could be classified in short or long

chain fatty acids. Analysis of fats for individual fatty acids has presented great problems in the past, but the introduction of gas chromatography technique has allowed determinations to be made more easily and accurately (Sinclair et al., 1982).

Nutritive value estimation

Factors related with nutritive value include digestibility, intake, palatability and energy content. The digestibility expresses the quantity of nutrients contained in the feed, which are degraded in the rumen and then absorbed by the intestinal mucosa. It is also expressed as the percentage ratio between the amount of digested (or absorbed) ingredients and the total amount of nutrients in the feed. This relationship takes the name of digestibility coefficient expressing the animal's ability to digest the food. The digestibility of a food is most accurately defined as the proportion, which is not excreted in the faeces and but absorbed by the animal. Digestibility may be determined directly, in animals (*in vivo*) or indirectly, in the laboratory (*in vitro*).

To evaluate the *in vivo* digestibility, it is necessary to know the amount of ingested feed administered to cows and the amount of nutrients excreted via with faeces in 24 h. The digestibility of feedstuffs for ruminants can be measured quite accurately in the laboratory (*in vitro*) by treating them first with rumen liquor and then with pepsin. *In vivo* digestibility trials are laborious to perform; however, it is generally more accurate than *in vitro* digestibility. The digestibility is closely related to chemical composition of

feed, in particular the fiber fraction has the greatest influence on diet digestibility. The digestibility of cell walls is highly variable and it depends on the degree of plant lignification, which in chemical terms is expressed as lignin content (NRC, 2001).

Intake can be considered like as the quantities of the feed that an animal can consume in a given period of time. Food intake in ruminants is controlled metabolically and it is limited by the rate at which food can be digested in the rumen. Forages with a high content of NDF are digested slowly, are low digestible and promote low intake. Features of environment, such as high temperature also influence intake (McDonald et al., 2010)

The term palatability is used to determinate the degree of readiness with which a feed is selected and eaten. Animals select forage on the basis of smell, feel and taste. Texture, leafiness, fertilization, moistness, pest infestation, or compounds can influence palatability; these factors cause forage to taste sweet or salty. Good quality forages are generally very appealing. Usually higher is the palatability of a forage such as higher is its intake. Palatability can be evaluated when several different feedstuffs are given to animals and their preference is for some of these. Palatability and feed intake are not synonymous (Bal et al., 2001).

The nutritional value expresses the amount of energy that a feed can actually make available for metabolism (maintenance and production) of the animal. Each feed contains a certain amount of energy in chemical form, which can be measured as the total amount of heat released by the

complete oxidation (combustion) of the organic compounds. This quantity is defined as gross energy (GE) and an adiabatic bomb can measure it. The digestible energy (DE) is obtained by subtracting to gross energy the energy lost with undigested nutrients excreted with faeces. Digestibility energy can be estimated in digestibility trials. Only a part of DE is available for the animal; rumen fermentation gas (carbon dioxide and methane) losses in ruminants (about 5–15% of GE) and nitrogen compounds from the catabolism of proteins eliminated in the urine (about 60 KJ of energy for g of N excreted) also need to be considered.

The metabolizable energy (ME) is the amount of actual energy utilizable in metabolism and its value can be obtained using metabolism cages. Subtraction of the heat increment of a feed (loss of heat as work, digestion, etc.) from its ME value gives the net energy (NE) value. The NE value is the energy that is available to the animal for useful purposes, i.e., for body maintenance and for various form of production. A feed, while providing a constant amount of ME, has a different nutritional value depending on the production target: the nutritional value is higher in lactation than for maintaining and it is higher for lactation compared to the growth-fattening.

Therefore, to know the real nutritional value of a feed for ruminants is also necessary to know the production target. The measurement of NE is quite complicate and needs of animal calorimeters. However, the different type of energy content of feed can be estimated by specific equations (INRA, 1988).

FACTORS AFFECTING THE QUALITY OF HAY

Appropriate rationing of animal is essential to obtain high performances, in terms of elevated weight gains, high milk yield and quality and reproductive efficiency. In this context, the forage quality assumes an important role. Forage quality is defined in several ways, but in the practice, it is often scarcely researched and understood. Forage quality varies greatly among and within forage crops depending of species and varieties, growth stage, climate condition, soil fertilization and storage methods. Legumes are normally higher in quality than grasses, but within each group there can be a wide range of quality. When both grasses and legumes are harvested at the proper stage of plant growth, legumes are usually higher in total digestibility, protein and many minerals and vitamins. A mixture consisting of grass and legume is usually of high quality when properly managed. Perennials, such as alfalfa, orchard grass, timothy, fescue and Bermuda grass, are usually more economical for hay crops than annuals, such as sorghum-Sudan grass hybrids, pearl millets, small grain, lespedeza and ryegrass. Maturity stage is the most important factor determining forage quality, because it declines with advancing maturity. The mature plants become more fibrous, NDF concentration increases and the intake dramatically drops. Structural carbohydrates are more difficult to digest than the non-fiber components; therefore, the digestion slows when forage becomes more mature. Digestibility is a parameter that greatly varies in forages: an immature plant may be digested at 80 to 90%, while a mature plant at less than 50% (Bal et al.,

2001). About climate condition, rain during field drying damages legume hay more than grass hay, because can cause loss of leaf nutrients. However, the effect of rain on nutrient content of hay depends on when rain occurred after cutting and on the intensity and frequency of rain. Similarly, sunlight can lower hay quality through lowering Vitamin A content (Bal et al., 2001). Fertilizing soil with nitrogen generally increases the crude protein level of grasses, but fertilization usually has little or no effect on the digestible energy of forage.

USE OF FORAGE AND FATTY ACIDS PROFILE IN ANIMAL PRODUCTS

The fats of terrestrial animals have a predominance of saturated fatty acids: in milk fat the proportion among SFA, MUFA and PUFA is 8.5, 3.3 and 0.3, while in meat it is 8.3, 8.3 and 2.0. Saturated fatty acids of foods are regarded as the cause of a high-risk pattern of blood lipoproteins; stearic (C18) and myristic (C14) acids, and all trans acids are considered to be the most damaging (Ulbritch and Southgate, 1991). With increasing consumption of SFA, blood levels of cholesterol and LDL are raised. Conversely, PUFA are judged to be beneficial, although the various families of PUFA differ in their effects; the n-6 PUFA (which occur mainly in plant lipids) reduce the blood concentration of LDL, and the n-3 PUFA (from fish lipids) reduce VLDL. It is considered desirable to have a balance in the diet of n-6 to n-3 PUFA: the recommended maximum ratio is 4:1 (Simopoulos, 2000). In the past 10-15 years there has been a great interest in modifying the fatty acid profile of meat and milk. The principal

objective was to increase the proportions of PUFA, especially eicosopentaenoic (EPA) and docosohexaenoic (DHA) acids, which are considered to have special benefits to human health. This objective can be achieved by increasing the proportion in dietary lipids of alpha-linolenic acid (LNA), which is the precursor of EPA and DHA. Changing the diet of ruminants from conserved forages and concentrates to fresh forages (i.e. grazed grasses and clovers) increases the intake of LNA and, despite rumen hydrogenation of PUFA, increases the proportion of long chain PUFA in meat and milk, and also lowers the n-6/n-3 ratio (McDonald et al., 2010). Conjugated linoleic acids make up a group of polyunsaturated fatty acids found in meat and milk from ruminant animals and exist as a general mixture of conjugated isomers of LA. Of the many isomers identified, the cis-9, trans-11 CLA isomer (also referred to as rumenic acid or RA) accounts for up to 80–90% of the total CLA in ruminant products (Nuernberg et al., 2002). Naturally occurring CLAs originate from two sources: bacterial isomerization and/or biohydrogenation of PUFA in the rumen and the desaturation of trans-fatty acids in the adipose tissue and mammary gland.

Milk

Tudisco et al. (2010) reported significant differences in fatty acid profile and CLA contents of milk yielded by goats raised according to the organic versus conventional system. In particular, on account of the alpha-linolenic acid, the nutritional quality of organic milk seems to be higher

than that of conventional milk. D'Urso et al. (2008) showed the positive effect of pasture on the nutritional value of milk from Cilentana goats has been demonstrated. Indeed, the pasture group showed significantly higher levels of unsaturated fatty acids and both the main CLA isomers.

Meat

Research spanning three decade supports the argument that grass-fed beef (on a g/g fat basis), has a more desirable SFA lipid profile (more C18:0 cholesterol neutral SFA and less C14:0 and C16:0 cholesterol elevating SFAs) as compared to grain-fed beef. Grass-finished beef is also higher in total CLA (C18:2) isomers, trans-vaccenic acid (C18:1 t11) and n-3 FAs on a g/g fat basis. This results in a better n-6/n-3 ratio that is preferred by the nutritional community (Daley et al., 2010). Maintaining the more favourable lipid profile in grass-fed beef requires a high percentage of lush fresh forage or grass in the ration.

The higher concentration of fresh green forages, the higher αLA precursor that will be available for CLA and n-3 fatty acids synthesis. Fresh pasture forages have 10 to 12 times more C18:3 than cereal grains. However, also dried or cured forages, such as hay, will have a slightly lower amount of precursor for CLA and n-3 fatty acids synthesis (Vahmani et al., 2015).

HAY QUALITY AND ENVIRONMENTAL SUSTAINABILITY

Global warming is principally due to human activity, which is responsible for sending in the atmosphere of large amounts of greenhouse gases (i.e. carbon dioxide, CO_2; methane, CH_4; nitrous oxide, NO_2). In the last years the interest to them was increased, in particular for CH_4 and relationship between methane emission and livestock. Greenhouse gases (GHG) caused unfavourable effects to the environment; some of these global changes are increasing temperatures of air and ocean, retreating glaciers, rising seas, and shrinking lakes.

Agricultural and livestock play an important role among the main sectors responsible for GHG emissions. It is estimated that 67% of GHG were emitted from beef and dairy cattle (Hristov et al., 2013). In particular, about 70% of CH_4 released derives from rumen fermentation. The FAO asserted that a copious (92–97% of the total CH_4) amount of total GHG emissions is from enteric methane emissions in dairy and beef cattle (Opio et al., 2013). Consequently, in recent years many strategies to reduce its production have generated great interest (Kumar et al., 2014).

Interest for methane also comes from the fact that its production from rumen is also associated with considerable losses of energy, which vary between 2 and 12% of gross energy consumption, (Johnson and Johnson, 1995) and leads to the decrease of energy gain and productivity. So, methane emission by the rumen reduces the energy utilization efficiency of energy content in feed. Many factors affect to the methane production in the rumen: depending by feed as relationship between forage and

concentrate in the diet, forage quality, type of carbohydrates fermenting (Moe and Tyrrell, 1979; Crutzen et al., 1986; Birkelo et al., 1986), by microbial variations in the rumen (i.e. number of protozoa, number and type of methanogens) (Makkar and Vercoe, 2007) or by animal (i.e. intake, digestibility, species, physiological status).

The proportion of the diet fodder is one of the most critical aspects: if the diet is rich in fiber, the production of CH_4 is more relevant, because the structural carbohydrates, such as cellulose and hemicellulose, are degraded with liberation of acetic acid and, in minor amounts, butyric acid, and hydrogen ions which represent the primary substrate utilized by methanogens for the production of methane (Janssen, 2010; Auguerre et al., 2011). For this reason, the extensive farms, which are often characterized by very fibrous diets and low production levels, impact environment more than intensive, which are more efficient from a manufacturing point of view (Compiani et al., 2015).

Regarding fodder, the two relevant aspects, which affect CH_4 production, are quality and digestibility. Hristov et al. (2013) reported that feeding animal with high quality forages, digestibility and ingestion level, increase the productivity, and, consequently, decrease methane production per unit of feed consumed and for amount of product. This is because high quality forage is generally little lignified (Wright and Klieve, 2011; Knapp et al., 2014). Also when animals are fed early vegetative stage forages, which contain more protein than fiber fraction, a reduction in methane

production is achieved, compared to the administration of advanced vegetative stage forages, that is more lignified (Janssen, 2010).

In vivo and in vitro methane determination

The quantification of methane emissions from ruminants can be detected with *in vivo* in many systems. Respiration chambers are the standard and the most used method, advantageous because measure all the CH_4 produced. Inside of them the volume of the expired gas is measured from the animals using the first principle of thermodynamics (Kebreab at al., 2006; Storm et al., 2012). They are generally made of metallic material with transparent walls, to facilitate the visual contact between animals, and are of variable size depending on the animal size (Storm et al., 2012; Johnson et al., 1994). To ensure animal welfare and the expression of his normal behaviour, temperature (18 ± 2 °C) and humidity (60 ± 10%) are controlled, the access to feed and water is easy guaranteed and the faeces and urine are removed (Johnson and Johnson, 1995; Johnson et al., 1994). This restriction is not optimal for ensuring animal welfare, the animals could change the behaviour in terms of voluntary intake and stress, so then also the emissions of CH_4 could be distorted and unreliable. As limiting aspects, respiration chambers allow to use only low number of animals. Furthermore, they are very expensive for climate regulation and internal circulation of air.

As alternative to respiration chambers there are the polytunnel that placed in the grazing areas allows the interaction between animals and

the maintenance of their normal social behaviour. The concentration of methane released inside the tunnel is detected by gas chromatograph (Kebreab et al., 2006). A newly commercial automatic feeding system *in vivo* measuring methane and carbon dioxide is the Greenfeed® patented by Zimmerman and Zimmerman (2012). This machinery measures repeatedly individual CH_4 emission for short-term (3–6 min) every time the animal visits the Greenfeed® (Hegarty, 2013). Air is continuously aspirated through the apparatus to quantify CH_4 and CO_2 emitted during eating (Storm et al., 2012). A limitation of the technique is that methane emissions are quantified only when the animals are eating.

Some authors (Boadi and Wittenberg, 2002; Pinares-Patiño et al., 2011) used the sulphur hexafluoride (SF6) tracer gas technique to *in vivo* measurement of CH_4. This method, which uses an inert tracer gas source placed in the rumen of the animal, allows direct measurement of CH_4 in unrestrained individual animals from samples of gases collected at the mouth and nose. This technique may be able to account for more than 95% of total CH_4 production. The SF6 tracer gas technique can be used on several animals simultaneously in their pens or while they graze, however, animals have to be trained to wear a halter and collection canisters.

The potential CH_4 production from feedstaff can be measured by the *in vitro* gas production technique (IVGTP). This technique was used for several years to assess the quality of individual feeds or diets, or to understand the mechanisms of the kinetics of microbial fermentation (Getachew et al., 1998; Calabrò et al., 2015; Musco et al., 2016). This

method has been improved over the years, using fully automatic system (Soliva and Hess, 2007). The basic principle underlying these techniques is to reproduce the rumen environment, incubating the feed or the diet, in a controlled environment at 39° C, together with a rumen bacterial *inoculum* for a period of time (24, 48, 72, 96 or 120 hours). Together with degradability of the material incubated, the gas produced during the fermentations can be analysed by gas chromatograph (Grazioli et al., 2016; Guglielmelli et al., 2011). This method is less expensive compared to an *in vivo* test and also less time-consuming. Other disadvantages of this method are the availability of fresh rumen liquor, any possible phenomena of adaptation of the long-term microbial population, there is not the growth of new microorganisms, especially fungi and protozoa; the waste products are not removed altering microbial activity, the incubated feed particles (1 mm) are much smaller compared to a real animal's diet and this can modify the activity of rumen microorganisms.

The *in vivo* methods allows to evaluate animals and feedstuffs in terms of methane emission, whereas the *in vitro* methods only the potential methane production by feedstuffs. In addition to the described methods used for the quantification of methane, in bibliography there are many equations to predict methane production by statistical or dynamical models (Johnson and Johnson, 1995; Kebreab et al., 2006).

CONCLUSION

Hay quality varies considerably due to numerous factors discussed previously. Hay of different quality should in turn be reflected in sale prices when hay is marketed, but this does not happen in practice in many countries in the world. Improving forage quality can result in many benefits that affect profit: animal welfare and health, reproductive efficiency, reduction or elimination of supplemental feeds, and finally, improve the product quality and limit the environmental impact.

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Chapter 2.

First Contribution
Determination of quality in forages
from Mediterranean Area preserved as hay

Part of the data were presented at the 67th Annual Meeting of the European Federation of Animal Science (EAAP), Belfast (UK), 29 August - 2 September 2016: Grazioli R., Musco N., Cutrignelli M.I., Tudisco R., Infascelli F., Calabrò S. (2016). Evaluation of hay for dairy cow produced in Mediterranean Area. Book of abstracts No. 22, Wageningen Academic Publishers, pp. 552.

ABSTRACT

In present study, a database containing information on mixed hays produced in some agricultural and livestock farms sited in Southern of Italy is presented. The mixed hays are used to formulating diets for dairy cow producing high quality milk (e.g. Latte Nobile®). The general intent was to characterize the local resources and, if possible, identify some critical points in the production steps in order to improve their quality. In particular, together with the chemical composition, an original method to evaluate the sensory characteristics of hay is proposed. All the hays sampled were also tested for the *in vitro* fermentation, including methane production. A cluster analysis was used to assess whether distinct groups could be created on the basis of chemical constituents. Overall, the hay produced in the Mediterranean Area considered are characterized by a medium quality due to the low crude protein level, the high structural carbohydrates content, low leafiness, but good softness. The sensory evaluation system proposed has demonstrated to be a useful tool for assigning a first quality judgment to the hay, which is often in line with the analytical characteristics determined in the laboratory.

Keywords: *sensory evaluation, in vitro gas production, methane, OM degradability, Cluster analyses*.

INTRODUCTION

Forage plays a key role in the ruminants diet, but to be available all year long it is necessary to preserve it. Hay is a grass crop, which is cut, dried in the sun, harvested and stored. In the Mediterranean Area haymaking is the most widely used methods to keep forage for dairy cow; because the climate condition does not allow to have pasture all months of the year, the administration of hays can guarantee balanced diets during full lactation period. The final hay quality varies widely in function of many factors: type of fresh forage utilized, soil fertilization, plant vegetative stage at cut, climatic conditions during haymaking, method of cut, overturn and harvest the forage mass and, finally, characteristics of the storage site (Suttie, 2000; www.fao.org; Grazioli, 2016). As widely reported, the nutritive value of hay is lower when compared to fresh forage, however the loses can be limited if the different phases are performed carefully (Kugler, 2004). Utilizing high quality forage in formulating ruminant diets means to guarantee animal products of high nutritional quality (i.e. meat, milk, dairy products), in terms of fat content, fatty acids profile and vitamins. This aspect is even more important if in the animal diet the proportion of forage (hay) is widely represented, as in the case of dairy cows raised in the small farm of Mediterranean Area producing milk and dairy products of high nutritional value (e.g. Latte Nobile®). The Latte Nobile® is a model of livestock development proposed by the “Associazione Nazionale Formaggi Sotto il Cielo” (ANFoSC) in 2010, in which livestock system, including consistent aspects of animal feeding,

in accordance with a disciplinary production, guarantees the elevated nutritional characteristics of the milk (i.e. omega 6/omega 3 ratio not higher than 4). In particular, forage (fresh or hay) has to represent at least the 70% of the total ratio, has to be constituted by different botanical specie (at least 4) and has to be of high quality (minimum score 70/100); silage, as well as genetically modified feed, are prohibited (www.lattenobile.it).

As hay presents an extremely variable composition and quality, should be very important to analyze it accurately and frequently. The direct evaluation in the field is often superficial and without an adequate tool. The possibility to evaluate the hay quality by a direct observation in the field before administering to the animals would be of great practicality and utility. A sensory evaluation of the hay is a combination of physical/sensory inspection, perceived through the senses of sight, smell and touch. The most important parameters to consider are evidenced by many authors (Troelsen et al., 1968; Taylor, 1998; Bal et al., 2001):

- *Color*: hay with a green color usually indicates that it was cut a relatively early stage of maturity, with no damage from weathering (i.e. rain, hail, drought, etc.) and rapidly cured, in absence of molds and overheating during the storage. Yellowing is due to rain or long stay in the field; browning is due to overheating.
- *Number of essence*: a several numbers of botanical species present in hay guarantees a better nutritive, in terms of

odoriferous compounds related to the intake as well as ensuring the animal welfare (Baumont et al., 2000). When hay is rich in essences with complementary characteristics (especially micro nutrients), its composition is more heterogenous

- *Dustiness*: the presence of dust in the hay is negatively evaluated as the palatability and the intake of the feed decreases. Dustiness can indicate quality problems often associated with haymaking (soil collection during cutting) or not good storage conditions.
- *Softness*: the tactile evaluation depends on plant stage of maturity and so on time of harvest. Softness usually results from early cutting and high leafiness content. The growth stage at harvest is very important. When the plant passes from the vegetative to reproductive stage, it can show higher fiber and lignin content and lower in protein concentration becoming woody and harsh. Forage quality declines with advancing maturity which causes reducing the use of its by animals.
- *Odor*: hay must have a fresh aroma, often associated to a green color, and it is influenced by the plant essences present; moldy smells is frequently connected to dustiness and can indicate others quality problems (i.e. rain damages or poor storage condition); a bad smell reduces palatability and feed intake.
- *Leafiness*: this parameter is related to the proportions of leaves to stems in the hay. A high content of leaves results in a high quality, because most of nutritive compounds (i.e. proteins, minerals,

vitamins) are present in leaves. Leafiness depends by plant species, stage of maturity at harvest, and by losses leaves (especially in legume hays) during haymaking.

Visual inspection can also detect foreign matters that are anything that has little or no feed value: sticks, rocks, wire, dead animals can be found in hay and they are obviously undesirable (Bal et al., 2001). For evaluating the quality of the hay the most reliable approach is a combination of sensory, chemical and biological examination.

The objective of the investigation was to create a database containing peculiar information on mixed hays produced in the agricultural and livestock farms of the Mediterranean Area sited in Southern of Italy and used for dairy cow producing high quality milk. The second aim was to identify the critical points in order to improve the quality of these local resources.

MATERIALS AND METHODS

Experimental design

Fifty-six samples of hay were object of study. They were evaluated for sensory characteristics in the field and analyzed for proximate analyses into the laboratory of “Feed analyses” of the DMVPA (University of Napoli Federico II, Napoli, Italy). The nutritive value was also estimated. In addition, the fermentation characteristics, including methane production, were evaluated using the *in vitro* gas production technique (IVGPT). The

cluster analysis was used to assess whether distinct groups could be created on the basis of chemical constituents.

Samples collecting

The samples were collected during the autumnal season (September-October) in three consecutive years (2014, 2015 and 2016). They were all produced by agricultural and livestock farms located in different areas of Central and Southern of Italy (Avellino, Benevento, Campobasso, Potenza, Roma and Salerno), as pictured in table 1 and more clearly illustrated in figure 1. For all the samples, the fresh forages were preserved using the local tradition system of haymaking: drying for 4-5 days in the field until reaching 850.0 g·kg of dry matter (DM).

Table 1.

Hays collected for the investigation classified for sampling area

Province	Longitude	Latitude	Altitude*	Temperature†	Rainfall†	Samples
	°E	°N	m	°C	mm	No.
Avellino	14°47′	40°54′	360	13.9	1354 mm	26
Benevento	14°46′	41°07′	154	15.1	787 mm	10
Campobasso	14°40′	41°33′	701	12.3	583 mm	2
Potenza	15°48′	40°38′	760	11.3	650 mm	7
Roma	12°28′	41°53′	21	15.7	798 mm	7
Salerno	14°45′	40°40′	2	24.0	29 mm	4

*a.s.l.: at sea level.
†Annual mean value

Figure 1.

Geographical map of the different sampling sites

Source: www.google.it/maps

Sensory evaluation

The sensory evaluation of the hay samples was carried out very accurately together by a group of five experts (i.e. agronomy, veterinary and researchers in animal nutrition) utilizing a score card reported in table 2, set up in collaboration with ANFoSC (www.anfosc.it). In a first phase of the investigation, the score card provided for the evaluation of color, number of essences, dustiness, softness, odor, *Graminae*: *Leguminosae*

ratio and leafiness; in a second time, the score card was simplified eliminating the *Graminae*: *Leguminosae* ratio. Each parameter is assigned a score ranging from a minimum to a maximum; the total score (from 0 to 100) for each hay sample give an idea of its overall rating.

Table 2.

Score card for the evaluation of hay quality

Parameter	Sensory evaluation	Possible score
Color	Brown or black	1
	Brown	2-3
	Straw Yellow	4-5
	Light Green	6-7
	Green	8-9
No. of essences	Natural Green	10
	From 1 to 3	until 6
	From 4 to 8	until 16
	From 9 to more	until 20
Dustiness	Lots of dust	1
	Medium dust	2-9
	Little dust	10-15
	No dust	20
Softness	Woody	1
	Harsh	2-9
	Slightly harsh	10-15
	Soft	16-20
Odor	Unpleasant, stool, fertilizer, urine, old, dust, moldy	2-8
	Fresh, clean, floral, herbaceous	10-14
	Pleasant, slightly persistent, slightly aromatic	16-20
Leafiness	Little leafy	2-6
	Medium leafy	8-12
	Very leafy	14-20

Chemical composition

Before the analyses, all the hay samples were ground to pass a 1 mm screen (SM 100, Retsch, Haan, Germany) and analyzed for dry matter (DM), crude protein (CP), ether extract (EE), crude fiber (CF) and ash according to official protocol by Association of Official Analytical Chemists (AOAC 2005; www.aoac.org) procedures (ID number: 2001.12, 978.04, 920.39 978.10 and 930.05 for DM, CP, EE, CF and ash, respectively). Neutral detergent fiber (NDF, with sodium sulphite and heat-stable α-amylase and expressed exclusive of residual ash), acid detergent fiber (ADF, expressed exclusive of residual ash) and acid detergent lignin (ADL, determined by solubilization of cellulose with sulphuric acid) were analyzed according to Van Soest et al. (1991).

Estimation of nutritive value

The nutritive value of the hay samples, expressed as net energy for lactation (Unité Fourragère du Lait, UFL), was calculated according INRA system (1988) utilizing the parameters of the chemical composition.

In vitro fermentation characteristics and volatile fatty acids

The *in vitro* fermentation characteristics were studied incubating all the hay samples at 39°C under anaerobic conditions with cow *inoculum* according to the protocol reported by Calabrò et al. (2015). In particular, the substrates were weighed (1.0082 ± 0.0081 g) in 120 ml serum bottles and 79 ml of anaerobic medium were added. The rumen fluid was

collected in a pre-warmed thermos at a slaughterhouse authorized according to EU legislation (Regulation EC No. 882/2004) from four dairy cows (mean body weight 680 kg) fed a total mixed ratio containing corn silage, oat hay and concentrate (CP 12 and NDF 43.5 % DM). The collected material was rapidly transported to the laboratory, where it was pooled, flushed with CO_2, filtered through cheesecloth and added to each bottle (10 ml). Gas production was recorded 24 times (at 2 to 24 h intervals) during the period of incubation using a manual pressure transducer (Cole and Palmer Instrument Co, Vernon Hills, IL, USA). The cumulative volume of gas produced after 120 of incubation was related to the incubated organic matter (OMCV, ml/g) and to degraded organic matter (Yield, ml/g). The fermentation was stopped at 120 h and the fermenting liquor was analyzed for pH (pH-meter ThermoOrion 720 A+, made in USA) and sampled for volatile fatty acids (VFA) determination. In particular, the fermenting liquor was centrifuged at 12,000 g for 10 min at 4°C (Universal 32R centrifuge, Hettich, Melle-Neuenkirchen, Germany) and 1 ml of supernatant was mixed with 1 ml of oxalic acid (0.06 mol). Volatile Fatty Acids were detected by gas chromatography (GC Focus AI 3000, Thermo Scientific, Waltham, MA USA) equipped with a fused silica capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness), using an external standard solution composed of acetic, propionic, butyric, iso-butyric, valeric and iso-valeric acids, as described by Musco et al. (2016). The extent of sample disappearance, expressed as organic matter degradability (dOM, %), was determined by weight difference of the

incubated OM and the undegraded filtered (sintered glass crucibles; Schott Duran, Mainz, Germany, porosity # 2) residue burned at 550°C for 5 h. For each gas run, three bottles were incubated without substrate (blanks) to correct dOM, OMCV and VFA.

Methane determination

The methane production was determined *in vitro* stopping three bottles for samples at 24 hours of incubation as proposed by Guglielmelli et al. (2011), sampling the head-space gas (3 ml) from each serum flask in duplicate with a gastight syringe. The analysis was carried out using a gas chromatograph (GC Trace 1310, Thermo Scientific, Waltham, MA USA) equipped with a loop TC detector and a packed column (HaySepQ SUPELCO, 3/16 inch, 80/100 mesh). At this time dOM was also measured.

Data processing

A preliminary Cluster analysis (method average) was used to assess whether distinct groups could be created on the basis of chemical constituents. As results, hay samples were clustered mainly on the similarity of CP and NDF content in three groups: high CP-low NDF (HL), medium CP-medium NDF (MM), low CP-high NDF (LH) following the pseudo-F statistics (table 3). On the basis of this results, further statistical analyses were carried out: i) one-way analysis of variance using the GLM procedure for sensory evaluation data, and ii) a mixed model analysis to compare the three clusters obtained for *in vitro* fermentation data,

including methane production. In this latter case, repeated measures of hays within cluster were used as random effect. For all the statistical analysis, the SAS System (2000) was utilized.

RESULTS AND DISCUSSION

Cluster membership due to the use of chemical constituents is reported in table 3. As previous mentioned, the Cluster analyses distinguished three groups, which were not equilibrated in terms of numerousness (Figure 2). In particular, HL group (high CP and low NDF content) showed the most favourable chemical composition, in terms of structural carbohydrates content (low value), crude protein and energy level (high value); MM group (medium CP and medium NDF content) showed CP and UFL content similar to HL group and NDF value in a medium position between HL and LH. In general, the crude protein is quite low also in the HL group and the NDF is rather high especially on LH group; this means that the quality of this forage is rather poor.

The results of sensory evaluation of the hays collected are pictured in table 4. The cluster groups have significant ($P<0.05$) effect only on leafiness, confirming the importance of this parameter in evaluating hay. As a whole, also from this evaluation, the hay quality appears rather poor; the parameters more responsible of this result are the low number of essence and the fairly low amount of leaf, whereas softness and odor show higher values. In particular, as mentioned earlier, an elevated number of essences improve the intake and animal welfare and also leads

agronomic advantages for the entire forage system. These data indicate that the preparation of hay can be improved. In particular, the choice of the essence to sown must be done accurately, considering the agronomic and climatic characteristics of the territory to allow all plants to grow properly.

Table 3.

Cluster membership due to the use of chemical constituent

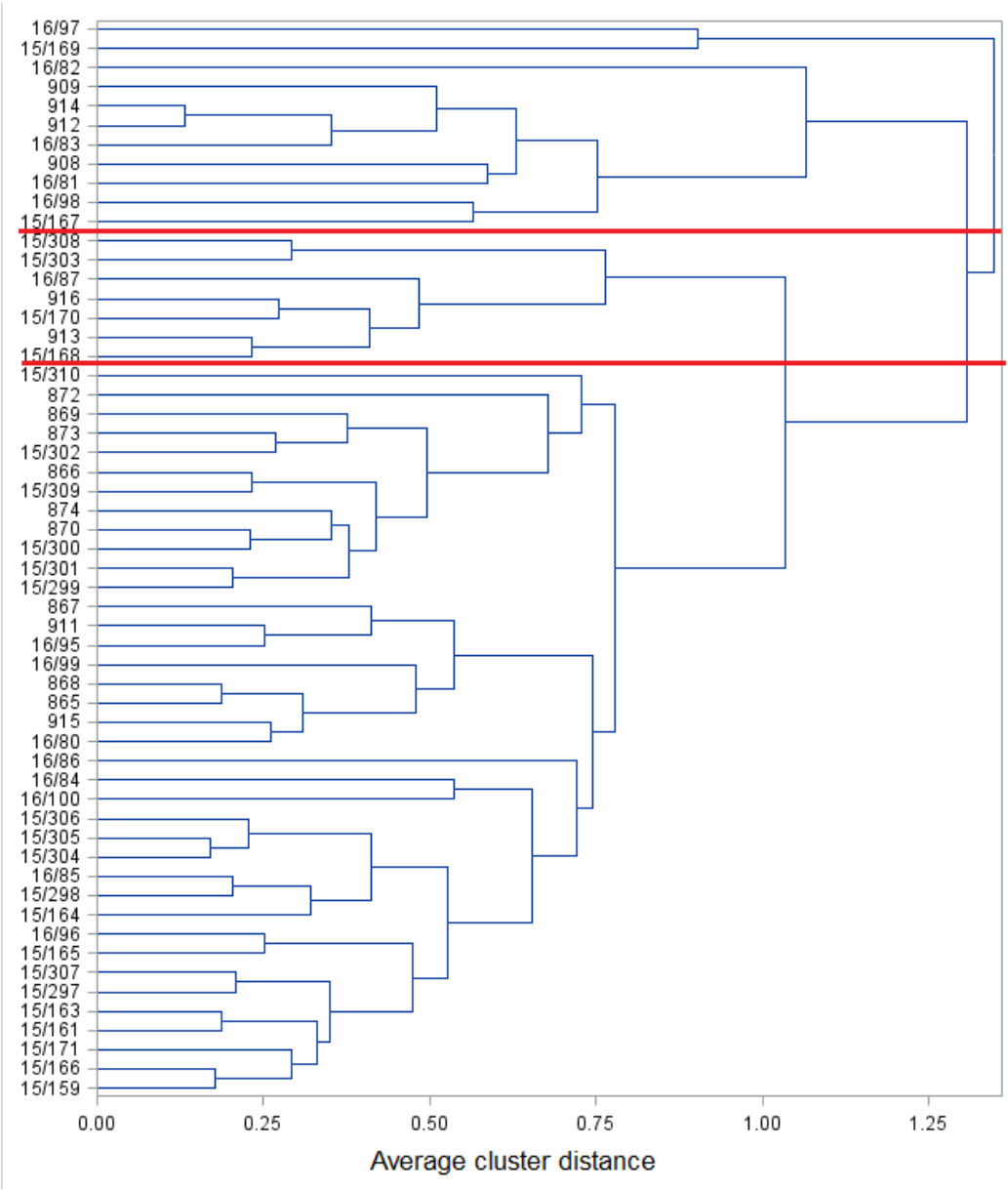
Cluster	Obs.	CP	NDF	ADF	ADL	Ash	UFL
	No.			*% DM*			*/kg DM*
HL	14	10.7	51.9	38.2	5.18	9.51	0.59
MM	76	9.37	55.7	38.4	5.51	9.70	0.58
LH	22	8.36	62.9	37.9	6.88	8.56	0.52

HL: high CP-low NDF; MM: medium CP-medium NDF; LH: low CP-high NDF. DM: dry matter; CP: crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber; ADL: acid detergent lignin; UFL: *Unité Fourragère du Lait* (INRA, 1988).

A wealth of botanical essences not only enriches the soil, but provides animals with a wide choice of molecules that will have a positive influence on the nutritional and organoleptic characteristics of the resulting products (i.e. milk, cheese). Moreover, the presence of leaves is essential to enrich the forage of nitrogen. The leave's leakage often occurs during the tedding phase, which must be performed gently, especially if excessive drying has been overcome due to not favourable environmental condition (extremely temperatures, wind and solar radiation).

Figure 2.

Cluster dendrogram for the tested substrates



The high value for odor evaluation indicates that the forage has been appropriately storage, even if in same cases it is very low (min value: 4/20). An others important factor influencing hay quality is the softness, which is strictly related to the structural carbohydrates content. It depends, mainly, at which stage is the plant at the time of cutting. The choice of the right time for cutting is not always easy for the farmer, mainly if different botanical species are present with different growing rate. Of course, the most affecting factor is the environmental condition: 3-4 days of favorable weather needs to dry the forage in the field. Due to the variable condition (risk of rain, excessive heat) during the cutting period (third week of May-June) in the Mediterranean area considered the cutting is often delayed. This result justifies the high content of structural carbohydrates, which however, are not excessively rich in lignin, probably due to the greater presences of Graminae, and therefore soft (ADL: 5.18, 5.51 and 6.88 %DM, for HL, MM and LH, respectively). The structural carbohydrates content is strictly related to maturity stage at harvest.

Table 4.

Sensory evaluation of the hays collected in the Mediterranean area

	Color	No. essences	Dustiness	Softness	Odour	Leafiness	Total score
	/10	/20	/20	/20	/20	/20	/100
Average	6.18	8.48	7.68	13.8	12.6	10.6	59.5
SD	± 1.87	± 4.01	± 3.29	± 3.77	± 3.36	± 4.04	± 11.7
Min value	1.00	1.00	2.00	2.00	4.00	4.00	30.0
Max value	9.00	16.0	20.0	20.0	18.0	20.0	82.0
			Cluster effect				
MSE	3.50	15.3	11.0	14.2	10.6	14.6	142
Sign. F	NS	NS	NS	NS	NS	*	NS

SD: Standard Deviation. MSE: mean square error.
* and NS: P<0.05 and not significant, respectively.

This is a most important factor determining forage quality because it declines with advancing maturity because when plants mature they become more fibrous. Also digestion slows dramatically. Maturity at harvest also influences forage consumption by animals, with advancing maturity forage intake drops dramatically (Bal et al., 2001).

Table 5.

In vitro fermentation characteristics after 120 h of incubation

Cluster	**dOM**	**OMCV**	**A**	**B**	**T_{max}**	**R_{max}**
	%	*ml/g*	*ml/g*	*h*	*h*	*ml/h*
HL	65.1^{ab}	265^{ab}	281^{b}	18.1^{c}	6.71	9.68^{a}
MM	67.2^{a}	253^{b}	275^{b}	20.60^{b}	6.99	8.49^{b}
LH	63.8^{b}	282^{a}	326^{a}	26.0^{a}	7.15	8.20^{b}
P-value	0.0039	<0.0001	<0.0001	<0.0001	0.747	0.0021

HL: high CP-low NDF; MM: medium CP-medium NDF; LH: low CP-high NDF. dOM: organic matter degradability; OMCV: cumulative gas production related to incubated organic matter; A: potential gas production; B: time at which A/2 was formed; T_{max}: time at which maximum rate was reached; R_{max}: maximum fermentation rate.
Along the column, different letters (a-c) means significant differences for P<0.05.

Table 5 showed the *in vitro* fermentation characteristics in the three cluster groups after 120 h incubation. All the parameters, except T_{max}, statistically ($P < 0.01$) vary in function of the groups. In particular, LH group, due to the less favorable chemical composition, showed the lowest dOM (63.8 %), and the slowest kinetics (R_{max}: 8.20 ml/h; T_{max}: 7.15 ml/h);

on the other hand, for HL group the fermentation process was faster (R_{max}: 9.68 ml/h; P>0.05) but the degradability was similar (65.1 %), due to the higher structural carbohydrates content caused by the vegetative state or by the prevailed presence of Graminae.

Table 6 reported pH value and volatile fatty acids production in the three groups after 120 h incubation. A significant (P<0.05) differences between groups only appear for pH value (mean value: 6.65, 6.31 and 6.47; for HL, MM and LH, respectively); however, the final pH values after 120 h of incubation showed that the buffering capacity of the medium was sufficient to maintain the pH within the 6.2 to 6.8 range necessary to ensure a linear relationship between VFA and gas production (Beuvink and Spoelstra, 1992). Total VFA recorded at 120 h, as well as the most representative acids (acetate, propionate, butyrate) did no differ between groups and are in line with previous data (tVFA: 53.67, 23.92 and 8.70 mmol/g incubated OM for acetate, propionate and butyrate, respectively) reported by Calabrò et al. (2006). The branched-chain fatty acids (iso-butyrate, iso-valerate), rising from the protein degradation, are significantly different (P<0.05) between groups and follows the protein content of the substrates: HL > MM > LH.

In Figure 3 mean value of methane production recorded after 24 h of incubation in the three cluster groups were depicted. Any significant (P>0.05) differences appear between the three groups. In particular, HL group showed the highest CH_4 production, either reported as percentage of total gas either on incubated OM, but the lowest value when related to

degraded OM (12.45 %, 11.96 ml/g, 22.15 ml/g, for pCH_4, iCH_4 and dCH_4, respectively). On the other hand, LH group showed the lowest CH_4, if reported as percentage of total gas and related to incubated OM as well, while the highest when related to degraded OM (11.29 %, 10.04 ml/g, 25.55 ml/g, for pCH_4, iCH_4 and dCH_4, respectively). This is probably due to the high cell wall content of LH hays that limit the rumen degradability. These results indicate that, for a forage to be eco-sustainable needs to produce few methane, but to be efficiently utilized by rumen microorganism it has to be highly degradable; so, the best group of hay, in terms of nutritive value and environmental impact is that one characterized by high protein level and low fiber content.

Producing hay of high quality is very important to guarantee a dietetic and nutritional quality in milk and dairy products. As known, fresh forage contains a very small percentage (1-3%) of lipids, in the form of glycolipids and phospholipids, and only a very variable part of these (from 20 to 60%) is represented by fatty acids. The administration of large amount of forage promotes, into the rumen, the growth of ruminal cellulosolytic bacteria. These bacteria are able to attack the lipid components and facilitate the hydrogenation of unsaturated fatty acids (UFA). Consequently, the concentration of short- and medium chain fatty acids (C12:0 to C16:0) in milk fat decrease for cows consuming pasture, while the UFA content increase (Kelly et al., 1998). Moreover, milk from grazing animals is characterized by a well-balanced n-6/n-3 ratio (Bailoni et al., 2005).

Table 6.

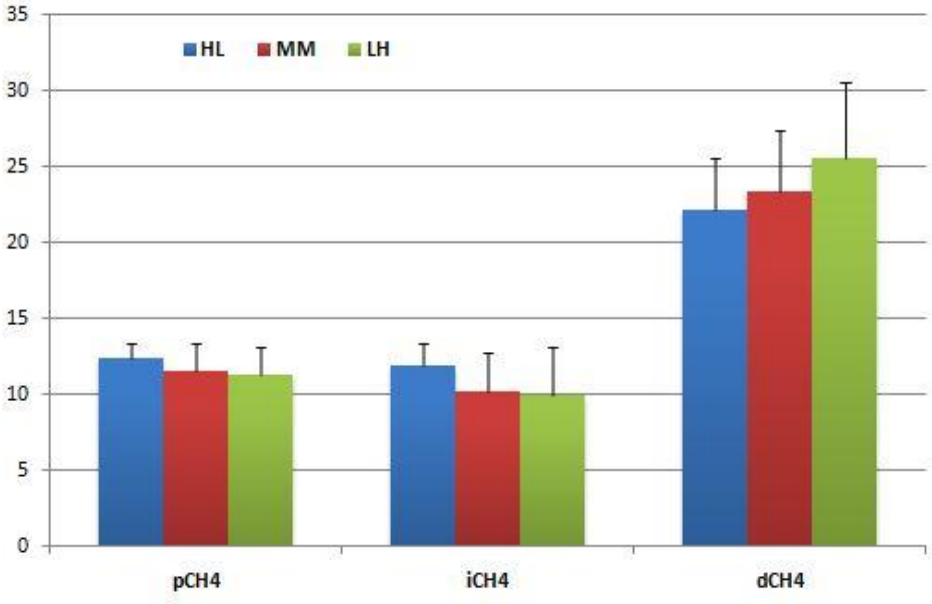
pH and volatile fatty acids after 120 h incubation

Cluster	pH	Acetate	Propionate	Iso-Butyrate	Butyrate	Iso-valerate	Valerate	tVFA
				mmol/g				
HL	6.65[a]	55.4	16.36	0.915[a]	6.22	1.58[a]	1.355[a]	78.7
MM	6.31[b]	58.3	16.5	0.709[b]	6.86	1.21[b]	1.004[b]	84.3
LH	6.47[a]	58.8	17.2	0.708[b]	6.68	1.20[b]	0.851[c]	80.2
P-value	<0.0001	0.527	0.4747	0.004	0.213	0.0069	<0.0001	0.2007

HL: high CP-low NDF; MM: medium CP-medium NDF; LH: low CP-high NDF.
tVFA: total volatile fatty acids (acetic + propionic + butyric + isobutyric + valeric + isovaleric).
Along the column: different letter a-c means significant differences for P<0.05.

Figure 3.

In vitro methane production after 24 h incubation



HL: high CP-low NDF; MM: medium CP-medium NDF; LH: low CP-high NDF.
pCH_4: methane production as percentage of total gas (%); iCH_4: methane production related to incubated organic matter (ml/g); dCH_4: methane production related to degraded organic matter (ml/g).
Error bars indicate the standard deviation for each parameter in every year.

It has also reported that, the CLA content increase when cows fed forage on pasture, compared to the administration of diet composed by fresh forage or hay with the same lipids content (Chilliard et al., 2003; Kelly et al., 1998; Lee et al., 2002). Compared to fresh forage hay have lower polyunsaturated fatty acids (about 70% less on the total in FA content), mainly due to the oxidative phenomena that start immediately after cutting in the field and proceed intensively during the haymaking. In

addition, during haymaking, a fraction of the leaves, the plant part richest in fatty acids, may lose.

CONCLUSIONS

Cluster analysis has proven to be useful in identifying groups of hays with similar nutritional characteristics, which have significantly influenced many of the *in vitro* fermentation parameters. The sensory evaluation system proposed has demonstrated to be a useful tool for assigning a first quality judgment at the hay, which is often in line with the analytical characteristics determined in the laboratory. Overall, the hay produced in the Mediterranean Area considered are characterized by a medium quality that can be improved. The most important aspect to look after is to increase the present forage essences and to anticipate the time of cutting.

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Chapter 3.

Second Contribution

Hay quality: comparison between mixed hays produced in different areas of Southern of Italy

ABSTRACT

The aim of this study was to characterize eight hays produced in four agricultural and livestock farms located in four Provinces (Avellino, Benevento, Campobasso and Potenza) of Southern of Italy, different for environmental condition. The sensory evaluation, nutritive value, *in vitro* fermentation characteristics and kinetics, including methane production were determined. The little differences observed between sampling areas showed evidence that the forage produced in Avellino area is the most interesting in terms of chemical composition, nutritive value, *in vitro* characteristics and environmental impact. Data obtained allows having more information about hays produced in the study area, useful for farmers to make balanced rations to maintain animal health and guarantee high level of production.

Keywords: *Mediterranean area, forage, in vitro fermentation kinetics, methane production, volatile fatty acids.*

INTRODUCTION

Administration of fresh forages for livestock is not possible during the whole year because their production is limited only to the most favorable seasons. Therefore, it is necessary to store forages in order to take advantage of stock throughout the year. Haymaking involves the transformation of grass into hay thanks to a progressive loss of moisture that reaches 15-20% (Suttie, 2000; www.fao.org). Haymaking is one of the

most popular and traditional systems of forage conservation in Italy. In particular, in many regions of Mediterranean area (Southern of Italy), the forage preserved as hay is produced in the hilly and mountain area of Appennino (Province of Avellino, Benevento, Potenza, Campobasso). This hay is utilized in ruminant farms, either extensive to produce principally high quality meat (i.e. Vitellone bianco dell'Appennino Centrale IGP (www.vitellonebianco.it) and milk and dairy products (i.e. Latte Nobile®, www.lattenobile.it); or intensive to produce mainly Mozzarella di Bufala DOP (www.mozzarelladop.it). As known, forages present extremely variable chemical composition depending on many factors (Buxton, 1996). A high influence is given by the environmental condition in which forages are grown, including geographical variation, climatic conditions (i.e. rainfall, temperature, humidity), soil type (i.e. sand, silt and clay) and characteristics (i.e. pH, water-holding capacity, fertility) and the stage of harvesting (Darby and Lauer, 2002). These variables affect, consequently, the quality of hay produced. Knowledge of hay nutritive value is important for balancing rations to maintain animal health and guarantee high level of production, in terms of quality and quantity. In addition, the use of locally produced hay compared to imported them (Ki et al., 2017) has economic advantages for the breeder but also environmental rewards in terms of global warming potential as reported in a Life Cycle Assessment (LCA) study (Ogino et al., 2007).

The objective of this investigation was to characterize forage preserved as hay produced in different agricultural and livestock farms of

Mediterranean area, in terms of sensory evaluation, nutritive value, *in vitro* fermentation characteristics, including methane production. The hypothesis is that different areas of sampling influence the hay characteristics due to environmental conditions.

Experimental design

For the study, eight mixed hay samples produced in four different areas of Southern of Italy [Avellino (AV), Benevento (BN), Campobasso (CB) and Potenza (PZ) provinces] were collected in November 2015; two samples for each area in two different agricultural-livestock farms were collected. The environmental conditions for each sampling area are reported in table 1. After collection, all samples were transported to the "Feed analyses" laboratory of the DMVPA (University of Napoli Federico II, Napoli, Italy), where they were analyzed for sensory evaluation and, after ground to pass a 1 mm screen (SM 100, Retsch, Haan, Germany) the chemical composition, *in vitro* fermentation kinetics and characteristics, including methane production were determined.

Sensory evaluation

The sensory evaluation was performed on the eight forages utilizing the score card (ANFoSC, www.anfosc.it) more explained in the first experimental contribute (Chapter 2.), that provided evaluation of color, number of essences, dustiness, softness, odor and leafiness. Each parameter is assigned a score ranging from a minimum to a maximum;

the total score (from 0 to 100) for each hay sample give an idea of its overall rating.

Table 1.

Geographical position, environmental condition of the hay sampling areas

Area	Longitude (°E)	Latitude (°N)	Altitude (m a.s.l.)	Temperature† (°C)	Rainfall† (mm)	Humidity† (%)
Formicoso (AV)	14.47	40.54	360	13.9°C	1354	60
Castelpagano (BN)	14.46	41.07	154	15.1°C	787	67
Campochiaro (CB)	14.40	41.33	701	12.3°C	583	68
Paterno (PZ)	15.48	40.38	760	11.3°C	650	76

*a.s.l.: at sea level.
†2015 mean value-

Nutritive value

The hay samples were analyzed for dry matter (DM), crude protein (CP), ether extract (EE), crude fiber (CF) and ash according to official protocol by Association of Official Analytical Chemists (AOAC, 2005; www.aoac.org) procedures (ID number: 2001.12, 978.04, 920.93, 978.10 and 930.05 for DM, CP, EE, CF and ash, respectively). Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were also determined (Van Soest et al., 1991).

The hays' nutritive value, expressed as net energy for lactation (Unité Fourragère du Lait, UFL), was calculated with two methods:

- according to INRA system as suggested by INRA (1988) utilizing the chemical composition parameters (DM, CP, ether extract, crude fiber, ash);
- as proposed by Menke and Steingass (1988) utilizing the 16e equation:

$$NE = 0.54 + 0.0959 \times GP24 + 0.0038 \times CP + 0.0001733 \times CP^2$$

where CP is expressed as g/kg DM and GP24 (gas produced after 24h of fermentation, see below) as ml/200 mg DM.

In vitro fermentation characteristics and volatile fatty acids

The *in vitro* fermentation characteristics were studied incubating the substrates at 39°C under anaerobic conditions with cow *inoculum* according to the protocol reported by Calabrò et al. (2015). In particular, the substrates were weighed (1.007 ± 0.0061 g) in 120 ml serum bottles and 79 ml of anaerobic medium were added. The rumen fluid was collected in a pre-warmed thermos at a slaughterhouse authorized according to EU legislation (Regulation EC No. 882/2004) from four dairy cows (mean body weight 680 kg) fed a total mixed ratio containing corn silage, oat hay and concentrate (CP 12 and NDF 43.5 % DM). The collected material was rapidly transported to the laboratory, where it was pooled, flushed with CO_2, filtered through cheesecloth and added to each bottle (10 ml). Gas production was recorded 24 times (at 2 to 24 h intervals) during the period of incubation using a manual pressure transducer (Cole and Palmer Instrument Co, Vernon Hills, IL, USA). The cumulative volume

of gas produced after 120 of incubation was related to the incubated organic matter (OMCV, ml/g) and to degraded organic matter (Yield, ml/g). The fermentation was stopped at 120 h and the fermenting liquor was analyzed for pH (pH-meter ThermoOrion 720 A+, made in USA) and sampled for volatile fatty acids (VFA) determination. In particular, the fermenting liquor was centrifuged at 12,000 g for 10 min at 4°C (Universal 32R centrifuge, Hettich, Melle-Neuenkirchen, Germany) and 1 ml of supernatant was mixed with 1 ml of oxalic acid (0.06 mol). Volatile Fatty Acids were detected by gas chromatography (GC Focus AI 3000, Thermo Scientific, Waltham, MA USA) equipped with a fused silica capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness), using an external standard solution composed of acetic, propionic, butyric, iso-butyric, valeric and iso-valeric acids, as described by Musco et al. (2016). The extent of sample disappearance, expressed as organic matter degradability (dOM, %), was determined by weight difference of the incubated OM and the undegraded filtered (sintered glass crucibles; Schott Duran, Mainz, Germany, porosity # 2) residue burned at 550°C for 5 h. For each gas run, three bottles were incubated without substrate (blanks) to correct dOM, OMCV and VFA.

Methane determination

The methane production was determined *in vitro* stopping three bottles for samples at 24 hours of incubation as proposed by Guglielmelli et al. (2011), sampling the head-space gas (3 ml) from each serum flask in

duplicate with a gastight syringe. The analysis was carried out using a gas chromatograph (GC Trace 1310, Thermo Scientific, Waltham, MA USA) equipped with a loop TC detector and a packed column (HaySepQ SUPELCO, 3/16 inch, 80/100 mesh). At this time dOM was also measured.

Data processing

To estimate the fermentation kinetics, for each bottle, the gas production profiles were fitted to the following model (Groot et al., 1996):

$$G = \frac{A}{(1 + \frac{B}{t})^C}$$

where G is the total gas produced (ml per g of incubated OM) at time t (h), A is the asymptotic gas production (ml/g), B is the time at which one-half of A is reached (h), and C is the curve switch. Maximum fermentation rate (R_{max}, ml/h) and the time at which it occurs (T_{max}, h) were calculated utilizing model parameters (Bauer et al., 2001):

$$T_{max} = C \times (\frac{B-1}{B+1})^{1/B}$$

$$R_{max} = \frac{(A \times C^B) \times B \times Tmax^{(B-1)}}{(1 + C^B) \times (Tmax^{-B})^2}$$

With the aim to evaluate the influence of the different sampling areas on sensory evaluation, chemical composition and *in vitro* fermentation characteristics, the statistical significance of the differences was determined by Student's t test (SAS, 2000) according to the model:

$$Y_{ij} = \mu + A_i + \varepsilon_j$$

where for each parameters (*Y*), μ represents the general mean, *A* (*i* = 1 – 4) the sampling area and ε the error term; the two farms were considered as repetition. The correlation between chemical composition, sensory evaluation and *in vitro* data were also studied using PRC CORR (SAS, 2000).

RESULTS AND DISCUSSION

Sensory evaluation and chemical composition

The results of sensory evaluation are reported in Table 2. Considering the singles parameters, sampling area influenced significantly the number of essences, the dustiness and the softness. The hays produced in PZ area has the highest total score (76/100) due to the elevated scores in all parameters, except for number of essences that was the lowest ($P<0.05$). All the other hays mainly due to a medium dust content, showed a scarce total score, under the limit (70/100) indicated by ANFoSC for the production of high quality milk (www.lattenobile.it). Hays produced in AV area showed the lowest total score (59/100), even if it was rich in number of essences (7.5/10) and very leafy (19/20).

In general, the botanical essences more representative individuated in the mixed hays were *Onobrychis viciifolia* and *Onobrychis montana, Hedysarum coronarium, Trifolium pratense, Trifolium subterraneum, Trifolium brachycalycinum, Medicago sativa, Medicago polymorfa*, for Leguminosae and *Lolium perenne, Lolium rigidum, Dactylis glomerata, Festuca arundinacea, Festuca pratensis, Poa pratensis, Phleum pratense, Phleum alpinum* for Graminae, that commonly are present in the mixed

hays produced in the areas of interest (Bullitta et al., 1991; Cavallero et al., 1992).

Table 2.

Sensory evaluation of mixed hays produced in the four areas

Area	Color	No. of essences	Dustiness	Softness	Odor	Leafiness	Total
	/10	*/10*	*/20*	*/20*	*/20*	*/20*	*/100*
AV	6.0	7.5^{ab}	8.0^{b}	8.0^{c}	10.5	19.0	59.0
BN	6.5	6.0^{b}	7.0^{b}	15.0^{ab}	14.0	10.5	59.0
CB	6.0	8.5^{a}	6.5^{b}	15.5^{ab}	16.0	10.0	62.5
PZ	7.5	3.5^{c}	18.0^{a}	18.0^{a}	16.0	13.0	76.0
Prob. T	NS	**	*	*	NS	NS	NS
MSE	2.179	0.612	2.475	2.372	2.850	2.750	7.425

AV: Avellino; BN: Benevento; CB: Campobasso; PZ: Potenza.
MSE: Mean square error. Different letters (a-c) within the column: differences statistically significant for P<0.05. **: P<0.001; *: P<0.05, NS: not significant.

This aspect has probably influenced the results obtained as the pedo-climatic characteristics of the area where fodder grows can also significantly affect nutritional parameters due to plant adaptability or resistance.

The chemical composition and nutritive value of the mixed hays is given in Table 3. All parameters resulted statistically influenced by sampling area, excepted ADL. In general, the crude protein level ranged from 7.92 to 14.20 % DM and NDF content between 42.13 and 58.63 % DM, indicating a medium quality as forage to including in ruminant diets. Considering the

sampling area effect, it is clear that hay produced in the area of Avellino evidenced the most favorable values in terms of CP, structural carbohydrates and UFL, even if the ash content was slightly high.

Table 3.

Nutritive value of the mixed hays produced in the four sampling areas

Area	DM	CP	NDF	ADF	ADL	Ash	UFL*	UFL†
	%			% DM			/kg DM	/kg DM
AV	89.84^{b}	14.19^{a}	42.13^{b}	29.83^{b}	5.17	11.99^{a}	0.65^{a}	0.66^{a}
BN	92.06^{a}	10.08^{ab}	54.05^{a}	38.69^{a}	5.81	9.25^{b}	0.41^{ab}	0.61^{ab}
CB	92.32^{a}	7.92^{b}	58.63^{a}	39.04^{a}	4.66	9.21^{b}	0.28^{b}	0.57^{b}
PZ	90.60^{ab}	12.59^{ab}	49.95^{ab}	35.12^{a}	5.01	8.60^{b}	0.56^{ab}	0.62^{ab}
Prob. t	**	*	**	***	NS	***	*	*
MSE	0.895	2.345	4.960	1.944	1.238	0.734	0.143	0.033

AV: Avellino; BN: Benevento; CB: Campobasso; PZ: Potenza. DM: dry matter; CP: crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber; ADL: acid detergent lignin.
*UFL calculated with 16e equation (Menke and Steingass, 1988). †UFL estimated as proposed by INRA (1988).
MSE: Mean square error. Different letters (a-b) within the column: differences statistically significant for P<0.05. ***: P<0.001, **: P<0.01, *: P<0.05,
NS: not significant.

On the other hand, the hay produced in Campobasso area showed the less advantageous values for the CP, NDF and UFL. The other two areas, BN and PZ, had intermediate values for all the chemical composition parameters. The nutritional value (UFL/kg DM) estimated using INRA

method is in every case higher than calculated using *in vitro* parameters; however, both system ranked the area in the same way (AV > PZ > BN > CB). The major differences emerged in samples with lower net energy content (BN and CB), in which UFL obtained with Menke and Steingass (1988) resulted very low (0.41 and 0.28, respectively); the gas produced after 24 h of incubation, quite low in these substrates, contributed to this result. As results, the nutritive value calculated with INRA system, only using chemical data, leads to an overestimation of energy availability, mainly in the forage of low quality, compared to a method that use a biological approach.

In vitro fermentation characteristics and methane production

The *in vitro* fermentation characteristics of mixed hays after 120 h of incubation are showed in Table 4, whereas the fermentation kinetics is illustrated in Figure 1. Comparing sampling area, statistically significant differences are observed for all parameters [dOM, B, T_{max} ($P<0.001$) and Yield, A, R_{max} ($P<0.05$)] except for OMCV, even if in CB area the value is clearly lower than in the other areas. In particular, hays produced in PZ area were more degradable (dOM: 70.29 %, $P<0.05$), characterized by a more rapid (R_{max}: 9.64 ml/h and B: 17.5 h, $P<0.05$) and more intense (OMCV: 260 ml/g, not significant) fermentative process. On the other hand, CB produced hays characterized by the lowest degradability (dOM: 60.77 %, $P<0.05$) and slower fermentation kinetics (R_{max}: 7.20 ml/h and B: 20.9 h, $P<0.05$). Regarding the *in vitro* fermentation kinetics (Figure 1) of

analyzed hays, the curves related to gas production and fermentation rate over time (Panel A and B, respectively) show different shape for AV and BN: BN showed a faster fermentation process compared to AV (T_{max}: 6.20 vs. 8.88 h, $P<0.05$; R_{max}: 8.60 vs. 7.64 ml/h, for BN and AV respectively). Some clear differences appear for PZ, characterized by a more rapid and intense fermentation process and for CB with a slower and less intense gas production.

Table 4.

In vitro fermentation characteristics of hays at 120 h

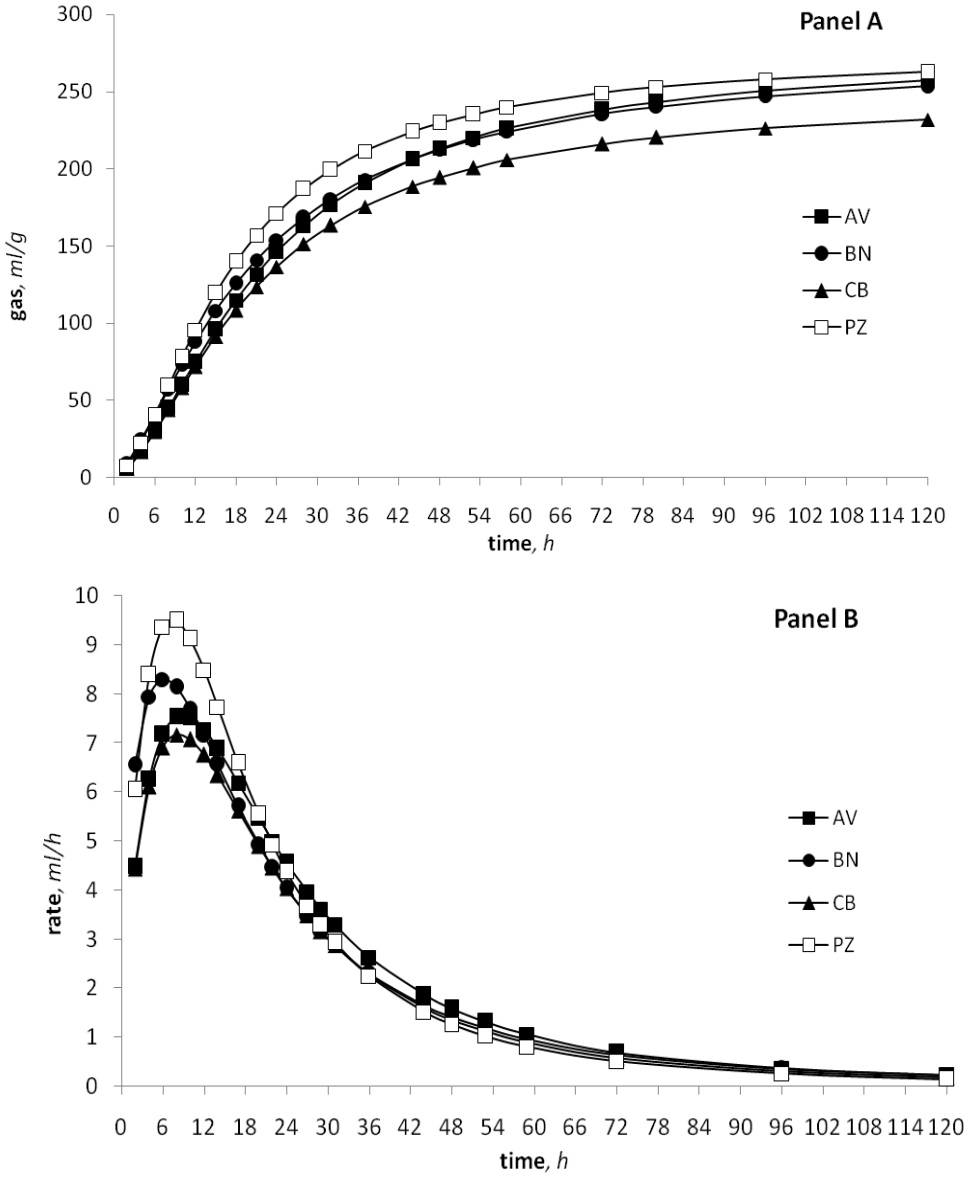
Area	dOM	OMCV	Yield	A	B	T_{max}	R_{max}
	%	*ml/g*	*ml/g*	*ml/g*	*h*	*h*	*ml/h*
AV	69.36^{a}	252	364^{ab}	274	22.1^{a}	8.88^{a}	7.64^{ab}
BN	60.77^{c}	250	413^{a}	273	20.1^{ab}	6.20^{b}	8.60^{ab}
CB	64.81^{b}	230	355^{b}	246	20.9^{a}	8.32^{a}	7.20^{b}
PZ	70.29^{a}	260	370^{ab}	274	17.5^{b}	7.38^{ab}	9.64^{a}
Prob. t	***	NS	**	**	***	***	**
MSE	3.57	461	1185	400	3.90	1.51	2.03

AV: Avellino; BN: Benevento; CB: Campobasso; PZ: Potenza. dOM: organic matter degradability; OMCV: cumulative gas production related to incubated organic matter; Yield: cumulative gas production related to degraded organic matter; A: potential gas production; B: time at which A/2 was formed; T_{max}: time at which maximum rate was reached; R_{max} = maximum fermentation rate.

MSE: Mean square error; Different letters (a-c) within the column: differences statistically significant for P<0.05. ***: P<0.001, **: P<0.01, NS: not significant.

Figure 1.

In vitro fermentation kinetics of mixed hays produced in different area



Panel A: gas production; Panel B: fermentation rate.
AV: Avellino; BN: Benevento; CB: Campobasso; PZ: Potenza.

Volatile fatty acids and pH measured after 120 h of incubation are showed in table 5. No statistical differences appear in hay samples of the sampling areas, except ($P<0.01$) for iso-butyric, iso-valeric and valeric acids. These three branched-chain fatty acids formed in the rumen, generally in small quantities, by deamination of amino acids: iso-butyric acid from valine, valeric acid from proline, 2-methyl butyric acid from isoleucine and 3-methyl butyric acid from leucine (McDonald et al., 2010). The total VFA are higher in AV and CB and lower in BN and PZ, and the same tendency is present in acetic and propionic acid. The pH values after 120 h of incubation is adequate for the fermentation of cellulolytic bacteria (Doane et al., 1997), even if slightly low for CB (pH: 6.08). The sampling area influenced significantly only some parameters recorded at 24 h (data not showed): dOM, OMCV and iCH_4. The highest methane production (pCH_4: 14.48 %; $P<0.05$) was measured in hays from PZ area, associated to the high acetate (58.5 mmol/g) and butyrate (7.56 mmol/g) production. On the contrary, CB area hay produced less methane (pCH_4: 11.72 %) associated to the highest propionate acid production (19.9 mmol/g). McAllister et al. (1996) and Moss et al. (2000), argued that in the *in vitro* study the methane production is related to volatile fatty acids, acetate and butyrate promote the methane production while the propionate formation can be considered as a competitive pathway for hydrogen use in the rumen. The data concerning methane production after 24 h of incubation are showed in Figure 2, together with dOM (24 h) and NDF content.

Table 5.

pH and volatile fatty acids after 120 h of incubation

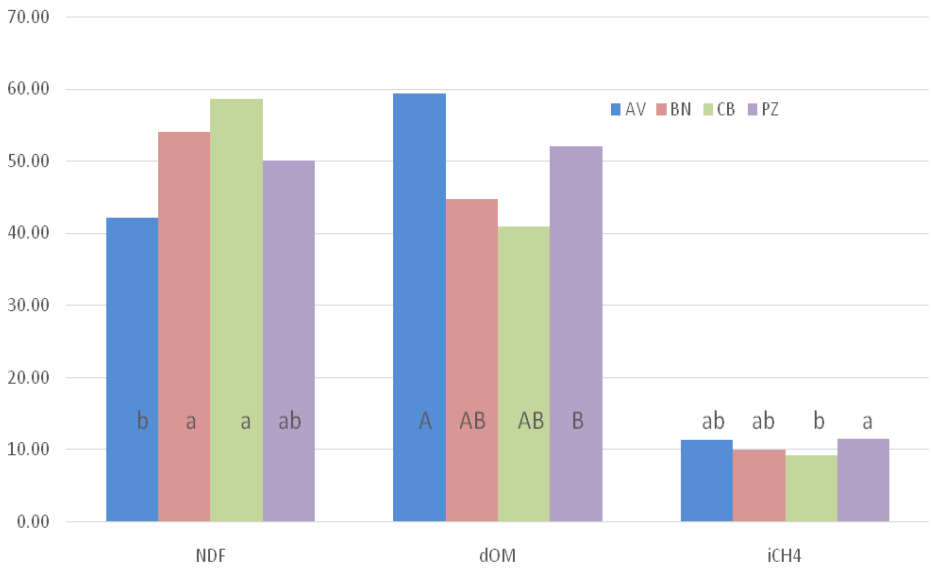
Area	pH	Acetate	Propionate	Butyrate	Iso-butyrate	Iso-valerate	Valerate	tVFA
					mmol/g			
AV	6.29	66.4	19.2	6.65	0.86^{a}	1.47^{a}	1.49^{a}	96.1
BN	6.47	58.0	16.8	5.63	0.82^{a}	1.34^{ab}	1.05^{b}	79.7
CB	6.08	70.3	18.4	5.80	0.56^{b}	0.81^{c}	0.92^{b}	96.8
PZ	6.45	54.0	15.2	5.39	0.52^{b}	0.87^{bc}	1.04^{b}	75.5
Prob. t	NS	NS	NS	NS	***	***	***	NS
MSE	0.109	124.93	8.59	0.646	0.007	0.087	0.046	254.55

AV: Avellino; BN: Benevento; CB: Campobasso; PZ: Potenza. tVFA: total volatile fatty acids.
MSE: Mean square error. Different letters (a-c) within the column: differences statistically significant for P<0.05. ***: P<0.001, NS: not significant.

The lowest methane production was measured in CB (iCH_4: 9.18 ml/g), which however, also showed the lowest dOM (40.9 %), probably due to the highest structural carbohydrates content (NDF: 58.63 % DM). On the contrary, AV showed the highest methane production (iCH_4: 11.25 ml/g), OM degradability (59.3 %) and a low NDF content (42.13 % DM). These results are also in line with the nutritive value (UFL/kg DM: 0.65 vs. 0.28, for AV and CB respectively) and maybe influenced by CP content (14.19 vs. 7.92 % DM, for AV and CB respectively); not so clear is the role of hemicellulose content, higher in CB and lower in AV (19.59 and 12.30 % DM, respectively).

Figure 2.

In vitro fermentation characteristics after 24 h of incubation



AV: Avellino; BN: Benevento; CB: Campobasso; PZ: Potenza.
NDF: Neutral detergent fiber (% DM). dOM: organic matter degradability (%);
iCH_4: methane production related to incubated OM (ml/g).
A-B and a-b: differences statistically significant for P<0.001
and P<0.05, respectively.

Relationship between chemical composition, sensory evaluation and in vitro data

The correlation results between chemical composition, sensory evaluation and *in vitro* parameters are presented in table 6. Not many parameters were statistically correlated, maybe due to the low number of observations (DF = 6). As expected, regarding chemical composition, protein and nutritive value were significantly and positively correlated with the leaf content (P<0.05 and P<0.01, respectively) whereas

negatively correlated to structural carbohydrates (P<0.001). The leafiness is also slightly (P>0.05) related to dOM, OMCV and CH_4. Some authors observed that the increased fiber concentration in plant tissues reduced DM degradation obtained *in vitro* and *in situ* techniques (Wilson et al., 1991) and influenced leaf: stem ratio reducing it (Hides et al., 1983). Moreover, protein content was related to gas and total VFA production (P<0.01), whereas nutritive value only with OMCV as well as structural carbohydrates (-0.77; P<0.05), which are correlated also with dOM, even if the correlation coefficient was not statistically significant (ADF: -0.68; P>0.05). Also Ahmed et al. (2014) reported a significant correlation between the crude protein content and the *in vitro* gas data, when corn hybrids were incubated. As reported by Getachew et al. (2004), significant relationship between chemical data and *in vitro* parameters were found, confirming that the IVGPT is a reliable method to describe feedstuff's characteristics. However, the correlations between OM degradability and chemical composition were poor and not significant. As reported by El Hassan et al. (1995), anti-nutritional factors, such as phenolics, can inhibit growth of rumen cellulolytic species of *Ruminococcus* and influence degradability. The configuration of cell-wall polysaccharides also influences the rumen microbial attachment and colonization of digesta particles (Cheng et al., 1984). Chemical composition is also correlated with methane production: CP and UFL (calculated with *in vitro* data) are positively correlated (P<0.05) while the NDF value is negatively correlated (P<0.05). Already many authors including Moss et al. (2000) discussed the

strong relationship between cell wall components and *in vivo* emission of CH_4. However, as suggested by Carulla et al. (2005), inhibition of methanogenesis is due to the reduction in fiber degradation, that limits the acetate production through the reduction in cellulolytic number (McSweeney et al., 2001). At the opposite, the soft consistency of the hay is significantly ($P<0.05$) related with the methane production. As expected, a statistically significant value was found in the relationship between gas and methane production ($P<0.05$). Not clear to understand, the statistically and negatively ($P<0.01$) correlations between the odorous component detected in the hay and the total volatile fatty acids production. Not easy to explain also the significant, but negative correlation between OMCV and tVFA, because many authors (Blümmel and Ørskov, 1993; Doane et al., 1997; Ahmed et al., 2014) reported that gas production is associated with volatile fatty acid.

Comparison between sampling area

In general, little differences among sampling areas were observed regarding all parameters considered (i.e. sensory evaluation, chemical composition, *in vitro* fermentation characteristics and methane production).

Table 6.

Correlating coefficients between chemical composition, sensory evaluation and in vitro parameters

	Softness	Odor	Leafiness	dOM	OMCV	Acetic	tVFA	pCH_4	iCH_4	dCH_4
CP	-0.45	-0.41	0.82	0.45	0.83	-0.67	0.71	0.41	0.78	-0.45
	NS	NS	*	NS	*	NS	*	NS	*	NS
NDF	0.55	0.44	-0.90	-0.46	-0.77	0.53	0.43	-0.29	-0.79	0.56
	NS	NS	***	NS	*	NS	NS	NS	*	NS
ADF	0.49	0.37	-0.94	-0.68	-0.40	0.18	0.10	-0.10	-0.62	0.45
	NS	NS	***	NS	NS	NS	NS	NS	NS	NS
UFL†	-0.57	-0.58	0.86	0.28	0.73	-0.60	-0.52	0.22	0.67	-0.57
	NS	NS	**	NS	*	NS	NS	NS	NS	NS
UFL*	-0.43	-0.39	0.83	0.46	0.83	-0.69	-0.60	0.39	0.77	-0.46
	NS	NS	**	NS	**	NS	NS	NS	*	NS
Softness	.	0.89	-0.62	-0.06	0.01	-0.27	-0.35	0.42	-0.03	0.77
	.	**	NS	NS	NS	NS	NS	NS	NS	*
Odor	.	.	-0.54	0.59	0.24	-0.88	0.36	0.77	0.65	-0.16
	.	.	NS	NS	NS	**	NS	*	NS	NS
Leafiness	.	.	.	0.52	0.49	-0.32	-0.24	0	0.60	-0.65
	.	.	.	NS	NS	NS	NS	NS	NS	NS

Table continued on next page

CP: crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber; *UFL calculated with 16e equation (Menke and Steingass, 1988). †UFL estimated as proposed by INRA (1988). tVFA: total volatile fatty acids. OMCV: cumulative gas production related to incubated organic matter; pCH_4: methane production at 24 h related as percentage of total gas; iCH_4: methane production at 24 h related to incubated organic matter; dCH_4: methane production at 24 h related to degraded organic matter.
DF: 6. ***: P<0.001, **: P<0.01, *. P<0.05, NS: not significant.

	Softness	Odor	Leafiness	dOM	OMCV	Acetic	tVFA	pCH_4	iCH_4	dCH_4
dOM	.	.	.	.	0.24	-0.15	-0.08	0.39	0.37	0.07
	.	.	.	.	NS	NS	NS	NS	NS	NS
OMCV	.	.	.	.	.	-0.88	-0.82	0.58	0.86	-0.21
	.	.	.	.	.	**	**	NS	**	NS
Acetic	.	.	.	.	.	.	0.99	-0.55	-0.65	0.04
	.	.	.	.	.	.	***	NS	NS	NS
tVFA	.	.	.	.	.	.	.	-0.50	-0.57	-0.01
	.	.	.	.	.	.	.	NS	NS	NS
pCH_4	.	.	.	.	.	.	.	.	0.65	0.55
	.	.	.	.	.	.	.	.	NS	NS
iCH_4	.	.	.	.	.	.	.	.	.	-0.16
	.	.	.	.	.	.	.	.	.	NS
dCH_4	.	.	.	.	.	.	.	.	.	.
	.	.	.	.	.	.	.	.	.	.

tVFA: total volatile fatty acids. OMCV: cumulative gas production related to incubated organic matter; pCH_4: methane production at 24 h related as percentage of total gas; iCH_4: methane production at 24 h related to incubated organic matter; dCH_4: methane production at 24 h related to degraded organic matter.
DF: 6. ***: P<0.001, **: P<0.01, *. P<0.05, NS: not significant.

However, the best area sampling, in terms of chemical composition (CP and NDF content) and nutritive value (UFL/kg DM) was the forage produced in Avellino area that also showed the most interesting *in vitro* characteristics (high OM degradability, gas and VFA production) and favorable environmental characteristics (low CH_4 production). On the other hand, the forage produced in Benevento area resulting medium in chemical composition, low in OM degradability and VFA production, and medium in CH_4 production; whereas Campobasso area produced forage with low environmental impact, but characterized also by a less favorable nutritive value. These considerations are in both case not in according to the sensory evaluation. The hay produced in Potenza province, showed better characteristics in terms of sensory evaluation and *in vitro* parameters, not completely in accordance with the chemical composition and environmental impact.

Considering the environmental condition of the areas covered by the studies, we can say that probably the low altitude (360 m a.s.l.) of this area, but also the high rainfall and intermediate temperature recorded in the sampling year (1354 mm and 13.9°C, respectively) in the Avellino area favored the production of a best quality hay compared to the other areas. On the contrary, the environmental condition of Campobasso area, sited in at higher altitude (701 m a.s.l.) and characterized in 2015 by low rainfall and intermediate temperature (583 mm and 12.3°C, respectively), are in part responsible of the lower quality of produced hay. In the area of Potenza, was produced a good quality forage probably due to high

altitude (760 m a.s.l.), not very rigid temperature (11.3°C) and low rainfall (650 mm). The most important environmental factors that influence forage quality are temperature, water deficit, solar radiation, and soil nutrient availability; of these factors, temperature usually has the greatest influence (Buxton, 1996). Optimal growth temperatures are near 20°C for cool-season species such as alfalfa, orchardgrass, and ryegrass (Buxton, 1996). At temperatures below the optimum for growth, soluble sugars accumulate because of the lower temperature sensitivity of photosynthesis compared with that of growth. A rise in temperature normally increases rate of plant development and reduces leaf/stem ratios and digestibility. Increasing temperature lowers forage quality even when compared at the same morphological stage. The depressed digestibility associated with elevated temperatures is usually attributed to higher NDF concentrations. Additionally, the NDF of forages grown under higher temperatures is usually less digestible than that of forages grown under lower temperatures because of increased lignification (Buxton, 1996).

CONCLUSIONS

Studied forages presented a rather variable chemical composition that could depend from many factors (i.e. environmental condition of the sampling area, during the plant growth and harvest, as well as the haymaking technique, but also the botanical species present in the hays),

which may have influenced the *in vitro* fermentation characteristics, including methane production.

Data obtained from this preliminary study provide some useful information about forages produced in some provinces of Southern Italy, where high quality dairy products are produced. The knowledge of these characteristics and the need to improve them, if necessary, is required by the farmers in order to make balanced rations to maintain animal health and guarantee high level of production, in terms of quality and quantity.

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Chapter 4.

Third Contribution

Assessing of *in vitro* methane production in mixed hays produced over a 3-years period

ABSTRACT

The objective of the investigation was to evaluate the environmental impact, in terms of *in vitro* methane production, of forages sampled over a 3-year period provided by a farm located in Southern of Italy and preserved as hay. The substrates to test were incubated *in vitro* using gas production technique, for 24 h with cow rumen fluid under anaerobic condition. The *in vitro* fermentation characteristics and kinetics, as well as chemical composition, including nutritive value and sensory evaluation were also determined. The methane was measured by gas-chromatography and estimated with stoichiometric calculation. The results evidenced that the climatic condition can influence hay quality. As expected, methane production is affected by some nutrients concentration. The methane estimation using volatile fatty acids needs better verification. Methane production related to the incubated OM linearly decreased moving from 2014 to 2016.

Keywords*: Mediterranean area, greenhouse gas, fermentation characteristics, stoichiometric calculation, volatile fatty acids.*

INTRODUCTION

Human activities are the main responsible for global warming for the emission into the atmosphere of large quantities of greenhouse gases (GHG), mainly carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O). The agricultural and livestock sectors, mainly ruminants (dairy,

beef, goats, and sheep), are the main responsible for the methane emissions (Koneswaran and Nierenberg, 2008), indeed, about 67% of the methane present in the atmosphere derives from rumen fermentation; for this reason, try to reduce its emission modulating feeding plan for animals have induced great interest in recent years (Grazioli, 2016). However, it is well known that CH_4 is also an energy loss of the animals, which is assumed to vary between 2 and 12% of gross energy ingested (Johnson and Johnson, 1995); its elimination represents a reduction of the productivity for the animal and, therefore, an economic loss (Johnson and Johnson, 1995; Hindrichsen et al., 2006).

Methane produced in the rumen during the normal fermentation of feedstuffs is then exhaled through the mouth and nose. The CH_4 emission varies based on the many factors, depending by animal (i.e. species, physiological phase, feed intake, etc.), by feed (i.e. composition, quality, processing, etc.) and by geographical location as well (Hook et al., 2010). The rumen ecosystem is an anaerobic environment, in which nutritional components are degraded by the different kind of microorganisms (bacteria, protozoa, fungi); the fermentation products are principally volatile fatty acids (mainly acetate, propionate, and butyrate), used as energy source by the host. During the fermentation the cofactors (NADH, NADPH, FADH) are re-oxidized (NAD-1, NADP-1, FAD-1) and in this oxidative process (dehydrogenation reaction) hydrogen (H_2) is releasing. The methanogenic Archaea (*Methanobacter* spp.) utilize H_2 to reduce CO_2

into CH_4, as their physiological end-product (Broucek, 2014), according to the following equation:

$$4 \cdot H_2 + CO_2 \rightarrow CH_4 + 2 \cdot H_2O$$

Direct quantification of CH_4 produced by animals requires complex equipment, is labour intensive, time consuming and expensive. Several studies on methane production by ruminants have been carried in the recent years using *in vitro* methods (Ramin and Huhtanen, 2012; Navarro-Villa et al., 2013; Patra and Yu, 2013; Cattani et al., 2014). The *in vitro* gas production technique (IVGPT) offer a valid alternative, allowing several diets and diet combinations to be evaluated simultaneously. Using the IVGPT, *in vitro* CH_4 production can be directly measured (Guglielmelli et al., 2011) or accurately estimated (Getachew et al., 2005).

Seasonal variation in environment (i.e. temperature, water deficit) alters forage quality, even when forages are harvested at similar maturity stages. Plant environment often exerts its greatest influence on forage quality by altering leaf/stem ratios, but it also causes modifications in plant development and changes in chemical composition of plant parts, with consequence on structural carbohydrate concentrations, lignification process and organic matter digestibility (Buxton and Fales, 1994). The main objective of the investigation was to evaluate the environmental impact, in terms of *in vitro* methane production, of forages sampled of a 3-year period. A second aim was to estimate the methane production using the stoichiometric calculation starting from volatile fatty acids

obtained *in vitro*. The hypothesis is that the environmental condition can influence the forage quality in terms of methane emission.

MATERIALS AND METHODS

Experimental design

Six samples of mixed hay were studied. After collection, for each sample a sensory evaluation was made in the field. Then, the samples were transported to the laboratory of "Feed analyses" DMVPA (University of Napoli Federico II, Napoli, Italy), where they were analyzed for chemical composition. The environmental impact was evaluated measuring the methane production after 24 hours of *in vitro* incubation with cow rumen fluid and estimated using stoichiometric calculation. The *in vitro* fermentation characteristics (i.e. pH, gas, degradability, end-products) were also determined after 120 h of incubation.

Samples collecting

The forage samples were collected directly from hay bale during the autumnal season (the first sample in September, the second one in October) in three consecutive years (2014, 2015 and 2016) in the same agricultural farm located in upland of Formicoso (Alta Irpinia area, province of Avellino, Southern of Italy) at 750 m a.s.l., with latitude 40°59'N and longitude 15°28'E, where the same typology of cultivated essences, the same agronomic and harvesting techniques have been maintained over the three years.

Sensory evaluation, chemical analyses, nutritive value

The sensory evaluation of the hay samples was carried out utilizing a scorecard (see chapter 2) set up in collaboration with ANFoSC (www.anfosc.it). Before the analyses, all the samples were ground to pass a 1 mm screen (SM 100, Retsch, Haan, Germany). The hay samples were then analyzed for dry matter (DM), crude protein (CP), ether extract (EE), crude fiber (CF) and ash according to official protocol by Association of Official Analytical Chemists (AOAC 2005; www.aoac.org) procedures (ID number: 2001.12, 978.04, 920.39 978.10 and 930.05 for DM, CP, EE, CF and ash, respectively). Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were also determined (Van Soest et al., 1991). The hays' nutritive value, expressed as net energy for lactation (Unité Fourragère du Lait, UFL), was calculated according to INRA system (1988) utilizing the chemical composition parameters (DM, CP, EE, CF, ash).

Methane determination and estimation

The methane production was determined *in vitro* after an IVGPT gas run stopped at 24 h according to the protocol proposed by Guglielmelli et al. (2011). At this aim six dairy cows (mean body weight 680 kg) fed a total mixed ration containing corn silage, oat hay and concentrate (CP 12 and NDF 43.5 % DM) were used as rumen fluid donors. In particular, equal volume of material was collected from each cow at a slaughterhouse authorized according to EU legislation (EU regulation No. 882/2004). The

collected rumen fluids were quickly transported to the lab in pre-heated thermos flasks, mixed, and strained through four layers of cheesecloths ensuring the temperature of 39°C and the anaerobic conditions, according to the protocol suggested by Calabrò et al. (2015). The rumen fluid (10 ml) was then mixed to anaerobic medium (75 ml) and reducing agent (4 ml) and dispensed anaerobically into 120 ml serum flasks containing 1.0201 ± 0.0279 g of substrate. The serum flasks were sealed and placed into an incubator at 39.0 ± 0.5°C for 24 h and were not agitated during incubation. After 24 h of incubation, the volume of gas accumulated in the headspace of each serum flask was recorded using a manual pressure transducer (Cole & Parmer Instrument Co., Barringhton, IL USA). Then the gas phase from each serum flask was sampled (3 ml) in duplicate with a gastight syringe; the analysis was carried out using a gas chromatograph (GC Trace 1310, Thermo Scientific, Waltham, MA USA) equipped with a loop TC detector and a packed column (HaySepQ SUPELCO, 3/16 inch, 80/100 mesh).

The organic matter degradability was determined by weight difference of the incubated OM and the undegraded residue throughout sintered glass crucibles (Schott Duran, Mainz, Germany, porosity #2). For volatile fatty acids determination, a sample of fermenting liquor was centrifuged at 12.000 × g for 10 min at 4°C (Universal 32R centrifuge, Hettich FurnTech Division DIY, Vlotho, Germany) and an aliquot (1 ml) of supernatant was mixed with 1 ml of oxalic acid (0.06 mol) VFA were measured by gas chromatography (GC Focus AI 3000, Thermo Scientific, Waltham, MA

USA) equipped with a fused silica capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness), using an external standard solution composed of acetic, propionic, butyric, isobutyric, valeric and isovaleric acids, as described by Musco et al. (2016).

Acetic, propionic and butyric acids (mmol) produced after 24 h of incubation were utilized to estimate CH_4 (mmol) production according to the following equations (Van Soest, 1994):

$$CO_2 = \frac{Acetic}{2} + \frac{Propionic}{4} + 3 \times \frac{Butyric}{2}$$

$$CH_4 = Acetic + 2 \times Butyric - CO_2$$

In vitro fermentation characteristics, final products, kinetics

Following the identical experimental conditions as just described above (Calabrò et al., 2015) the same substrates were also incubated with the aim to study, after 120 hours of incubation, the fermentation characteristics [OMCV, total volume of gas accumulated in the headspace related to incubated OM (ml/g); dOM, organic matter degradability determined for filtration (%)] and final products [(VFA, volatile fatty acids as described above (mmol/g)]. During the period of incubation, the gas production was recorded 24 times (at 2 to 24 h intervals) using the manual pressure transducer (Cole and Palmer Instrument Co., Vernon Hills, IL, USA). For each bottle, the gas production profiles were fitted to the following model (Groot et al., 1996):

$$G = \frac{A}{(1 + \frac{B}{t})^C}$$

where G is the total gas produced (ml per g of incubated OM) at time t (h), A is the asymptotic gas production (ml/g), B is the time at which one-half of A is reached (h), and C is the curve switch. Maximum fermentation rate (R_{max}, ml/h) and the time at which it occurs (T_{max}, h) were calculated utilizing model parameters (Bauer et al., 2001):

$$T_{max} = C \times (\frac{B-1}{B+1})^{1/B}$$

$$R_{max} = \frac{(A \times C^B) \times B \times Tmax^{(B-1)}}{(1 + C^B) \times (Tmax^{-B})^2}$$

Statistical analyses

The effects of the year collection were statistically evaluated for the parameters obtained related to chemical composition (DM, CP, NDF, ADF, ADL, ash and UFL), *in vitro* fermentation and kinetics (dOM, OMCV, model parameters, end-products) and methane production (CH_4 determined and estimated) with the following model:

$$Y_{ij} = \mu + CY_i + + \varepsilon_j$$

where for each parameters (*Y)* μ represents the general mean, *Y* the year collection (2014, 2015, 2016) and ε the error effect. The two samples taken each year in September and October represent the replications. When comparing different preservation method, differences were statistically assessed at 5% using Student's t-test (SAS, 2000).

RESULTS AND DISCUSSION

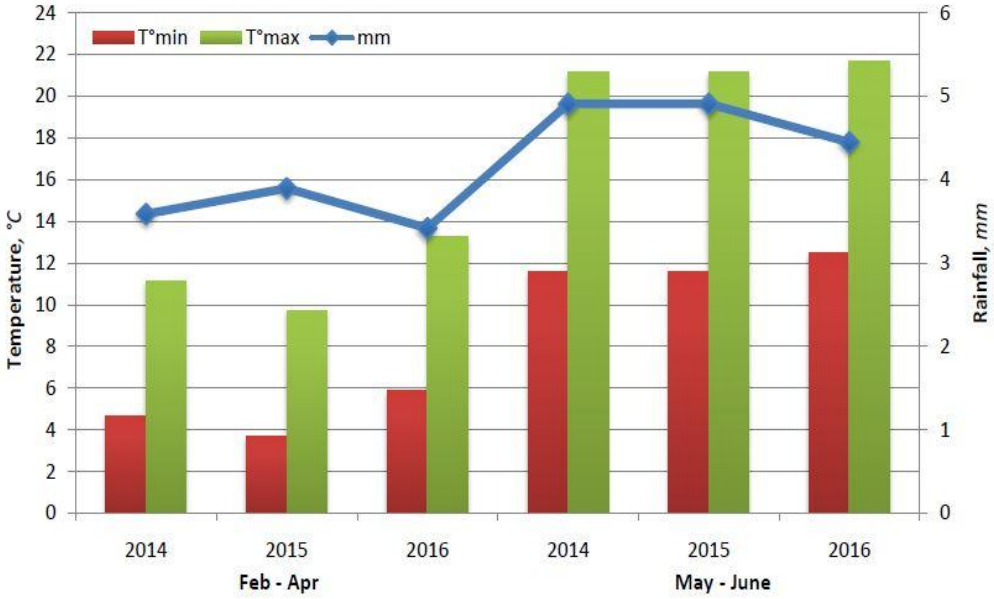
Comparing year of sampling for hay quality

The environmental conditions in the area sampling (upland of Formicoso, AV) of hay production during the critical periods corresponding to the first vegetative stage (February, March, April) and the cutting, curing and raking phases (May, June) in the three years of sampling are reported in Figure 1. During the first vegetative stage of the plants, when the plant should be enriched with leaves, and therefore proteins, the minimum temperatures were quite rigid (many values under 0°C) in 2015 and the precipitation, lower but snowy, in 2016. On the other hands, during the haymaking process, when low moisture favour drying process but too high temperatures occur in high structural carbohydrate concentration (Suttie, 2000; www.fao.org; Poethig, 2013), few differences appear, only 2016 was slightly less rainy.

The sensory evaluation evidenced the best quality of hay produced in 2014 compared to the others two (mean value: 80/100 vs. 65/100 and 66/100, for 2014 vs. 2015 and 2016 respectively). The chemical analyses and nutritive value of the mixed hays collected over the 3-period year is presented in Table 1. It is clear that all parameters resulted highly influenced by sampling year. The best chemical composition (in terms of high CP and UFL values, and low NDF values) was observed in the hay produced in 2014 and 2015, and, at the opposite the hay produced in 2016 showed the worst characteristics (CP: 15.16, 14.18 and 12.43 %DM; NDF: 52.27, 40.76 and 58.03 %DM; 2014, 2015 and 2016 respectively).

Figure 1.

Environmental conditions during the critical period of hay production in the three years



Source: www.3bmeteo.com

In figure 2, *in vitro* fermentation process, gas production and fermentation rate over time, are pictured for hay samples collected in the three years. According to the chemical composition, the most rapid *in vitro* fermentation kinetics (in terms of high R_{max} and low T_{max}) is showed in hay collected in 2014. On the other hands, the lower fermentation process is evidenced in hay sample collected in 2015; maybe the unfavorable climatic condition (low temperature) during the vegetative stage of the plants contributed to these results.

Table 1.

Effect of year sampling on chemical composition and nutritive value

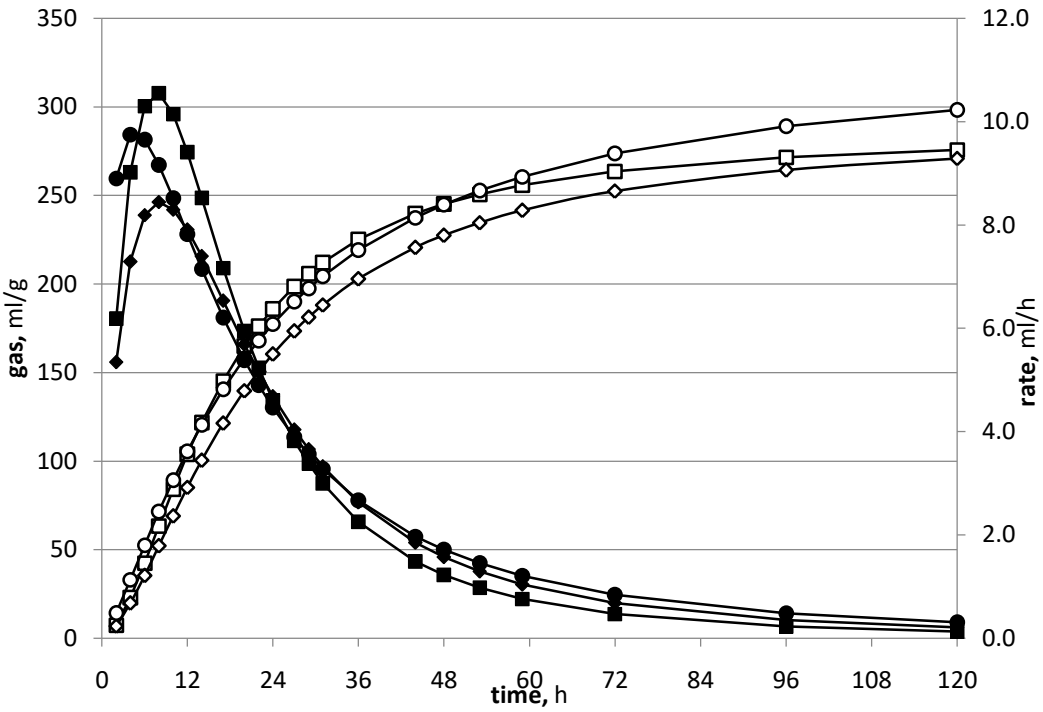
Year	DM	CP	NDF	ADF	ADL	Ash	UFL
	%			*% DM*			*/kg DM*
2014	90.20^{a}	15.16^{a}	52.27^{b}	32.54^{b}	5.33^{ab}	10.35^{b}	0.73^{a}
2015	88.76^{c}	14.18^{b}	40.76^{c}	29.12^{c}	4.56^{b}	12.39^{a}	0.65^{b}
2016	89.74^{b}	12.43^{c}	58.03^{a}	41.96^{a}	7.43^{a}	9.55^{c}	0.53^{c}
P value	0.0001	0.0001	0.0001	0.0006	0.0002	0.0008	0.0001
MSE	1.069	0.857	3.416	2.997	0.843	1.005	0.037

DM: dry matter; CP: crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber; ADL: acid detergent lignin. UFL: Unité Fourragère du Lait.
MSE: Mean square error. Different letters (a-c) within the column: differences statistically significant for P<0.05.

The fermentation characteristics measured after 120 h of incubation are reported in figure 3. The trend of these parameters can be explain as follows: the high CP content in hay produced in 2014 favor the OM degradability (73.30%), but not gas and volatile fatty acids production (OMCV: 274 ml/g and tVFA:75.45 mmol/g); the low ADL (4.56 %DM) content favored high total VFA production (96.97 mmol/g) in sample collected in 2015.

Figure 2.

In vitro fermentation kinetics in the three years



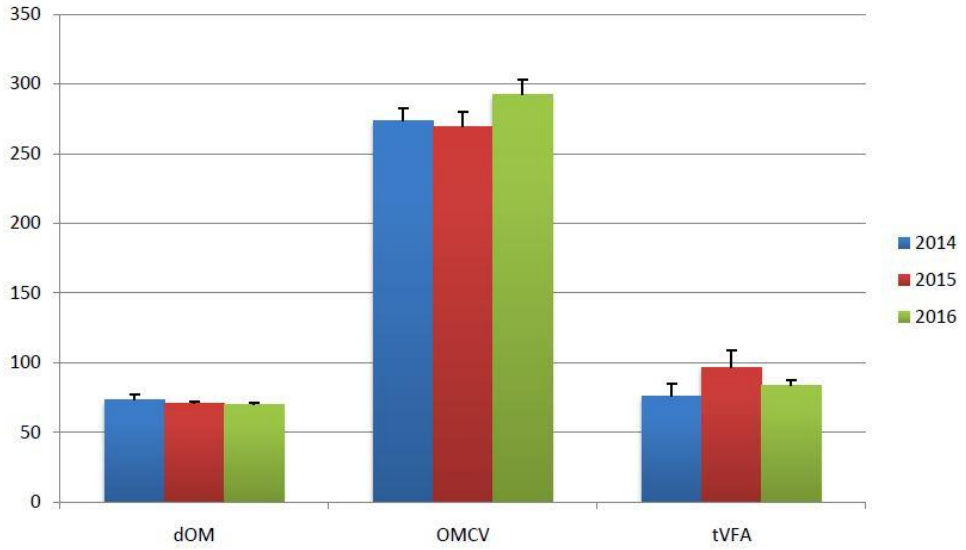
Gas production: white symbol. Fermentation rate: black symbol. 2014: square □, 2015: rhombus ◇, 2016: circle ○.

As tradition in this area the fodder production is almost exclusively destined to become hay. The productivity of grassland follows a seasonal pattern where harsh winters and dry summers characterize the climate. Autumn-winter grasses are cultivated almost exclusively in primary culture, in rotation with cereal crops. The sowing time is clearly influenced by weather conditions: the seeds are planted between late October and early November. The cutting is, certainly, the most important

step of the entire process, the fresh forage mown is dried for 4-5 days in the field, until reaching 900 g kg of dry matter.

Figure 3.

In vitro fermentation characteristics after 120 h of incubation



dOM (%): organic matter degradability; OMCV (ml/g): cumulative gas production related to incubated organic matter; tVFA (mmol/g): total volatile fatty acids (acetic + propionic + butyric + isobutyric + valeric + isovaleric) related to incubated OM.
Error bars indicate the standard deviation for each parameter in every year.

At this stage the rain is definitely the most dangerous enemy. The forage harvesting is carried out using rectangular collectors and hay can be made into round or square bales. The most used in this area are the bales, this is due to the fact that the bales are wrapped in a mesh network more or less tight in plastics, have the possibility to stay on the field even during a few days of rain without this affects too much quality.

Methane production

Regarding methane production, the parameters recorded at 24 h of incubation are showed in Table 2. The year of sampling clearly affected the reported parameters. As expect, the highest methane production is related to highest structural carbohydrates content, however, if the plant cell wall is lignified the degradability is compromised with consequently lower methane production, as the case of samples collected in 2016, which showed the highest ($P<0.01$) NDF content (58.08 ml/g), but the lowest ($P>0.05$) OM degradability (70.68 %) and consequently the lowest ($P<0.05$) CH_4 production (9.23 ml/g). On the other hand, the highest ($P<0.05$) CH_4 production in samples collected in 2014 (13.0 ml/g) could be due to the higher content in hemicellulose (19.73, 11.64, 16.07 % DM, in 2014, 2015 and 2016, respectively) that in the first hours of incubation favors CH_4 formation; also, the high protein content of this substrate (15.16 % DM; $P<0.05$) may have undirectly contributed to methane production favouring the growth of methanogens bacteria. Quantity and quality of feed utilized by ruminants have a key effect on the methane production and is due to the individual volatile fatty acids proportion in the rumen, which is influenced by forage: concentrate ratio (Fahey and Berger 1988), nature and rate of fermentation of carbohydrates (Johnson et al., 1996). Roughage-based diets favor acetate production and increase methane emission per unit of fermentable organic matter (Johnson and Johnson, 1995).

In all cases, methane estimated data are always higher compared to those determined; however, the two parameters ranked the substrates in the same order (2014 > 2015 > 2016). Getachew et al. (2005), incubating commercial total mixed ratios with cow rumen fluid, found estimated values for total CH_4 production, calculated from VFA production, only slightly higher than those measured (about an 8.6 % of difference), in our case the differences are much higher (about 60%). On the contrary, Eun et al. (2004) in their study have evaluated the effects of dilution rate and forage-to-concentrate ratio on gas production by rumen microbes and have reported that CH_4 stoichiometrically calculated was poorly correlated with measured values. Methane from agriculture arises primarily from enteric fermentation; therefore, ruminants (especially beef and dairy cattle) are mainly responsible for its enteric emissions (Kebreab et al., 2006). Reducing carbon conversion of ruminally degraded feed into methane increases feed efficiency and reduces emission of this potent GHG into the environment. In the past, many investigations of enteric CH_4 production were basing on measurements of emission from animals in respiration chambers under strictly controlled environments (Murray et al., 1999). The *in vitro* methods have the advantage not only of being less expensive and less time-consuming, but also it is possible to maintain experimental conditions more precisely than *in vivo* trials (Getachew et al., 1998). *In vitro* techniques, using rumen fluid as an inoculum, can be considered as models of *in vivo* rumen digestion (Moss and Givens, 2002). The similarity between measured and calculated CH_4 values with IVGPT

suggests that its production can be calculated if only gas and VFA production is measured (Getachew et al., 2005); this is important since many laboratories only have VFA analysis capability.

Table 2.

Effect of year sampling on methane recorded at 24h

Year	**determined**						**estimated**
	pCH4	**iCH4**	**dCH4**	**Ace***	**Prop***	**But***	**CH_4**
	%	*ml/g*	*ml/g*	*mol*	*mol*	*mol*	*ml/g*
2014	11.36[b]	13.00[a]	24.96[a]	3.11	0.90	0.33	36.88[a]
2015	13.00[a]	12.25[a]	19.71[b]	3.13	1.31	0.47	31.23[b]
2016	10.79[b]	9.56[b]	25.76[a]	2.96	0.94	0.45	30.50[b]
P value	0.0056	0.0001	0.0034	-	-	-	0.0004
MSE	0.245	0.311	0.876	-	-	-	3.076

pCH_4: methane production related as percentage of total gas; iCH_4: methane production related to incubated organic matter; dCH_4: methane production related to degraded organic matter.

*Acetic, Propionic and Butyric acid used to calculate methane production.

MSE: Mean square error. Different letters (a-c) within the column: differences statistically significant for $P<0.05$.

CONCLUSIONS

In this study, the methane production, determined using the *in vitro* technique, provide data in agreement with some nutrients concentration. However, the methane estimation using volatile fatty acids produced *in vitro* needs better verification. Despite the low number of samples, a linearly tendency in decreasing methane production related to incubated OM moving from 2014 to 2016 can be observed. Many authors reported that, methane estimated by IVGPT is very close to that measured *in vivo*, demonstrating that the system could be used to estimate the potential CH_4 production from feedstuffs to generate a database in order to plan mitigation strategies in ruminants to improve their performance as well as to reduce the GHG. In this study, notwithstanding the estimated data are similar to some authors as well as the determined one to other references. In addition, the relationship between chemical composition and methane production is not always clear. Consequently, further *in vitro* researches are recommended in this area to increment the testing forage, better if characterised by elevate differences in nutritional profiles, in order to identify those ones better either for the nutritional (high degradability) either eco-sustainability (low methane) characteristics.

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Chapter 5.

Fourth Contribution

Effect of haymaking on *in vitro* fermentation characteristics

ABSTRACT

The aim of this investigation was to study on the effect of haymaking in three mixed forages, on the *in vitro* fermentation characteristics, including methane production. Samples of three mixed forage, fresh and after haymaking, were collected from three different farms located in the Mediterranean area. For all the samples, the fermentation characteristics and kinetics were studied with the *in vitro* gas production technique using a manual system and cow rumen fluid as *inoculum* source. Methane production was measured after 24 h of incubation by gas-chromatography. As results, a worsening of the *in vitro* fermentation characteristics emerged as direct consequences of haymaking process. Methane production also increases moving from fresh to hay.

Keywords: *Mediterranean area, gas production, degradability, methane, volatile fatty acids.*

INTRODUCTION

In most parts of the world, forage conservation is a key element for productive and efficient ruminant livestock system. Forage conservation methods such as ensiling and haymaking allow a more reliable supply of quality feed when forage production is low or latent. The conservation of forage is a way to reduce the availability variations due to the seasons. Haymaking is the most traditional and widespread method to preserve the fodder. Haymaking offers a number of advantages: harvesting can

largely be mechanized, hay stores well when adequately protected and hay can satisfy the nutritional requirements of most classes of livestock (Lacefield et al., 1999). Forage cannot be preserved as harvested, but must be appropriately transformed, as the grass, due to its high-water content, would be rapidly deteriorated. Grass transformation must be as fast as possible in order to obtain a stable product that retains the nutritional qualities. Crop growth is influenced by the weather condition, but green fodder is only available at certain times of the year, and shorter is the growing season, more uneven is its distribution in time. The conservation of forage and crop is a traditional way to reduce seasonal variations in availability (Suttie, 2000; www.fao.org). Haymaking involves the drying of the forage thanks to the action of solar radiation (traditional haymaking) or by ventilation systems with hot air. The final quality of the hay depends on the quality of fresh forage (related to botanical species and cultivar), mowing season, climatic conditions during haymaking, cutting, weaning, turning, harvesting and storage. The quality of the hay depends significantly on the stage of maturity at harvest and the care taken in drying and storage. The object of preservation is to conserve nutrients as efficiently as possible; the grade of preservation depends on a rapid drying (Van Soest, 1982).

During the drying process, from fresh grass to hay, there are some losses of nutrients that derive from the action of plant enzymes, chemical oxidation and mechanical damage. After the cut of grass the plant the enzyme activities continue, the main changes involve the soluble

carbohydrates and nitrogenous component (McDonald et al., 2010). During the periods of drying, as a result of respiration, there is an increase in the concentration of constituents in the plant, especially the cell-wall components, which are reflected in the neutral-detergent fiber content (NDF). Furthermore, proteases present in the plant cells rapidly hydrolyze the proteins, hydrolysis being followed by some degradation of specific amino acid (McDonald et al., 2010). So, the effect of haymaking is reflected, principally, in crude fiber and crude protein contents. During the haymaking, a certain amount of oxidation occurs, the carotene, for example, may be reduced (from 150-200 mg/kg in the fresh grass to 2-20 mg/kg in the hay) (McDonald et al., 2010). Leaf loss, during haymaking, due to an excessive mechanical handling, is liable to cause a loss of nutritive value because the leaves are richer in nutrients than the stems, the resultants hay may be of low quality.

Many studies have highlighted how forage preservation (silage and haymaking) causes a considerable variation in their chemical composition, especially in the carbohydrate fractions, that decrease the nutritive value (Bonsembiante et al., 1982; Bittante and Andrighetto, 1982; Pinosa et al., 1995). Others studies also demonstrate the influence of haymaking on the *in vitro* fermentation characteristics and kinetics of carbohydrate fractions (Doane et al., 1997; Calabrò et al., 2005; Calabrò et al., 2006).

Aim of the present study was to investigate on the effect of haymaking on the *in vitro* fermentation characteristics, including volatile fatty acids and methane production. We hypothesized that the *in vitro* gas production

technique (IVGPT) can put in evidence the changes due to the preservation method.

MATERIALS AND METHODS

Collection and preparation of the forage samples

In three farms located in Castel Pagano (Benevento Province, Southern of Italy), longitude 41°24'N latitude 14°48'E at 630 m a.s.l., three samples of fresh forage (F1, F2, F3) were collected at random in the field at the moment of cut during the first operation of haymaking (May 2016). All the forages were a mix (about 70/30) of *Gramineae* (*Lolium* spp., *Avena sativa* L., *Phleum pretense*, *Dactilis glomerata* L.) and *Leguminosae* (*Trifolium* spp., *Hedysarum coronarium* L., *Vicia sativa* L.).

After four months (September 2016) three samples of the same forage preserved as hay (H1, H2, H3) were collected in the same farms. All the samples were transported to the laboratory of "Feed analyses" of the DMVPA (University of Napoli Federico II, Napoli, Italy); the fresh samples were oven dried at 105°C for 24 h and the moisture was registered. All the samples were prepared for the analysis grinding to 1 mm (SM 100, Retsch, Haan, Germany).

Chemical composition

All the forages samples were analyzed for chemical composition including dry matter (DM), crude protein (CP), ether extract (EE) and ash according to official protocol by Association of Official Analytical Chemists (AOAC

2005; www.aoac.org) procedures (ID number: 2001.12, 978.04, 920.39 and 930.05 for DM, CP, EE and ash, respectively). Neutral detergent fiber (NDF) was determined according to Van Soest et al. (1991) and non-structural carbohydrates (NSC) calculated as follows: 100 – CP – ash – EE – NDF.

In vitro fermentation characteristics, kinetics, end-products

The substrates were incubated at 39°C under anaerobic conditions with cow *inoculum* according to Calabrò et al. (2015). In particular, the substrates were weighed (1.0184 ± 0.024 g) in 120 ml serum bottles and 79 ml of anaerobic medium were added. The rumen fluid was collected in a pre-warmed thermos at a slaughterhouse authorized according to EU legislation (Regulation EC No. 882/2004) from four dairy cows. The collected material was rapidly transported to the laboratory, where it was pooled, flushed with CO_2, filtered through cheesecloth and added to each bottle (10 ml). Gas production was recorded 24 times (at 2 to 24 h intervals) during the period of incubation using a manual pressure transducer (Cole and Palmer Instrument Co., Vernon Hills, IL, USA). The cumulative volume of gas produced after 120 h of incubation was related to the incubated OM (OMCV, ml/g) and to degraded OM (Yield, ml/g). The fermentation was stopped at 120 h and the fermenting liquor was analyzed for pH (pH-meter Thermo Orion 720 A+, made in USA) and sampled for volatile fatty acids (VFA) determination. In particular, the fermenting liquor was centrifuged at 12,000 g for 10 min at 4°C (Universal

32R centrifuge, Hettich, Melle-Neuenkirchen, Germany) and 1 ml of supernatant was mixed with 1 ml of oxalic acid (0.06 mol). VFA were measured by gas chromatography (GC Focus AI 3000, Thermo Scientific, Waltham, MA USA) equipped with a fused silica capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness), using an external standard solution composed of acetate, propionate, butyrate, iso-butyrate, valerate and iso-valerate, as described by Musco et al. (2016). The extent of sample disappearance, expressed as organic matter degradability (dOM, %), was determined by weight difference of the incubated OM and the filtered undegraded OM (sintered glass crucibles; Schott Duran, Mainz, Germany, porosity # 2) residue burned at 550°C for 5 h. Three bottles were incubated without substrate (blanks) to correct the organic matter degraded, and gas and VFA production.

Methane (CH_4) production was determined in three bottles after 24 hours of incubation as proposed by Guglielmelli et al. (2011). Immediately after 24 h of incubation, total volume of gas (OMCV, ml/g) accumulated in the headspace of each serum flask was recorded using a manual pressure transducer (Cole & Parmer Instrument Co., Barringhton, IL USA). Then the gas phase from each serum flask was sampled (3 ml) in duplicate with a gastight syringe; the analysis carried out using a gas chromatograph (GC Trace 1310, Thermo Scientific, Waltham, MA USA) equipped with a loop TC detector and a packed column (HaySepQ SUPELCO, 3/16 inch, 80/100 mesh). Methane production was reported as percentage of total gas produced in the bottle after 24 h of incubation, and as amount (ml)

related to incubated (g) and degraded (g) organic matter (pCH_4, iCH_4, dCH_4, respectively).

Data processing

For each bottle, the gas production profiles were fitted to the following model (Groot et al., 1996):

$$G = \frac{A}{(1 + \frac{B}{t})^C}$$

where G is the total gas produced (ml per g of incubated OM) at time t (h), A is the asymptotic gas production (ml/g), B is the time at which one-half of A is reached (h), and C is the curve switch. Maximum fermentation rate (R_{max}, ml/h) and the time at which it occurs (T_{max}, h) were calculated utilizing model parameters (Bauer et al., 2001):

$$T_{max} = C \times (\frac{B-1}{B+1})^{1/B}$$

$$R_{max} = \frac{(A \times C^B) \times B \times Tmax^{(B-1)}}{(1 + C^B) \times (Tmax^{-B})^2}$$

In order to evaluate the influence of the haymaking (H) and the producing farm origin (F) on the *in vitro* fermentation characteristics, all obtained parameters (OMCV, Yield, dOM, VFA, pH, A, B) were subject to the analysis of variance utilizing the PROC GLM (SAS, 2000) according to the model:

$$Y_{ijk} = \mu + H_i + F_j + (H \times F)_{ij} + \varepsilon_k$$

where for each parameters (*Y)* μ represents the general mean, *H* the preservation method (fresh vs. hay), *F* the agricultural farm (1, 2, 3), *H x F* the first order interaction and ε the error effect. When comparing preservation method, mean differences were statistically assessed at 5% and 1% level by Tukey's test.

RESULTS AND DISCUSSION

Chemical composition

The chemical composition of the six forages sampled in three farms - fresh and hay - is shown in Table 1. In general, either for fresh forage and hay, the values are in the range of mixed forages (www.feedipedia.org), even if few data are presented in bibliography relatively to the involved regions with whom to make a comparison (Martinello, 2004). In particular, the protein content is quite relevant (mean value: 16.6 and 12.7 %DM, in fresh and hay respectively) and structural carbohydrates level is moderate (NDF mean value: 34.4 and 49.2 %DM, in fresh and hay respectively). However, some differences appear between the three farms. Farm 2 produced lower quality forage (fresh and hay) in terms of crude protein and NDF, compared to the other farms.

In vitro fermentation characteristics and methane production

The *in vitro* fermentation characteristics and methane production are reported in Table 2. The two main factors (farm and preservation

methods) showed no significant effect, except for dOM; whereas the interaction significantly influenced dOM, Yield, and CH_4 (data not showed).

Table 1.

Chemical composition of the six forages analyzed

Sample	DM	Ash	CP	NDF	NSC
	%	*% DM*	*% DM*	*% DM*	*% DM*
F_1	95.87	9.33	18.28	32.26	38.43
F_2	93.68	8.54	15.02	38.23	36.51
F_3	94.73	8.51	16.61	32.74	40.44
H_1	91.38	10.06	14.83	46.09	26.78
H_2	91.04	9.81	10.59	54.61	23.29
H_3	91.05	9.93	12.77	46.77	28.83

F1, F2, F3: fresh forage sample of farm 1, 2, 3 respectively.
H1, H2, H3: hay sample of farm 1, 2, 3 respectively.
DM: dry matter; NDF: neutral detergent fiber;
NSC (non-structural carbohydrates): 100 – CP – ash – EE – NDF.

The interaction farm x preservation methods was not significant (P>0.05) for all parameters. In general, organic matter degradability (dOM) ranged between 60 to 76 % and gas production (OMCV) is around 270 ml/g. These values are comparable with previous results obtained for forage samples with similar in chemical composition and evaluated in analogous *in vitro* experimental condition (Calabrò et al., 2001; Calabrò et al., 2006). Similarly, methane production also falls in a range reported by other authors that incubated *in vitro* for 24 h with bovine rumen fluid,

feedstuffs with similar NDF content 15.0 ml/g DM and 10.6 % total gas produced. Soliva et al. (2008) found 15.0 ml of CH_4/g of incubated DM and 16.6 % of reported as percentage of total gas produced; Xu et al. (2010) found the following value for the same parameters: 16.9 ml/g DM and 12.1%. Cattani et al. (2014) obtained similar methane value incubating *in vitro* meadow and ryegrass hay for 24 h with cow rumen fluid: 17.1 and 12.6 ml/g DM and 12.8 and 13.1 ml/100 ml, respectively.

Table 2.

Effect of haymaking on in vitro fermentation characteristics

	dOM	**OMCV**	**Yield**	**pCH_4**	**iCH_4**	**dCH_4**
	%	*ml/g*	*ml/g*	*%*	*ml/g*	*ml/g*
Fresh	76.76	282	361	12.18	14.18	25.10
Hay	61.59	273	444	12.38	13.49	28.63
Prob. t	***	NS	NS	NS	NS	NS
MSE	1.64	18.46	69.90	0.334	0.602	10.08

dOM: organic matter degradability at 120 h; OMCV: cumulative gas production at 120 h related to incubated organic matter; Yield: cumulative gas production at 120 h related to degraded organic matter; pCH_4: methane production at 24 h as percentage of total gas; iCH_4: methane production at 24 h related to incubated organic matter; dCH_4: methane production at 24 h related to degraded organic matter.
MSE: Mean square error. NS: not significant, ***: P<0.001.

End-products measured after 120 h of incubation are showed in table 3. Except pH and valeric acid, all the parameters were significantly (P<0.05) affected by farm, while preservation method only affects pH (P<0.001)

and total volatile fatty acids (P<0.01). The interaction farm x preservation methods was significant (P<0.05) only for acetic acid. The pH values after 120 h of incubation for all substrates ranged between 6.54 and 6.80 indicating that the buffering capacity of the medium was always able to guarantee an adequate fermentation environment for cellulolytic bacterial activity (Doane et al., 1997). The main end-products of carbohydrates fermentation by rumen microorganism are acetate, propionate, butyrate, carbon dioxide and methane. The composition of end-products formed influence the amount of gas produced, high acetate and butyrate production is associated with high gas production (Calabrò et al., 2001). In this study, not significant differences appear in gas, acetate and butyrate production. However, total volatile fatty acids are slightly lower compared to our previous results (Calabrò et al., 2001; Calabrò et al., 2006), maybe due the different carbohydrates content.

Effect of haymaking

As expected, comparing chemical composition of fresh and hay forage (table 1), crude protein values decreased (mean value: from 16.6 to 12.7 %DM), while ash and structural carbohydrates increased (mean value from 8.8 to 9.9 and from 34.9 to 49.2 %DM, for ash and NDF, respectively) due to the preservation method.

Table 3.

Effect of haymaking on in vitro fermentation end-products at 120 h of incubation

	pH	Acetic acid	Propionic acid	Butyric acid	Isobutyric acid	Isovaleric acid	Valeric acid	tVFA
					mmol/g OM			
Fresh	6.58	56.96	17.40	6.77	0.982	1.658	1.305	85.07
Hay	6.77	53.54	16.88	7.23	1.007	1.787	1.142	73.07
Prob. t	***	NS	NS	NS	NS	NS	NS	**
MSE	0.0016	9.083	0.986	0.503	0.014	0.049	0.026	13.56

tVFA: total volatile fatty acids (acetic + propionic + butyric + isobutyric + valeric + isovaleric) related to incubated OM.
MSE: Mean square error. NS: **, ***: not significant, P<0.01, P<0.001, respectively.

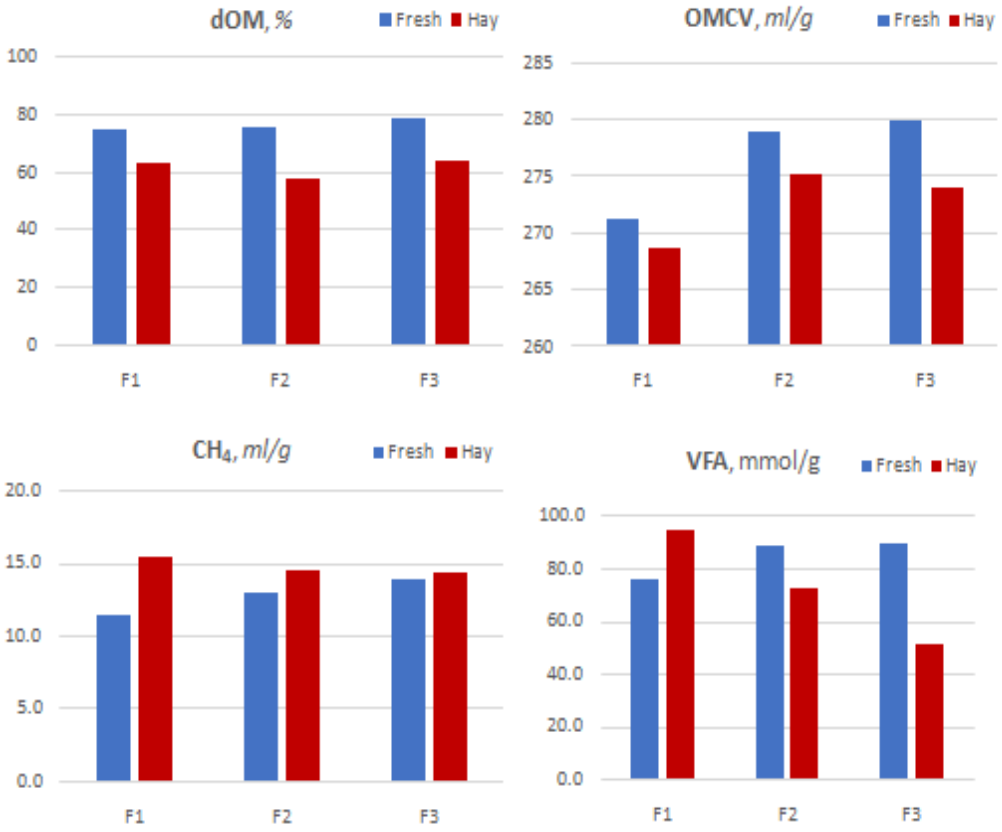
Comparing fresh and hay mean values after 120 h of incubation (Figure 1), a significant ($P<0.001$) decrease of dOM and tVFA was observed in each farm; for OMCV, the same trend was observed the same even if the differences were not statistically significant ($P>0.05$); methane production (CH_4, ml/g of incubated OM) after 24 h of incubation showed an increased production due to the haymaking, even if the differences between fresh and hay were significant only for farm 1 ($P<0.01$) and 2 ($P<0.05$).

Total volatile fatty acids resulted significantly ($P<0.05$) higher in fresh compared to hay samples (85.1 vs. 73.1 mmol/g); this trend is more evident for the fatty acids more represented (acetic and propionic acids).

Concerning the *in vitro* fermentation kinetics (Figure 2) of the analyzed forages, the curve related to gas production (Panel A) and fermentation rate (Panel B) over time show similar shape, even if some clear differences appear. In every farm, hay forage showed a fermentation process lower and slower compared to fresh forage. In particular, the time at which maximum fermentation rate is reached resulted always higher in fresh compared to hay (T_{max} mean value: 9.27 vs. 5.97 h; $P<0.001$), as well as the maximum fermentation rate (R_{max}: 10.8 vs. 11.0 ml/h) even if in the latter case the difference was not statistically significant ($P>0.05$).

Figure 1.

Effect of haymaking on in vitro fermentation characteristics

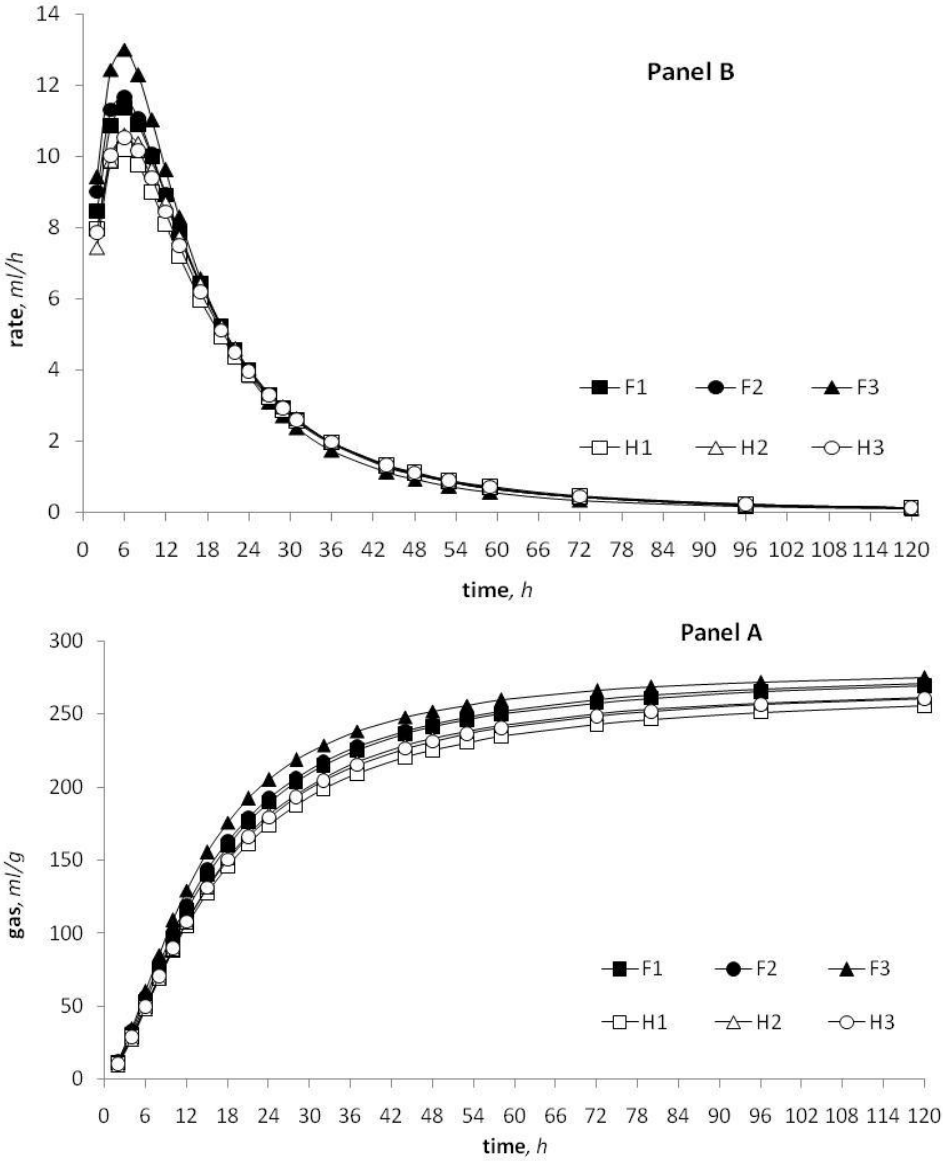


F1, F2, F3: agricultural farm 1, 2 and 3, respectively. dOM: organic matter degradability at 120 h; OMCV: cumulative gas production at 120 h related to incubated organic matter; CH_4: methane production at 24 h related to incubated organic matter; tVFA: total volatile fatty acids (acetic + propionic + butyric + isobutyric + valeric + isovaleric) production at 120 h.

All these results are surely due to the losses during the different steps of haymaking; in particular, the protein loss occurs mainly during the mechanical operation (i.e. tedding and field handling, but also collection, transport and baling) that causes the leaf loss of overturning.

Figure 2.

In vitro fermentation kinetics of the six forages analyzed over time



Panel A: gas production; Panel B: fermentation rate.
F1, F2, F3: fresh forage sample of farm 1, 2, 3 respectively.
H1, H2, H3: hay sample of farm 1, 2, 3 respectively.

The soluble sugar content reduced during the last phase of cellular respiration and causes a concentration of structural carbohydrates; indeed, the NSC fraction is reduced by about of 30% in hay compared to fresh for all samples. This last effect has a clear consequence on OM degradability, gas and VFA production, fermentation kinetics and also methane emission.

The ash increasing, probably due to the presence of soil during the harvesting of fodder mass, could be also responsible of reducing OM degradability and gas production. Similar results were found in previous study (Calabrò et al., 2005) when *Avena sativa* L., fresh, and preserved as hay and silage, was incubate for 120 h with buffalo rumen fluid. Also Pinosa et al. (1995) obtained a reduction of degradability due to haymaking comparable effect for *Festuca arundinacea* L. using the *in vivo* method on sheep. The same effect in hay compared to fresh forage on fermentation kinetics, degradability and VFA production is also reported by Calabrò et al. (2006) when NDF fraction of two intercropped forages *Hordeum vulgare* + *Vicia faba minor* and *Vicia sativa + Avena sativa* were incubated *in vitro* for 120 h with sheep rumen fluid.

CONCLUSIONS

In general, all the data obtained *in vitro* are in agreement with the results of chemical composition and show an expected tendency: a worsening of the *in vitro* fermentation characteristics and kinetics as a direct consequences of haymaking process. Regarding methane production, it is also clear that the worsening of the forage quality increasing its production. It is not possible to identify the best hay because the values obtained, in terms of chemical composition, *in vitro* fermentation and methane production, do not follow always the same tendency.

However, quantifying losses due to haymaking, in order to contain them, is very useful for improving the hay quality, albeit when this is used in large amounts for the production of high quality dairy products.

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Web sites consulted

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Chapter 6.

Main Conclusion

Final consideration of the results obtained

As as widely described in the thesis, the quality of forages preserved as hay varies considerably because of numerous factors. Improving forage quality can result in many benefits that can affect profit: animal welfare and health, reproductive efficiency, reduction or elimination of supplemental feeds, and finally, improvement of product quality.

Based on the three years of research activity and on the results obtained in the four experimental contributes, the following main conclusions can be drawn.

For the methodological point of view, the sensory evaluation system proposed could be taken into consideration as a valuable, practical and low-cost method for assigning a first quality judgment at the hay, directly applicable in the field, in order to provide effective indications to the farmers and optimize the use of local feed resources. The method has been validated by comparison with laboratory methodologies those are undoubtedly more accurate, but also more expensive and time-consuming and therefore, for some aspects, less suitable for use in an "on-site" technical assistance system for the breeders. The method is an original protocol mainly designed to evaluate forages for dairy cows characterised by a limited milk production and able to better enhance the local forages, especially in marginal environments. Further studies could include a "validation" of the method, also indicating repeatability and reproducibility parameters that could allow a verification, for example, of the variability of the results related to the "evaluator" effect.

Moreover, as reported by many authors, methane estimated by IVGPT is very close to that measured *in vivo*, demonstrating that the system could be used to estimate the potential CH_4 production from feedstuffs; this can be useful to generate a database to plan mitigation strategies in ruminants, improve their performance and reduce GHG. In this study, notwithstanding the estimated and determined methane value are similar to data found by other authors, the relationship between chemical composition and methane production is not always clear. In general, the database should be increased in order to improve their understanding, using very diversified forages in qualitative terms. Consequently, further *in vitro* researches are recommended to increment the forages data-base in this area, better if characterized by elevate differences in nutritional profiles, with the aim to identify forages with the best nutritional value (high degradability) and eco-sustainability characteristics (low methane). In general, all the data obtained *in vitro* are in agreement with the results of chemical composition and show an expected tendency: a worsening of the *in vitro* fermentation characteristics and kinetics as a direct consequences of haymaking process.

Regarding methane production, from the results obtained it emerges that the worsening in forage quality increases its production. However, it is not possible to identify the best hay because the values obtained, in terms of chemical composition, *in vitro* fermentation and methane production, do not follow clearly the same tendency.

Final consideration of the results obtained

The data collected in this study allow making some considerations on the description of forages made in some provinces of Mediterranean Area, on which the literature is limited. In general, the tested hays are characterized by a fairly medium quality and surely it is necessary try to improve them. The most important aspect to be considered is to increase the presence of botanical essences and to anticipate the time of cutting. In the future, it would be interesting to study the effect of the cutting period (i.e. growth stage) in the same environmental conditions.

Quantifying losses due to haymaking, in order to contain them, is very useful for improving the hay quality, albeit when this is used in large amounts for the production of high quality dairy products. In general, the in the studied forages the chemical composition resulted variable in function of climatic condition that can change in the different years; this factor also influences *in vitro* fermentation characteristics, including methane production.

In conclusion, the results of the research could have a practical effect elaborating a sort of guidelines (i.e. “Best Practices”) for the cultivation and conservation of forages in this particular area. The knowledge of all the investigated characteristics is required by the farmers in order to make balanced rations to maintain animal health and guarantee high level of production, in terms of quality and quantity.