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FORAGE EVALUATION AND FACTORS AFFECTING RUMEN FERMENTATION

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1. Introduction

Ruminants have played a major role in farming production for thousands of years, and have provided mankind with meat, milk and clothing. They can adapt to all kinds of climates and, above all, they can obtain feed on all kinds of temperate and tropical vegetation from lichens to trees. The ability to digest vegetation is conferred by a part of the digestive tract, the rumen. Indeed, they cannot breakdown plant fibre auto-enzymatically, and they rely on the fermentation of fibrous components by rumen microorganisms, which yield the short chain fatty acids that are absorbed and metabolized by the host (Stevens and Hume, 1998).

The essential role of microorganisms in the alimentary tracts of grazing mammals has been increasingly appreciated ever since Sprengel (1832), who reported that acetic and butyric acids were product of the breakdown of plant materials in the rumen. Protozoa and bacteria were soon identified as abundant inhabitants of alimentary tracts of cattle and horses and, after Pasteur recognized the alcoholic fermentation by yeast as an anaerobic equivalent of respiration, the rumen acids were similarly interpreted as metabolites of an anaerobic rumen microbiota. Zuntz (1879) cited evidence that the acids were absorbed and oxidized by the animal to meet its energy requirements, and formulated the fermentation hypothesis to explain the host-microbe symbiosis. This was then

confirmed and demonstrated experimentally, and numerous laboratories and investigators over the world have continued to report important new knowledge of rumen microbiology and physiology and its significance in ruminant production (Hungate, 1988).

Feeds administered to animals affect the complex ruminal system. Forage is defined as "a crop of cultivated plants or plant parts, other than separated grain, produced to be grazed or harvested for use as feed for animals" (definition given by the Forage and Grazing Terminology Committee, 1991; revision 2008). Since a wide range of feeds are included in this definition, with composition and nutritive value extremely variable, it is important to consider that different forages can make very different contribution to production system, varying from feeds not capable of supporting animal maintenance to those with a high digestibility and energy concentrations. So, adequate knowledge and better use of forage sources, would play a major role in the economic strategy of farms. In the years, estimative methods have been developed and refined to provide information on forage quality and to obtain information about animal response particularly to reduce the waste of time and money necessary for performing *in vivo* tests. Some of these have a chemical basis. Measurement of crude fibre (Weende method as described by Association of Official Agricultural Chemists, 1980) and cell walls method according to Van Soest *et al*. (1991) were used widely as a basis for predicting diet characteristics affecting digestibility and food intake. *In situ* technique measure feed degradation in nylon or polyester bags, directly suspended in the animals' rumen (Mehrez and Ørskov, 1977). Other possibilities are *in vitro* techniques, such as the

two-stage technique developed by Tilley and Terry (1963) and the gas production technique (Menke and Staingass, 1988) based on incubation of forage with rumen fluid. Recently, a filter bag technique for analyzing *in vitro* dry matter and neutral detergent fibre digestibility was developed by ANKOM Technology Corporation. A less laborious procedure that does not required rumen fluid as inoculum is the NIRS (near infrared reflectance spectroscopy) technology. It is a rapid, sensitive and accurate method for the nutritive evaluation of feedstuffs, such as forages (Norris *et al*., 1976), not requiring the use of animals.

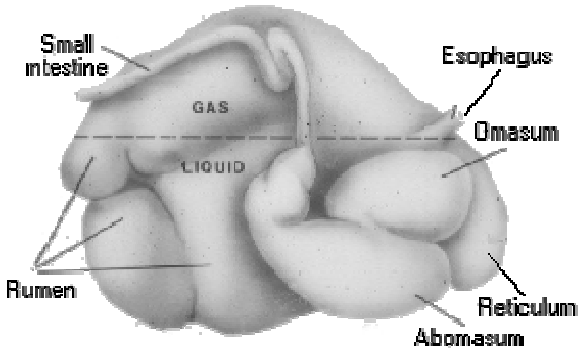
In some cases, such as high-production system, the high-dietary energy inputs can alter the digestive microbial balance, creating problems to the animal. To prevent health disorders and to improve performance, some additives were used in ruminant nutrition, such as *Saccharomyces cerevisiae* yeast. Although initially it was used mainly in the form of yeast culture, that include the yeast and the medium it was grown on, in the following years the attention has turned to live yeast products that maintain a high live cell count without the culture medium. Responses of the addition of *S. cerevisiae* on ruminal fermentation and microorganisms have been variable during the several studies.

With the previous considerations, the general objective of this study was to evaluate the nutritive value of representative forage sources from Piemonte (N-W Italy) testing different analytical methods, and to investigate the effect of inactivated cells of *Saccharomyces cerevisiae* vs. live cell yeast culture on ruminal fermentation.

2. Rumen system

2.1 Digestive anatomy in ruminants

The stomach of ruminants has four compartments: the rumen, reticulum, omasum and abomasum, as shown in the following diagram:



Collectively, these organs occupy almost 3/4ths of the abdominal cavity, filling virtually all of the left side and extending significantly into the right. The reticulum lies against the diaphragm and is joined to the rumen by a fold of tissue. The rumen, far and away the largest of the forestomachs, is itself sacculated by muscular pillars into what are called the dorsal, ventral, caudodorsal and caudoventral sacs. In many respects, the reticulum can be considered a "cranioventral sac" of the rumen; for example, ingesta flows freely between these two organs. The reticulum is connected to the spherical omasum by a short tunnel. The abomasum is the ruminant's true or glandular stomach. Histologically, it is very similar to the stomach of monogastrics. The interior of the rumen, reticulum and omasum is covered exclusively with stratified squamous epithelium similar to what is observed in the oesophagus. Each of these organs has a

very distinctive mucosa structure, although within each organ, some regional variation in morphology is observed. The anatomic features described are exemplified by cattle, sheep and goats. Certain other animals are also generally called ruminants, but have slightly different forestomach anatomy. Camelids (camels, llamas, alpacas, vicunas) have a reticulum with areas of gland-like cells, and an omasum that is tubular and almost indistinct. These animals are occasionally referred to as pseudoruminants or as having "three stomachs" rather than four. Stratified, squamous epithelium such as that found in the rumen is not usually considered an absorptive type of epithelium. Ruminal papillae are however very richly vascularized and the abundant volatile fatty acids produced by fermentation are readily absorbed across the epithelium. Venous blood from the forestomachs, as well as the abomasum, carries these absorbed nutrients into the portal vein, and hence, straight to the liver.

3. Nutritive value of feed

The efficiency of feed utilization is an important area of investigation and application, that includes the comparison among productive efficiency of animals and the evaluation of diverse feed resources. Feeds supply energy and essential nutrients to animals to support their different requirements (such as maintenance, growth, reproduction and lactation); the response depends on the nutritive value of feeds and the interactions among nutrients (Van Soest, 1994).

As reported by Raymond (1969), the nutritive value is conventionally defined by ruminant nutritionists into three general components:

- *digestibility,* described as the balance of matter lost in the passage through the digestive tract of animal. It is possible to separate the apparent digestibility, as the balance of the feed less the faeces, and the true digestibility represented by the balance between the diet and the respective feed residues, without the metabolic products. The true digestibility is important because represents the only part of feed available for digestion by animal, rumen bacteria and microbial enzymes. The estimation of this parameter are usually made by in vitro laboratory procedures that, since are not able to appreciate the faecal endogenous matter, are related more to true digestibility.
- *feed consumption*, or *ad libitum* intake, is a particularly complicated factor to define feed quality, because it depends on animal response, forage palatability, and forage selection.

The animal response can vary depending on the species, its status, its energy demand, and even its sex. The measurement of *ad libitum* consumption is usually conducted in a digestion stall.

- *energetic efficiency*, used for evaluating feed and expressing requirement in animal nutrition. Commonly it refers to metabolizable energy (the quantity of metabolizable nutrients expressed as energy, and calculated as the subtraction of urine and methane losses from total digestible energy). Although for monogastric animals this determination is relatively easy, for ruminants this assessment may present some problems, in view of large and variable losses characteristic of this biological suborder.

The importance of these parameters in animal nutrition has been recognised since long time, but digestibility can be considered the most important for its great influence both on feed ingestion and feed efficiency (Raymond, 1969).

3.1. Rumen fermentation approach

The rumen is a fermentation vat par excellence, providing an anaerobic environment, with constant temperature, physiologically optimal pH, and good mixing. Well-masticated substrates are delivered through the oesophagus on a regular schedule, and fermentation products are either absorbed in the rumen itself or flow out for further digestion and absorption downstream. A variety of techniques have been developed to simulate the digestion process that occurs in the rumen. *In*

vivo techniques are considered time-consuming, laborious, expensive and unsuitable for large-scale evaluation (Coelho *et al*., 1988; Carro *et al*., 1994), so many attempts have been made to predict digestibility using laboratory techniques. Much effort has been directed towards the development of regression equations to predict digestibility from forage chemical composition, but it is not so simple to obtain a regression equation which satisfactorily predicts a wide range of forages (McLeon and Minson, 1971; Van Soest, 1994). Therefore, the digestibility of feeds can be estimated by biological methods which simulate the digestion process by *in vitro* systems. With i*n vitro* rumen procedures (anaerobic fermentation of a sample substrate with medium and rumen liquor, followed by an end point measurement) it is possible to determine the extent of digestion or substrate utilization. The function of medium, usually a buffer solution with macro and micro elements, is to simulate the ruminant saliva (although without continual supply as in the rumen) and to provide the nutrient necessary for the proper functioning of the system. Although the chemical procedures are faster and offer better replication than i*n vitro* methods, the latter reflect better the biological process of digestion that occurs in the rumen environment, since microorganisms and enzymes are more sensitive to factors influencing the rate and extent of digestion (Van Soest, 1994).

The major digestion techniques are so summarized.

- The Tilley and Terry method (1963) was used in many laboratories for its convenience, particularly in large-scale testing. Samples are incubated for 48 h in buffer solution mixed to rumen fluid, and then subjected to another 48 h incubation with pepsin diluted in acid solution that allows to

solubilise feed and microbial protein. This technique had a great validation with in vivo values (Van Soest, 1994), since ruminant faeces are essentially composed by indigestible residue of plant cell walls and microorganisms such as the *in vitro* residue. A modification was introduced by Goering and Van Soest (1970) to estimate true dry matter digestibility: the residue after 48 h of incubation in rumen fluid is treated with neutral detergent solution. This solution is used to extract microbial mass and plant cellular content that are not fermented, but is assumed to be completely digested. In this way the final residue consists only in not digested plant cell walls. In both procedures samples are incubated in glass tubes and indigestible residue is obtained through centrifugation. Although both *in vitro* procedures allow to obtain a good estimation of *in vivo* digestibility (Van Soest, 1994), there are some disadvantages. It is necessary to have rumen fistulated animals that, besides ethical issues, introduce variability in the test due to animal differences and the administered diet. In addition, this method is an end-point measurement and does not provide information on the kinetics of forage digestion; moreover, the residue determination destroys the sample, and a large number of replicates are needed.

- To cover some of these deficiencies, many laboratories prefer to use the enzymatic method developed by Jones and Hayward (1975) and its modifications. Samples are initially incubated for 24 h in pepsin diluted in acid solution to remove proteins, and then treated with cellulase solution for

48 h. This technique, as well as giving accurate results, is relatively quick and inexpensive (Kitessa *et al*., 1999). Despite that, comparative studies have shown that the accuracy of this method is higher than the estimates obtained with different chemical fraction and comparable with the Tilley and Terry method. Obviously the accuracy of the method varies depending on fungal species chosen for the production of enzymes, and those obtained from *Trichoderma viridae* have shown the more close enzymatic digestibility data to those of dry matter in vivo digestibility (Coelho *et al*., 1988; Jones and Hayward, 1975).

- *In situ* procedures are accepted as referential methods since provide estimates of rate and extent of disappearance of feed constituents (Mehrez and Ørskov, 1977). The method involves the introduction of nylon (or other synthetic fiber) bags containing feed samples into the rumen fistulated animals, which will be recovered at different times to know the amount of feed lost through the pores as a result of digestion that occurs in the rumen (Hvelplund and Weisbjerg, 2000; Nocek, 1988). The estimation of rumen degradation using bags incubated into the rumen was used initially by Quin *et al*. (1938), but only the introduction of mathematical tools from Ørskov and McDonald (1979) has allowed to assess the data obtained together with the fractional rate of passage and, therefore, transform the values of digestibility in effective digestibility. Knowledge of effective digestibility is important because it takes into account the different rate of

passage in which each constituent leaves the rumen. The *in situ* method is subject to certain limitation as reported by Huntington and Givens (1995), Michalet-Doreau and Ould-Bah (1992), Nocek (1988), Vanzant *et al*. (1998). The following are critical points to be considered: soluble and degradable nutrients can be different; chose the most appropriate kinetics model for the degradation of the feed under study; loss of material through the pores of bags; contamination of residue of incubation with microbial material. The *in situ* method also requires surgically modified animals (White and Ashes, 1999) and only a small number of forage samples can be assessed at any one time (De Boever *et al*., 1997b). Although this process has been accepted as the reference method, the routine application involves a laborious and time-consuming work. For this reason it has been necessary to find another technique with sufficient predictive power to assess adequately the kinetics parameters of forage degradation. For this purpose the technique of *in vitro* gas production has seen a significant increase in recent years.

3.1.1. In vitro gas production measurements

The origin of the rumen fermentative gas measuring technique started in the early ‘40s (Quin, 1943), but it was considered as a routine method of feed evaluation after the work of Menke *et al*. (1979), where a high correlation between *in vitro* gas production and *in vivo* apparent digestibility was reported. Most rumen microorganisms obtain energy

initially from the fermentation of sugars, secondly from starch hydrolysis and finally by the hydrolysis of cellulose and of the remaining cell wall polysaccharides. Carbohydrates are so fermented to gases, mainly CH_4, CO_2 and H_2, that are expelled from rumen by eructation, as well as volatile fatty acids, which are absorbed through the walls of rumen, reticulum and omasum (Jarrige, 1990). Gas production is basically the results of carbohydrates fermentation to short chain fatty acids: acetate, propionate and butirrate (Blümmel and Ørskov, 1993; Wolin, 1960). Gas production from protein fermentation is relatively small (Wolin, 1960), and the contribution of fat is negligible (Getachew *et al*., 1998b; Menke and Steingass, 1988). So the gas production is related to the rumen fermentation.

The gas production in the *in vitro* technique includes a direct fraction (CO_2 and CH_4) as a result of fermentation, and an indirect fraction (CO_2) from the buffering of short chain fatty acids. Depending on the diet, variable amounts of indirect fraction were observed, corresponding to approximately 60% in concentrate diets and 50% in roughages (Getachew *et al*., 1998a). The highest production of gas is obtained when the substrate is fermented to acetate and butyrate. Even the molar proportion of different short chain fatty acids produced depends on the type of diet (Beuvink and Spoelstra, 1992), therefore the molar ratio of acetate to propionate was used to evaluate differences in the substrate. When slowly fermentable carbohydrates are incubated, acetate production is higher than propionate, and the reverse takes place with rapidly fermentable carbohydrates.

The measure of gas production from a batch culture to determine the fermentability of feed and, consequently, the potential rumen degradability, was continued by McBee (1953) and Hungate (1966). Trei *et al*. (1970) modified the technique by attaching a water displacement manometer to each vessel to measure the gas produced. Another modification was done by Jouany and Thivend (1986), and Beuvink and Spoelstra (1992), which determined the volume of water displaced using inverted measuring cylinders, then detected by an automatic system (Beuvink *et al*., 1992). Direct displacement of an handle by fermenting a feedstuff within a glass syringe was developed by Czerkawsky and Breckenridge (1975); the apparatus was the basis for the method developed by Menke *et al*. (1979). The syringe technique was originally developed to determine end-point fermentability of samples but, recording handle displacement at more frequent intervals, the kinetics of the fermentation profile were also determined.

Different approaches have been developed for the kinetics of fermentation *in vitro*, including measuring the gas produced in a sealed vessel by a pressure transducer that detects the accumulation of pressure in the vessel headspace. This method has been widely adopted for its simplicity and sensivity, and can be performed either by manual measurement (as described by Theodorou *et al*., 1994) or with a semi- and full automatic data collection system (Cone *et al*., 1996; Mauricio *et al*., 1999; Davies *et al*., 2000).

This technique has gained general recognition over the last decades, being used increasingly for feed evaluation, to investigate mechanisms of microbial fermentation, and for studying the mode of action of anti-nutritive factors, additives and feed supplements (López *et al*., 2007).

Some factors can affect rumen microorganisms fermentation and consequently *in vitro* gas production: it is necessary to assure the proper conditions of anaerobiosis and temperature, suitable pH and adequate buffering (Getachew *et al*., 1998a); even animals (Trei *et al*., 1970) and batch of inoculum (Beuvink and Spoelstra, 1992) have a considerable influence.

Table 3.1
Summary of the common methods used in the in vitro gas production technique. Source: Rymer et al., 2005.

	Menke et al. (1979)	Blümmel and Ørskov (1993)	Theodorou et al. (1994)	Mauricio et al. (1999)	Pell and Schofield (1993)	Cone et al. (1996)	Davies et al. (1998)
Incubation conducted in:	Syringes kept in rotor (50 cm diameter, 1 rotation/min)	Syringes kept in waterbath	Bottle in incubator	Bottle in incubator	Bottle and stirrer (48 rpm) kept in incubator	Bottle in shaking water bathe (50 oscillations/min)	Bottle in incubator
Microbial inoculum	Liquid phase	Liquid and solid phase	Liquid and solid phase	Liquid phase	Liquid phase	Liquid phase	Liquid and solid phase
Rumen fluid collection time	Before feeding	Before feeding	Before feeding	Before feeding	2 h after feeding	2 h after feeding	Before feeding
Inoculum volume (ml)	10	20	10	10	2	33	10
Incubation volume (ml)	30	30	100	100	10	100	100
Medium reference	Menke et al. (1979)	Menke et al. (1979) + trypticase	Theodorou (1993)	Theodorou (1993)	Goering and van Soest (1970)	Steingass (1983)	Theodorou (1993)
Sample (mg)	200–300	200	500–1000	1000	100	400–500	1000
Sample container (ml)	100	100	125	125	50	250	125
Number of fermentation vessels	60	–	As required	As required	15	12	50
HCO_3 mmol/g sample	7.2	7.2	9.3	9.3	9.3	11.7	9.3

When a pressure transducer is used, it is important to define the relationship between pressure and gas volume. López *et al*. (2007) analyzed the calculation to estimate the gas volume from pressure measurement applying Boyle's law, which states that volume (V, ml) is inversely proportional to pressure (P, psi) at a given temperature (°C). This relationship means that pressure increases as volume decreases, and *vice versa*, and that the product of pressure and volume at a given temperature is constant.

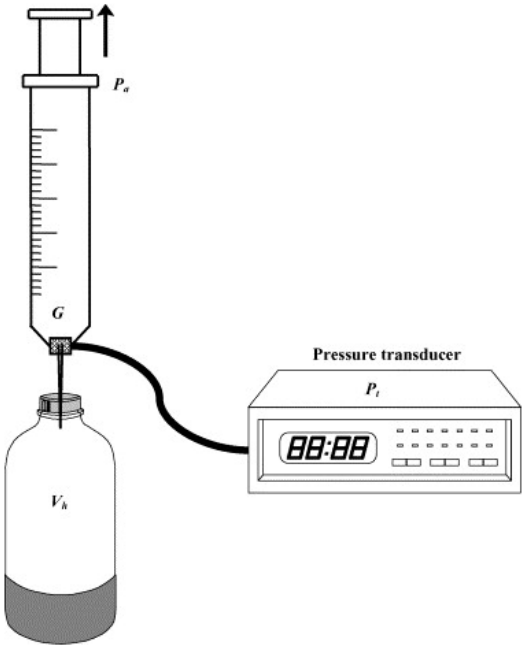


Figure 3.1
Measurement of gas production in gas-tight culture bottles by measuring changes in pressure in the headspace using a pressure transducer (*G*, volume of gas produced recorded in the syringe; V_h, headspace volume; P_a, atmospheric pressure; P_t, pressure measured by the transducer above P_a). Source: López *et al*., 2007.

With recoverable data from the method, gas volume (G) is thus related to pressure recordings (P_t) by a coefficient that can be calculated as the ratio between head space (V_h) and atmospheric pressure (P_t). Following the assessment made by Lopez *et al*. (2007), which evaluated and validated the expression using 26924 gas production data recorded in the same place, it was concluded that the value of the coefficient can be derived theoretically considering the atmospheric pressure at each site, with high degree of concordance with values estimated from linear regression of gas volume against pressure data measured experimentally.

To obtain the kinetics of fermentation, the gas production must be determined at different incubation times to fit the pool of data in a mathematical model. Several models have been proposed for describing kinetics gas production data, varying in complexity from single pool models assuming a variable fractional digestion rate (France *et al*., 1993) to empirical multipool models (Groot *et al*., 1996).

3.1.2. In vitro digestibility – Ankom method

As described above, the ethic, high costs and labour intensiveness of determining the digestibility of feeds *in vivo* have led to use alternative laboratory based techniques. The *in vitro* batch cultures proposed by Tilley and Terry (1963) and Van Soest *et al*. (1966) were widely used for the relatively accurate estimates of the digestibility of feedstuffs. In both procedures samples were incubated in glass tubes and the indigestible residue was obtained through centrifugation or filtration. The Daisy procedure of Ankom Technology Corporation (Ankom, 1997) proposed further modifications, allowing a large number of samples to be analyzed

in a short time with a fairly simple incubator. With this technique an amount of ground sample is weighted into a porous polyester bags and the residue of incubation is easily obtained by washing the bags. Several studies demonstrated that digestibility values from Ankom method are comparable to those obtained with traditional procedures (Holden, 1999; Mabjeesh *et al*., 2000; Wilman and Adesogan, 2000; Spanghero *et al*., 2003), although some limitations are present. Wilman and Adesogan (2000) compared conventional *in vitro* dry matter degradability estimates of 72 forage samples from two forage species to values obtained using the Daisy technique and found that, although the conventional technique is likely to give more precise results than the Daisy, the use of the latter gave acceptable digestibility estimates for forage when the emphasis was on saving labour. Furthermore, Adesogan (2002) noted that the results obtained with the new method can be affected by sample size and processing method, the proximity of the incubation jars to the heat source, and the extent to which individual bags are submerged throughout the incubation. In addition, as suggested by Adesogan (2005), the method include the potential for losses of undigested, soluble or fine particulate material through the pores of the bags which may overestimate digestibility. There are also potential associative effects between different feeds incubated in the same culture jar which may result in different digestibility values from those measured in tubes. Not many digestibility data on *in vivo* vs. Daisy are available. Demiran *et al*. (2008) showed that digestibility values estimated by Daisy and *in situ* technique were correlated (r^2 = 0.58-0.88) with values estimated by conventional *in vitro* and *in vivo* techniques, although in most cases Daisy and *in situ* techniques overestimated dry matter and neutral

detergent fibre digestibility. As suggested by the authors the practicability and accuracy of Daisy technique can be increased by sieving off of different size particles in the ground forage sample, correcting for the fine particle losses from the filter bag during digestion, washing and grouping the feeds into categories based on cell wall structure, and applying a corresponding correction factor.

3.1.3. Near infrared reflectance spectroscopy

Since Norris *et al*. (1976) first introduced the near infrared reflectance spectroscopy (NIRS) equations for predicting forage quality, the number of NIRS analytical application has greatly expanded. The parallel development of computer, optical devices and calibration software has stimulated this application in feed analysis. This technology is based on the vibration of molecular bonds in the NIR electromagnetic region (750-2700 nm) containing hydrogen (H) attached to atoms such as nitrogen (N), oxygen (O) and carbon (C) (Murray, 1993; Deaville and Flinn, 2000), that makes it very feasible for measurements to be made in organic and biological system. The advantage of this method is that it is a sensitive, fast and non-destructive analytical technique, with simplicity in sample preparation (Osborne *et al*., 1993).

When a molecule absorbs energy in the form of infrared radiation, the vibratory and rotational states are modified (Murray and Williams, 1987; Robinson, 1974). There are various forms of vibration for two atoms of a molecule against a third, each of which absorbs radiation at different wavelengths. Depending on the vibration of the atoms of the molecule, it will turn around its molecular axis, and this complexity of

movement generates not a simple absorption line for each group of atoms within the molecule but an absorption band covering a wide range of wavelengths (Robinson, 1974). It may also happen that a photon causes two or more vibration simultaneously, generating the so-called combination bands (0.7-1.8 μm), which increase the infrared spectral complexity (Clark and Lamb, 1991; Murray and Williams, 1987). In any case, only the vibration capable of generating a rhythmic change in dipole moment of a molecule can absorb energy in the infrared region (Bokobza, 1998; De Boever *et al*., 1994; Wetzel, 1998). On the other hand, the overtones (1.8-2.7 μm) include a wider spectral region than fundamental absorption, and therefore tend to overlap. For this reason it is difficult to isolate and interpret the spectrum in the near infrared zone (De Boever *et al*., 1994; Wetzel, 1998).

In the past, some features such as the lower intensity of overtones compared to fundamental absorptions, combination bands and overlapping phenomenon were considered drawbacks, preventing the development of this spectral region for use as analytical technique (Bokobza, 1998). Currently, not only it is not considered a problem but has a great advantage of providing greater selectivity of the technique (De Boever et al., 1994). So, the overlapping overtone between 1100 and 2500 nm corresponds to the vibration of light atoms with strong molecular bonds or with high frequency vibration (Bokobza, 1998; Wetzel, 1998), which corresponds to C-H, O-H and N-H groups of organic compounds that form part of plant and animal tissue (Foley *et al*., 1998). For this reason, samples spectra contain detailed information

about the chemical composition of material analyzed (Shenk and Westerhaus, 1994).

NIR spectroscopy has revolutionized the estimation of nutritive value of forages for ruminants, because not only is enable to obtain data on the wide range of organic constituents in agricultural products (Díaz-Güemes *et al*., 1980; Lila and Furstoss, 2000; Masoero *et al*., 1993; Walshaw *et al*., 1998), but also allows to estimate parameters related to animal response, as degradability and digestibility of feed. This is possible because the infrared absorption that occurs in the region 1650-1670 nm and 2260-2280 nm causes vibrations in the C-H bonds associated with lignin and cellulose, forage constituents which affect its digestive use (Albanell *et al*., 1995 and 1997; Aufrère *et al*., 1996; Baker *et al*., 1994; Clark and Lamb, 1991; De Boever *et al*., 1998; Deaville and Givens, 1998; García-Ciudad *et al*., 1993; Murray, 1993; Norris *et al*., 1976). Deaville and Givens (1998) also showed that samples absorbance in the infrared spectral region situated between 1430-1630 nm and 2020-2230 nm corresponded to sugars, cellulose and starch associated with the loss of carbohydrates during microbial degradation in the rumen.

By NIRS technology it has been possible to estimate some parameters related to feed digestibility, such as *in vivo* dry matter digestibility (De Boever *et al*., 1997a; Kitessa *et al*., 1999; Park *et al*., 1997) and *in vivo* organic matter digestibility (Baker *et al*., 1994; Barber *et al*., 1990; Brichette *et al*., 2000; Coleto *et al*., 2000; De Boever *et al*., 1996; Givens *et al*., 1991 and 1997; Gordon *et al*., 1998; Windham and Coleman, 1989). Nevertheless, for making an estimate it is necessary to

analyze a large number of samples by *in vivo* experiments, where feed is evaluated for a particular species or breed, concrete productive state and under a defined management (Murray, 1993). The alternative is therefore to obtain NIRS calibration from data of dry matter digestibility estimated using one of the *in vitro* methods, such as Tilley and Terry (Boller *et al*., 1998; Brown and Moore, 1987; Castro and Oliveira, 1994; De Boever *et al*., 1986), Goering and Van Soest (Jung *et al*., 1998), and enzymatic procedures (Aufrère *et al*., 1996; De Boever *et al*., 1994; Van Waes *et al*., 1997).

Several studies have shown that the kinetics parameters of cell wall and crude protein degradation of different feeds can be estimated using NIRS technology (Barrière *et al*., 1998; Berardo *et al*., 1993). Deaville and Givens (1998) tried to predict parameters of rumen degradability of 113 silages by NIRS and concluded that the possibilities of this method to estimate the potential and effective degradability were promising, although it did not happen for lag time and rate of degradation. The *in vitro* gas production was also estimated with good approximation (Herrero *et al*., 1996 and 1997), which is of great interest considering the correlation between gas production at 24 h and metabolizable energy content of feeds (Menke *et al*., 1979; Menke and Steingass, 1988). It is interesting to highlight how the major regions of infrared spectrum associated with the gas production (1664-1696 nm) coincide with the wavelength of cellulose and lignin and thus with the *in vivo* dry matter digestibility (Herrero and Jessop, 1998).

The technique has been used not only for quantitative determination but also for selecting and discriminating plant varieties (Albanell *et al*., 1995; Cozzolino *et al*., 2000; Edney *et al*., 1994; Jung *et al*., 1998; Sinnaeve *et al*., 1994), allowing an easy and quick rating procedure (Julier *et al*., 1999).

4. *Saccharomyces cerevisiae* supplement in the ruminant system

The growing interest in the relationship between diet and human and veterinary health care, has led to an increasing demand for food products that support health as well as provide nutrients. Probiotics, which are just opposite to antibiotics that destroy beneficial bacteria, are components that can be incorporated into foods. Health benefits are related to their interaction with the gastrointestinal tract. By definition of the International Live Science Institute (Ashwell, 2002), a probiotic is "a live microbial food ingredient that, when ingested in sufficient quantities, exerts health benefits on the consumer". Similarly, the Joint Food and Agriculture Organization/World Health Organization Working Group on drafting "Guidelines for the Evaluation of Probiotics in Food" (2002), defines probiotics as "live microorganism which, when administered in adequate amounts, confer a health benefit on the host". Probiotic supplements are composed of single or multiple strains of microbial cells, which can survive or establish in the intestine of the host. Certain strains of yeast (*Saccharomyces cerevisiae*) and fungi (*Aspergillus oryzae*) are also included under probiotics. Instead, the concept of prebiotic refers to "non digestible food ingredient, that beneficially affects the host by selectively stimulating the growth and/or activity of limited number of bacteria in the colon, and thus health", as defined by Gibson and Roberfroid (1995). The purpose of prebiotic is therefore to stimulate the growth of one or a limited number of the potentially health promoting indigenous microorganisms, thus modulating the composition of the natural ecosystem. Among prebiotics, as well as some peptides, proteins

and certain lipids, can be included microorganisms such as the inactivated form of *Saccharomyces cerevisiae*, characterized by immediate availability of the substances contained within the cell. All these microbial products are considered food supplements, and their use is subjected to the rules required for animal feed additives, such as the European Reg. 1831/03/CE. According to this Regulation, microorganisms as pro-prebiotics belong to the category of "zootechnical additives", listed as "stabilizer of intestinal flora". The legislation requires specific labelling requirements:

- expiry date of the guarantee, or the storage life from the date of manufacturing;
- instructions for use;
- identification number of the strain;
- number of colony forming units per gram (CFU/g).

The European authorization is granted only after careful evaluation of its performance characteristics and safety. The evaluation criteria for microorganisms were determined by SCAN (Scientific Committee for Animal Nutrition) in 2001. The aim is to differentiate chemicals from microorganisms and enzyme additives and provide answers on efficiency and concerns health (safety, man, animal and environment).

4.1. Generality and physiological properties of the yeast

As cited by Viljoen and Heard (1999), the most useful yeast that probably was used since ancient time for brewing, wine-making and baking bread is *Saccharomyces cerevisiae*. It is also the most studied as

an important model system for basic research into the biology of the eukaryotic cell. *Saccharomyces cerevisiae* is the type species of the genus *Saccharomyces*, introduced by Meyen in 1938 and defined by Rees in 1870, whereas Hansen described the beer yeast, *S. cerevisiae*, in 1888. The species belongs to the family *Saccharomycetaceae* and the subfamily *Saccharomycetoideae*; it is a unicellular fungus, reproducing vegetatively by multilateral budding, and sexually by means of ascospores. The asci are persistent and contain one to four globose ascospores. The vegetative cells are globose, ovoidal or cylindrical and appear butyrous and light cream-coloured, while the surface is smooth and flat (Figure 4.1). Actually the genus *Saccharomyces* encompasses 16 species, while the species *S. cerevisiae* includes 95 synonymisms.

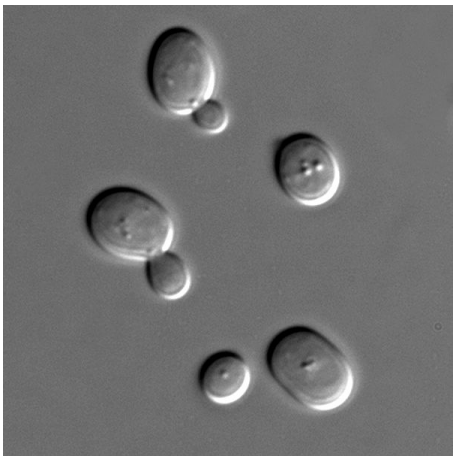


Figure 4.1
***S. cerevisiae* under differential interference contrast microscopy**

Saccharomyces cerevisiae can grow within a wide range of temperature (between 0°C and 40°C), but the optimum range for

maximum growth rate is 28-35°C, or slightly lower if the cell yield is considered. Although *S. cerevisiae* can grow under microaerophilic conditions, oxygen is essential to maintain cell viability, because under anaerobic conditions the synthesis of some cellular constituents such as fatty acids and sterols, ceases and the yeast cells stops growing. The species shows a great tolerance to pH, but prefers a slightly acid medium with an optimum pH between 4.5 and 6.5.

With regard to some biochemical properties, the species is able to ferment the hexose sugars D-glucose (normally the fastest fermentation), D-fructose and D-mannose. Other sugars that can be fermented by some strains of *S. cerevisiae* include sucrose, maltose and D-galactose, while dextrine and starch are rarely fermented, and lactose is not fermented. Other non-fermentable compounds such as lactic acid, other organic acids and polyhydroxy alcohols can be readly metabolized. Among strains of the yeast there is difference in using sources of nitrogen; while many inorganic ammonium salts can promote the growth, some strains show different abilities to use free aminoacids, and some nitrates are not utilized. On the contrary, some yeast strains can utilize urea as a source of nitrogen.

Saccharomyces cerevisiae is important in the food industry for the production of fermented beverages and breads, in the food spoilage, in the food waste processing and in the production of food ingredients; but it has aso assumed importance as a probiotic and prebiotic in animal nutrition.

4.2. Effects on ruminal fermentation

Yeast products have been used in ruminant diets from many years and the three main pourposes, as suggested by Wallace and Newbold (1995) and Chaucheyras-Durand *et al*. (2008), were:

- enhance the development of the adult rumen microflora (young animal);
- stabilize the ruminal pH and intestinal flora;
- stimulate the dry matter intake and productivity, increasing the fibre degradation.

In recent years, the European Union has banned the use of antibiotics and synthetic chemical products as feed additives for the growing concern about safety and quality of animal products, and environmental issues (Anadón, 2006). Consequently, the use of "natural" additives has increased, also for reducing the risk of animal digestive transfer of potential human pathogens and for limiting the excretion of pollutants (Chaucheyras-Durand *et al*., 2008).

4.2.1. Impact on rumen maturity

Young ruminants receive ruminal microflora and microfauna in the first days of life, when the mother licks them and when they themselves lick the saliva of the mother, combined with the rumen fluid regurgitated during rumination. The newborn animals receive the microorganisms present in the maternal rumen, especially unicellular species which are characteristic of the rumen and strictly anaerobic; this starting inoculum of organisms play an important role in influencing the biotype of the

adult microbiota. Only when the young animal eats forage partially chewed by an adult, ruminal population is completed (Mariani and Podestà, 1995). Therefore the development of rumen function and immune system results from the establishment of a complex microbial ecosystem. Some experiments reported results that support the hypothesis that yeast supplementation accelerates the condition for a greater maturation of the rumen microbial ecosystem. Chaucheyras-Duran and Fonty (2001) showed that the use of *S. cerevisiae* increased the establishment and stability of cellulolytic bacteria in lambs, and allowed a faster appearance of ciliate protozoa in the yeast treated than in no-supplemented lambs (Chaucheyras-Duran and Fonty, 2002). The results obtained in the experiment of Callaway and Martin (1997), suggested that yeast culture provides soluble growth factors (i.e., organic acids, B vitamins and amino acids) that stimulate the growth of ruminal bacteria that utilize lactate and digest cellulose. However, Newbold *et al*. (1995) suggested that is necessary to take care in the selection of commercial yeast culture, because even if the stimulation of the total and cellulolytic bacteria is the central mechanism in the action of yeast, not all strains have the same capability of stimulating bacterial numbers in the rumen. Indeed in another experiment conducted by Chiquette (1995) the addition of direct-fed microbials in steers did not affect bacterial counts or bacterial colonization of fibrous feed in the rumen.

4.2.2. Variation of ruminal pH

The readily fermentable carbohydrates in the high-dietary energy inputs cause typically lower pH, that is produced by a higher volatile fatty acids concentration in the rumen due to an increase of microbial

activity. Owing to the drop in pH, microbial ecosystem is altered: as shown by Martin *et al*. (2006) microbial diversity is reduced and protozoa number may decline. For example the fibre-degrading species, such as *Fibrobacter succinogenes*, *Ruminococcus albus* and *R. flavifaciens*, are particularly sensitive to low pH and the number may reduce (Russell and Wilson, 1996). Also, the lactate-producing bacterial species such as *Streptococcus bovis* may outnumber the lactate-utilizing species *Megasphaera elsdenii* and *Selenomonas ruminantium*, causing an accumulation of lactate in the rumen (Chaucheyras-Duran *et al*, 2008).

Several experiments were carried out to study the effect of *Saccharomyces cerevisiae* on the stabilization of ruminal pH. Thrune *et al*. (2009) conducted an experiment in control vs. yeast-supplemented cows and observed a greater mean, minimum and maximum ruminal pH in the yeast treatment compared with the control. As suggested by authors, the changes in rumen pH may also be due to different eating behaviour to which the animal were subjected. Bach *et al*. (2007) obtained a similar response in ruminal pH when cows were supplemented with the active yeast product. In the study of Guedes *et al*. (2008), it was observed that the positive effect of the yeast on pH was consistent with the reduced lactate concentration in the rumen. On the contrary, Chiquette (1995) obtained lower pH in animal receiving *S. cerevisiae* in combination with *Aspergillus oryzae*. Other experiments (Putnam *et al*., 1997; Miller-Webster *et al*., 2002; Lila *et al*., 2004; Erasmus *et al*., 2005) obtained no results on ruminal pH when yeast culture were administered. The comparison between live yeast and dead yeast products was conducted by Lynch and Martin (2002), which obtained that the

incubation of soluble starch or alfalfa hay with dead yeast preparation decreased the rumen pH, whereas it increased with supplementation of a live yeast product.

4.2.3. Impact on fibre degradation

Among farmed livestock, ruminants are the best adapted to digest plant cell walls, a great renewable carbon source further to carbon fixation in photosynthesis of plants. The hydrolysis of plant cell walls is permitted by ruminal microorganisms that synthesize and secrete the β 1-4 cellulase enzyme complex. However the conversion of feed, especially fibrous forages, to animal products is not always so efficient (Varga *et al*., 1997) . Principal factors that regulate the fibre digestion in ruminant, as suggested by Cheng *et al*. (1991), are:

- plant structure and composition;
- nature and amount of fibre-digesting microorganisms;
- factors affecting the adhesion and hydrolysis by hydrolytic enzyme of microbial populations;
- animal factors that increase the availability of nutrients trough mastication, salivation and digesta kinetics.

Among factors such as intake, dietary interactions and feeding strategy, also feed additives can influence microbial growth and subsequently fibre digestion. *Saccharomyces cerevisiae* has been shown to have effect on the stimulation of rumen fungus *Neocallimastix frontalis* enhancing the colonization of plant cell walls (Chaucheyras-Duran *et al*., 1995), and also on the activities of fibrolytic bacteria. Girard and Dowson (1995) reported that yeast has stimulated growth of *Fibrobacter succinogenes* and reduced the lag time for growth of *Ruminococcus albus*,

Ruminococcus flavefaciens and *Butyrivibrio fibrisolvens*. Again with *in vitro* trials, *S. cerevisiae* could accelerate rate, but not extent, of cellulose filter paper degradation by *F. succinogenes* and *R. flavefaciens*. Studies conducted *in vivo* reported positive results on improvement of ruminal fibre degradation by yeast (Plata *et al*., 1994; Miranda *et al*., 1996; Schwartz and Ettle, 2002), as well as no effects (Angeles *et al*., 1998; Corona *et al*., 1999). Jouany (2006) observed that the greater fibre digestion usually was associated to increased DM intake. The same author, in a previous study (Jouany *et al*., 1998), suggested that yeast in live form could create more favourable environment for growth and activity of the anaerobic autochthonous microbiota, in part due to effect on oxygen consumption (dissolved oxygen can be detectable *in situ* owing to water intake, rumination or salivation, as observed by Newbold, 1995), but also to growth factors released by yeast cells to closely associated microflora.

4.3. Considerations on the use of *S. cerevisiae* supplement

Although the use of microbial feed additives has been found useful in ruminant nutrition, especially when digestive microbiota was changing due to feed transition, high-dietary energy inputs and stress period for the animal, variable and inconsistent results were achieved in the numerous research activities conducted over the years. Because the conditions under which experiments were conducted were numerous, part of these differences have been attributed to the type and strain of yeast being used, animal factors (for example age or stage of lactation), and feeding strategy. Future studies will therefore focus on identifying and better

understanding the specific characteristics and behaviour of yeast cells, and to select the right target of additives for improved benefits in ruminant systems.

5. Aim of work and experimental trials

The overall objective of this doctoral thesis was to assess the nutritive value (chemical composition, *in vitro* degradability, *in vitro* fermentation kinetics and NIRS) of a representative forages source of the Piemonte region (N-W Italy), and then consider more specifically how experimentally controlled conditions in the management of maize silage can affect such characteristics. The thesis also attempted to act through manipulation of ruminal environment in order to improve the parameters that could influence the feed intake and milk production in dairy cattle.

The experimental plan was divided into three different trials. To provide a more clear and understandable reading, each contribution was written as a scientific paper, consisting of introduction, material and methods, results, discussion and conclusion.
For an overall reading of the scientific issues considered, please refer to the final conclusions.

The **first trial** was partially funded by the Regione Piemonte project "Extension Foraggi Piemonte", whose goal was to estimate chemical composition and *in vitro* digestibility of hays and silages commonly used in dairy cattle farms located in the Piemonte region (N-W Italy).
During a 9 month period spent at the Department of Animal Science of the University of León (Spain), it was possible to expand the investigation on the *in vitro* degradability analysis and gas production measurements, and NIRS spectra. Aim of the research was to use the set of results to discriminate the nutritive quality of different categories of forages with different botanical composition, method of conservation and

maturity stage. The results of this trial have been published on the CIHEAM Journal "*Options Méditerranéennes"*, and they were presented as a poster in León, Spain, October 14th-16th, 2009, at the 13th Seminar of the FAO-CIHEAM, Sub-Network on Sheep and Goat Nutrition.

The **second trial** was partially funded by the Regione Piemonte project "Extension Foraggi Piemonte". The goal of the trial was to estimate chemical composition, *in vitro* digestibility and gas production parameters of corn silage under experimental conditions at the research centre "Tetti Frati" of the Facoltà di Agraria of the University of Torino. Variables tested were two FAO class hybrids, sown in three different times and collected at two maturity stages. During the 9 months period spent at the University of León, it was possible to obtain more information analyzing the variability within experimental thesis tested. In addition, the impact of rumen degradability of NDF on estimated milk production was evaluated through the use of the Milk2006 model. This trial has been submitted to *Livestock Science*.

After extending the knowledge on characteristics of the main Piedmontese sources of forages, the same were included at different percentage into total mixed rations (TMRs) and tested in the **third trial.** The objective was to investigate the interaction of diet characteristics and yeast products (*Saccharomyces cerevisiae*) on ruminal fermentations. The effects of an innovative inactivated cell product was also tested against a live cell product to evaluate if the potentially positive effects of *S. cerevisiae* could be attributed to the yeast's viability or to its content of

nutrients. This trial has been submitted to *Animal Feed Science and Technology*.

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6. Discrimination of different categories of forages harvested from North-western Italy according to near infrared reflectance spectroscopy, chemical composition and *in vitro* digestibility

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6.1. Abstract

Near infrared reflectance spectroscopy (NIRS), *in vitro* digestibility and fermentation kinetics data were used to discriminate categories of forage quality, based on the method of conservation, forage species, maturity stage or harvest season of 64 forages commonly used in ruminant production systems. Forages used in the study included 40 hay (H) and 24 silage (S) samples of *Lolium multiflorum* L., *Medicago sativa* L., *Zea mays* L. and grassland herbage harvested from 20 dairy cattle farms in Piemonte (N-W Italy). All the samples were scanned in duplicate (400 to 2500 nm) with a NIRSystems 6500 scanning monochromator, and the absorbance data were transformed by a second order derivative before being used in the principal component analysis (PCA). The chlorophyll absorbance in the visible region (650-690 nm) explained most of the variance in the spectra, so maize silage samples were classified apart from grass silage and hay samples. A similar classification was obtained when just the infrared region (1100-2500 nm) was used in the PCA; however, in this latter case the variables contributing to explain most of the response variation along the first two principal components were related to the starch and protein absorbance (2280-2300 nm). Hay samples were highly heterogeneous (different stages of maturity, different harvest seasons or cutting dates within a season, with simple or complex botanical composition), thus precluding discrimination or clustering according to the botanical composition when the absorbance data were used in the PCA. All forage samples could be classified correctly according to the type of conservation (H vs. S) when chemical composition data were used in the PCA. However, in agreement with the results obtained for the absorbance data, with the

information used in the PCA (gas production, *in vitro* digestibility or chemical data) it was not possible to discriminate different clusters according to the botanical composition, and no categories could be identified within each method of conservation. A proper discrimination of the botanical groups was not possible owing to the high heterogeneity of the samples included in the study, thus causing the overlapping of different clusters.

Keywords: NIRS – forage – *in vitro* digestibility – fermentation kinetics.

Title et Résumé (French version)

Différenciation entre diverses catégories de fourrages, récoltés dans le Nord-Ouest de l'Italie, sur la base de la spectroscopie à réflectance infrarouge (NIRS), la composition chimique et la digestibilité in vitro.

La spectroscopie à réflectance infrarouge (NIRS), la digestibilité in vitro et les données cinétiques de fermentation ont été utilisées pour analyser la qualité de 64 échantillons de fourrages communément employés dans l'alimentation des ruminants. Les fourrages se distinguaient selon les espèces fourragères, les systèmes de conservation, le stade physiologique et la période de récolte. Les fourrages utilisés dans cette étude ont été: 40 foins (H) et 24 ensilages (S) de *Lolium multiflorum* L., *Medicago sativa* L., *Zea mays* L. et d'herbe de prairies récoltés dans 20 exploitations de vaches laitières du Piémont (N-O de l'Italie). Tous les échantillons ont été soumis deux fois au scanner (de 400 à 2500 nm) avec

le NIRSystems 6500 scanning monochromator. Les données de l'absorbance ont été transformées par une dérivée de second ordre avant d'être utilisées dans l'analyse des composantes principales (PCA). L'absorbance de la chlorophylle dans la région visible (650-690 nm) explique bonne partie de la variance du spectre, c'est pourquoi, les échantillons d'ensilages de mais ont été classifiés séparément des échantillons d'ensilages d'herbe et des foins. Une classification semblable a été obtenue dans la PCA avec l'emploi de la région infrarouge (1100-2500 nm); cependant, dans ce deuxième cas, les variables qui ont contribuer à expliquer bonne part de la variation le long des deux premières composantes principales ont été l'absorbance de l'amidon et de la protéine (2280-2300 nm). Les échantillons de foins étaient très hétérogènes (différents stades de maturation, différentes périodes de récolte ou de dates de coupe dans la même période de récolte, composition botanique simple ou complexe). Cela a empêché une discrimination ou un ensemble cluster sur la base de la composition botanique avec les données de l' absorbance dans la PCA. En utilisant la PCA, tous les échantillons de fourrages auraient pu être correctement classifiés selon le système de conservation (H vs. S) avec les données de la composition chimique. Toutefois, en accord avec les résultats obtenus pour les données d'absorbance, aucune des informations utilisées dans la PCA (production de gaz, digestibilité *in vitro* ou données chimiques) ne portaient à clusters différents sur la base de la composition botanique, et aucune catégorie ne pouvait être distinguée selon la méthode de conservation. La discrimination de groupes botaniques n'a pas été possible à cause de la grande hétérogénéité des échantillons utilisés dans l'étude ce qui a causé la juxtaposition des différents clusters.

Mots.clés: NIRS – fourrage – digestibilité *in vitro* – cinétique de la fermentation.

6.2. Introduction

Forage quality may have a major effect on animal performance, affecting the voluntary intake and digestibility and, consequently, milk yield and growth of ruminants (Minson, 1990, Getachew, 1998) and influencing excretion of undigested nutrients and emission of gases that may have an important impact on the environment (Getachew, 2005). Laboratory methods have been developed and refined to provide information on forage quality and to obtain accurate predictions of intake and digestibility from *in vitro* procedures. Recently, a filter bag technique for analyzing *in vitro* dry matter and neutral detergent fibre digestibility was developed by ANKOM Technology Corporation, allowing a large number of samples to be analyzed in a short time (Damiran et al., 2008). Kinetics of rumen degradation of feedstuffs can be studied *in vitro* by means of the gas production technique (Mauricio et al., 1999). These procedures are laborious and require rumen fluid as inoculum. In contrast, NIRS (near infrared reflectance spectroscopy) is a rapid, sensitive and accurate method for the nutritive evaluation of feedstuffs, such as forages (Norris et al., 1976), not requiring the use of animals. This technique is based on the molecular vibrations in the NIR electromagnetic region where the hydrogen is bound to carbon, nitrogen or oxygen, and requires calibration equations to correlate the spectral response to defined reference methods, in order to estimate chemical composition (Norris et al., 1976; García-Ciudad et al., 1993), digestibility

(Park et al., 1998) and *in vitro* gas production parameters (Murray, 1993; Andrés et al., 2005). This method can be used as a qualitative tool to discriminate and classify different feedstuffs on the basis of its spectral features (Lister et al., 2000; Prieto, 2008).

The aim of this study was to discriminate the nutritive quality of different categories of forages with different botanical composition, method of conservation and maturity stage, by means of near infrared reflectance spectroscopy, chemical composition and *in vitro* digestibility.

6.3. Material and methods

The study was carried out with samples of 64 forages commonly used in ruminant production systems. Forages included 40 hay (H group) and 24 silage (S group) samples of *Lolium multiflorum* L., *Medicago sativa* L., *Zea mays* L. and grassland herbage (grass hay 1^{st}, 2^{nd} or 3^{rd} cut, hay single cut or grass silage), collected from 20 dairy cattle farms located in Piemonte region (N-W Italy) during 2008. Samples were oven dried at 60°C for 48 h, then ground in Buhler mill to pass 1 mm screen and analysed for dry matter (DM), ash, crude protein (CP) and ether extract (EE) following the methods of AOAC (2000). Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined with the ANKOM fibre analyser (Ankom Technology Corp., 1997), following the procedure of Van Soest et al. (1991). For the *in vitro* assays six rumen-fistulated Merino sheep were used as donors of ruminal inoculum, fed alfalfa hay and with free access to water. Ruminal contents were collected before the morning feeding in thermos flasks, taken to laboratory, strained through cheesecloth, kept at 39°C under a CO_2 and

diluted (1/4 v/v) with a culture medium containing mineral and buffer solution as described by Van Soest et al. (1966). *In vitro* dry matter digestibility (IVDMD) was determined using the ANKOM-DAISY procedure following the approach proposed by Van Soest et al. (1966). Samples (0.25 ± 0.01 g) were weighed into F57 Ankom bags with a pore size of 25 µm, heat-sealed and then placed into a jar (5 l volume) containing 2 l buffered rumen fluid. The jars were placed in a DaisyII Incubator (ANKOM Technology Corp., Fairport, NY, USA) at 39°C, with continuous rotation. After 48 h of incubation the jars were emptied and the bags were gently rinsed and dried in an oven at 60°C. Bags were then washed with a neutral detergent solution at 100°C during 1 h and rinsed with distilled water into the fibre analyzer. Considering the amount of NDF incubated, *in vitro* NDF degradation (IVNDFD) could be estimated. Four incubation runs were carried out in different weeks giving four single observations per sample. *In vitro* gas production measurements were conducted using a pressure transducer as described by Theodorou et al. (1994), in which 0.50 ± 0.01 g of sample was incubated in a 120 ml serum bottle containing 50 ml of diluted rumen fluid. Bottles were sealed, shaken and placed in the incubator at 39°C. The head-space gas pressure released upon fermentation of feed was measured using a transducer, at incubation times of 3, 6, 9, 12, 16, 21, 26, 31, 36, 48, 60, 72, 96, 120 and 144 h after inoculation time. Gas volume was estimated from pressure measurements using the equation suggested by Lopéz et al. (2007). Three incubation runs were performed in different weeks, using in each of them two bottles per sample. Blanks were used to compensate for gas production in the absence of substrate. ANKOM-DAISY procedure with 144 h of continuous incubation was

used to estimate the potential DM disappearance (D144). The exponential model proposed by France et al. (2000) was fitted to gas production data: $G = A\ [1 - e^{-c(t-L)}]$, where G (ml/g DM) is the cumulative gas production at time t, A (ml/g DM) the asymptotic gas production, c (h^{-1}) the fractional rate of fermentation and L (h) is the lag time. The extent of degradation in the rumen (ED), for a rate of passage (k) of 0.033 h^{-1} (characteristic of sheep fed a forage diet of maintenance level), was estimated using the equation suggested by France et al. (2000): $ED = (D144 \times c \times e^{-kL}) / (c + k)$.

All samples were subsequently scanned in duplicate using a scanning monochromator NIRSystems (FOSS, Silver Spring, MD, USA) and spectra were collected in the visible region (vis) and NIR region (400-2500 nm) at 2 nm intervals. Absorbance was calculated as log (1/R), where R is measured reflectance. The mean spectrum was used for each sample, and different mathematical treatments of the spectra, based on first or second order derivatives, were applied. Raw spectra were noticeably improved when using the second derivative transformation, so that finally these data were used in the principal component analysis (PCA).

6.4. Results and discussion

Ranges of values on chemical composition of forages are presented in Table 6.1. The dry matter content was different for both methods of conservation, with values ranging between 819 and 929 g/kg in hays and from 210 to 639 g/kg in silages, although some hays and silages were prepared from the same parental material. The CP, NDF and ADF

contents differentiated maize silage apart from grass silages and hay samples, with narrower ranges of 68-88 g CP/kg DM, 361-528 g NDF/kg DM and 207-343 g ADF/kg DM compared to the other forages. Alfalfa forage, either as hay or silage form, showed a higher lignin content than other forages, with values ranging from 53 to118 g/kg DM.

Ranges of *in vitro* digestibility coefficients and parameters of fermentation are presented in Table 6.2. All variables observed did not discriminate the forages according to the botanical composition and type of conservation, because all the forages showed similar ranges of values, regardless their botanical composition or method of conservation.

Data of chemical composition, *in vitro* digestibility and gas production kinetics (a), and those derived from NIR spectra of samples ($1/R$), improved by second-order derivative to obtain a better resolution of raw spectra (b), were used in the principal component analysis (PCA) to obtain the corresponding scores of samples for the first two principal component (PC1-PC2) in a coordinate axis system, as is shown in Fig.6.1.

Table 6.1

Ranges in chemical composition (g/kg DM) of forages

Forages	n	Dry matter	Ash	Crude protein	Ether extract	NDF	ADF	ADL
Alfalfa hay	8	870-929	62-93	81-170	10-21	542-694	409-526	53-118
Italian ryegrass hay	6	819-915	67-88	64-89	13-22	583-690	344-413	33-58
Grass hay 1st cut	14	822-913	64-104	58-132	10-31	542-668	325-522	26-103
Grass hay 2nd cut	6	851-887	56-91	73-158	15-36	544-676	294-404	25-48
Grass hay 3rd cut	3	848-869	69-101	132-157	25-31	591-624	333-360	34-47
Grass hay single cut	2	876-909	60-81	44-127	10-29	633-638	383-399	45-47
Italian ryegrass silage	4	285-639	98-149	96-166	16-29	512-660	279-415	24-55
Grass silage	3	210-539	107-158	113-129	21-36	567-614	315-401	37-47
Maize silage	17	252-375	39-48	68-88	28-45	361-528	207-343	17-38
Alfalfa silage	1	428	141	145	22	550	363	84

NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin.

Table 6.2

***In vitro* digestibility (g/g DM) and parameters of fermentation (ml/g DM)**

Forages	n	*In vitro* digestibility (ranges)				Parameters of fermentation (ranges)		
		IVDMD	IVNDFD	D144	ED	G24	*A*	*c* (h^{-1})
Alfalfa hay	8	0.595-0.688	0.361-0.532	0.618-0.740	0.363-0.453	162-199	239-292	0.034-0.066
Italian ryegrass hay	6	0.641-0.855	0.480-0.757	0.683-0.871	0.336-0.440	153-205	284-322	0.032-0.049
Grass hay 1st cut	1	0.566-	0.341-	0.619-	0.306-	90-	211-	0.024-
	4	0.796	0.654	0.848	0.474	212	331	0.049
Grass hay 2nd cut	6	0.668-0.775	0.401-0.621	0.711-0.835	0.386-0.480	169-220	283-331	0.037-0.047
Grass hay 3rd cut	3	0.533-0.772	0.252-0.615	0.587-0.791	0.267-0.460	118-200	243-300	0.028-0.046
Grass hay single cut	2	0.698-0.720	0.523-0.561	0.729-0.747	0.382-0.396	168-209	297-343	0.040-0.036
Italian ryegrass silage	4	0.642-0.831	0.300-0.697	0.710-0.857	0.361-0.488	108-211	191-327	0.035-0.049
Grass silage	3	0.712-0.734	0.530-0.538	0.780-0.822	0.431-0.463	172-198	275-305	0.041-0.045
Maize silage	1	0.616-	0.128-	0.715-	0.375-	188-	310-	0.043-
	7	0.761	0.443	0.812	0.455	252	372	0.059
Alfalfa silage	1	0.677	0.412	0.728	0.434	169	245	0.049

IVDMD, in vitro dry matter digestibility; IVNDFD, in vitro neutral detergent fibre digestibility; D144, in vitro dry matter disappearance after 144 h of incubation; ED, extent of degradation; G24, cumulative gas production at 24 h; A, asymptotic gas production; c, fractional rate of fermentation.

When combined data of chemical composition, *in vitro* digestibility and gas production kinetics were used in PCA analysis two different clusters could be observed (Fig.6.1 – a), one for hays (identified as H group) and another one grouping silage samples (S group). This discrimination could be attributed to differences between both types of forages in chemical composition according to data presented in Tables 6.1 and 6.2. Discrimination of forages with different to botanical composition was not observed. The grouping based on absorbance data with second-order derivative transformation is presented in Fig. 6.1 – b. The only well defined discrimination that could be observed was between maize silage (A group) and the rest of samples (B group), without a clear separation of samples in B group associated with their differences in forage conservation (hay vs. silage) or in botanical composition. Analysing the spectra profiles, it seemed that the one of the variables explaining a greater proportion of the variance was the colour, with chlorophyll absorbance at 650-690 nm (in the visible region). A similar classification was obtained when just the infrared region (1100-2500 nm) was used in the PCA; however, in this latter case the variables contributing to explain most of the response variation along the first two principal components were related to the starch and protein absorbance (2280-2300 nm).

a)

b)

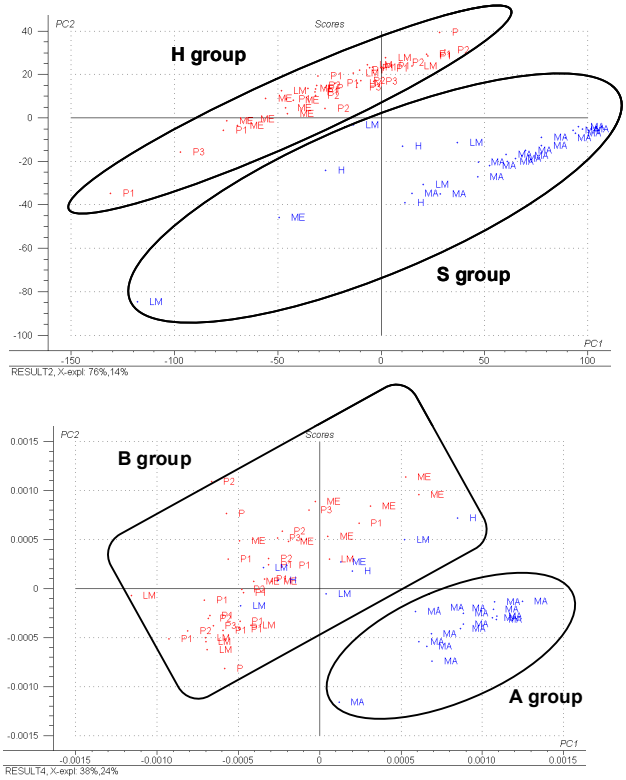

Figure 6.1

Plots of samples in the principal components (PC1 – PC2) calculated using either (a) data of chemical composition, *in vitro* digestibility and parameters of fermentation kinetics, or (b) second-order derivative data from average NIR spectra. Forages were alfalfa (ME), Italian ryegrass (LM), grass hay from 1st (P1), 2nd (P2), 3rd (P3) or a single (P) cut, grass silage (H) and, maize silage (MA).

6.4. Conclusions

Forage samples could be classified correctly according to the type of conservation (H vs. S) when chemical composition data were used in the PCA. However, using absorbance data in the PCA analysis it was not possible to discriminate different clusters according to the botanical composition, and no categories could be identified within each method of conservation. A proper discrimination of the botanical groups was not possible owing to the high heterogeneity of the samples included in the study, thus causing the overlapping of different clusters.

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7. Influences of hybrid, sowing date, and maturity at harvest on *in vitro* gas production, *in vitro* digestibility and estimated milk yield of fresh and ensiled whole-plant maize

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7.1. Abstract

Two maize hybrids, an early maturing Cisko 300 and a late maturing Arma 700, were sown on three different dates (March, April or May) and harvested at two stages of maturity (kernel milk line scores of 1/4<ML<1/3 or 1/2<ML<2/3). Yield, *in vitro* digestibility, energy value were measured and milk production from silage were predicted. Hybrid and sowing date influenced the DM yield of whole-plant maize, with DM yield being 40% higher with Arma than with Cisko, and DM yield decreasing with more advanced sowing dates. Advanced maturity increased DM concentration at harvest for both hybrids, due to kernel development. The NDF content numerically declined and starch and lignin values increased ($P < 0.001$ and $P < 0.01$, respectively) due to dilution by grain of more mature plants. Digestibility was estimated *in vitro* following the Ankom procedure. Rate and extent of ruminal degradation were estimated from gas production profiles during incubation in diluted rumen fluid. Hybrid, planting date and maturity had no effect on *in vitro* DM digestibility of pre-ensiled maize, but following 240 days of fermentation significant differences ($P < 0.05$) between hybrids in digestibility were detected. Gas production kinetic parameters differed between hybrids for pre-ensiled maize, with Cisko having higher ($P < 0.01$) asymptotic gas production but lower ($P < 0.01$) fractional rates and longer ($P < 0.01$) lag times than the Arma hybrid. Gas production volumes were greater as sowing or harvest date was delayed. Energy value and milk production were estimated using the Milk2006 Model. In pre-ensiled maize, a 38% greater ($P < 0.001$) milk yield per hectare was found with Arma than with Cisko hybrid, matching the 40% greater DM yield. The same trend was observed in maize silage, where hybrid and

planting date affected milk production per hectare, with greater ($P < 0.001$) values for Arma than for Cisko hybrid and lower values for the latest planting date ($P < 0.01$). Optimal management practices, including decision making on planting and harvest time and hybrid selection, all can influence the yield and nutritive value of maize silage.

Keywords: Whole-plant maize; Sowing; Maturity; *In vitro* digestibility; Gas production technique.

Abbreviations: A, asymptotic gas production; c, fractional rate of gas production; D144, dry matter disappearance after 144 h of incubation; DM, dry matter; ED, extent of degradation; G24, cumulative gas production at 24 h of incubation; IVDMD, in vitro dry matter digestibility; IVNDFD, in vitro neutral detergent fibre degradability; L, lag time; ML, milk line; NDF, neutral detergent fibre; NEL-3x, net energy of lactation at 3x of maintenance; SEM, standard error of the mean.

7.2. Introduction

High production potential, high concentrations of nutrient, good ensiling properties, and feasibility for incorporation into total mixed rations (TMR), have made maize silage (*Zea mays* L.) a widespread forage for feeding dairy cows. Factors such as total yield of DM and amount of grain produced, initially considered reliable indicators for choosing a hybrid, in recent years have been partly displaced by other qualities that may be associated with performance of farm livestock, e.g., high fibre and starch digestibility, in an attempt to maximize milk

production per hectare or milk production per Mg of silage (Barriere et al., 1995; Neylon and Kung, 2003). One factor affecting the nutritive value and digestibility of silage is the stage of maturity at harvest (Johnson et al., 1999); this can be modified by management practices like harvest methods and hybrid selection (Xu et al., 1995). Wiersma et al. (1993) suggested that harvesting too early can be detrimental, not only due to effluent losses of nutrients from the silo, but also because low energy content of feed due to incomplete starch accumulation in kernels. In contrast, mature maize silage harvested at the black layer (BL) stage may be low in nutritive value because of reduced starch and fibre digestion (Wiersma et al., 1993). Bal et al. (1997) suggested that 2/3 milkline (ML) was the optimum maturity stage for harvesting maize for silage to be fed to lactating dairy cows (similar results were found by Johnson et al., 2002a). In their review, Johnson et al. (1999) highlighted the negative interaction between grain development and stover quality associated with maturity of maize silage. The decline in digestibility of the stover component with progressive maturity is mainly due to decreasing non-structural carbohydrates and increasing fibre concentration. The concentration of neutral detergent fibre (NDF) in the whole plant decreases as maturity advances from early milkline (1/3 ML) to 2/3 ML, then remains constant untill the BL stage of maturity. The *in situ* degradability of whole plant DM, that has not been kernel processed, decreases progressively from early to late maturity stages despite the decline in NDF content (Johnson et al., 1999). Xu et al. (1995) suggested a strategy to improve maize silage quality through the selection of maize hybrids that maintained stover quality with advancing maturity. Furthermore, the NFD intake is closely related to the chewing activity,

the increase of rumination time, and the buffering capacity of the rumen in dairy cows (Allen, 1997). However, high levels of NDF could be detrimental to intake and production, since NDF content is negatively correlated to the whole-plant *in vitro* DM digestibility (Cox et al., 1994), and can limit intake through ruminoreticular fill. When the fill is a limiting factor, one approach to increase dry matter intake (DMI) is to increase NDF digestibility, which increases the rate of NDF clearance from the rumen thereby creating additional space. Oba and Allen (1999) reported that a one-unit increase in forage NDF digestibility was associated with a 0.17 kg increase in DMI and a 0.25 kg increase in 4% FCM yield. Laboratory methods have been developed and refined to provide information on forage quality and to obtain accurate predictions of DMI and digestibility (Damiran et al., 2008). Recently, a filter bag technique for analyzing *in vitro* DM digestibility (IVDMD) and NDF digestibility (IVNDFD) was developed by ANKOM Technology Corporation. This new technique allows a large number of samples to be analyzed in a short time; and estimates of digestibility agree with those other conventional *in vitro* techniques (Damiran et al., 2008). Undersander et al. (1993) proposed an index of forage quality based on ADF and NDF analyses to measure the milk yield per Mg of forage DM. This index was modified for maize silage by Schwab and Shaver (2001) using modified NRC energy values (NRC, 2001) and clarify better predictions of DMI and NDF digestibility. An updated version of this index (Milk2006, Shaver et al., 2006) became a focal point for maize silage hybrid-performance trials and hybrid-breeding programs in academia and in the seed-maize industry. Milk2006 uses updated

information and user-defined input flexibility to more closely predict actual milk produced per Mg of maize silage.

In addition to IVNDFD, dynamics of rumen degradation, studied by means of the gas production (GP) technique, can be used to rapidly assess fermentation of feed by accumulation of fermentation gases (Mauricio et al., 1999). Because *in vitro* GP has been used to evaluate effects of varieties, growing sites and different cereal grain species (Opatpatanakit et al., 1994), and to compare maize grain and methods of processing (DePeters et al., 2007), the assessment of differences within hybrids characterized by different FAO maturity class can be of significant importance.

The aim of this study was to compare two different FAO class hybrids sowed on three different dates and harvested at two different stages of maturity. This comparative study considered: 1) *in vitro* dry matter digestibility (IVDMD) and *in vitro* neutral detergent fibre digestibility (IVNDFD) before and after ensiling; 2) parameters of fermentation and extent of dry matter fermentation determined using *in vitro* gas production before and after ensiling; 3) estimated NE_L and milk production before and after ensiling from Milk2006 model.

7.3. Materials and methods

7.3.1. Experimental procedure

The trial was conduced in 2008 at the experimental farm of the University of Turin in the western Po plain, Italy (44°50’ N, 7°40’ E,

altitude 232 m above see level), characterized by sandy loam soil, with a subalcaline pH. The climatic patterns (1978-2008 period) were characterized by mean daily temperature increases of 0.6°C in January to 22.3°C in July, and an average maximum July temperature of 28.8°C. The monthly means of air temperature and accumulated rain, as well as the annual mean temperature and the annual accumulated rain of the experimental year from January to December and the 31-year mean values are reported in Table 7.1. The field was divided into three blocks; each block was subdivided into three (90 m x 12 m) plots giving three replications per treatment (one in each block). Within each block sowing dates were randomly assigned to the plots. Within each plot, maize variety was assigned as the subplot factor and the harvest date was assigned as the sub-subplot factor within each subplot. Maize hybrids of two different FAO maturity classes (Cisko, FAO class 300 and Arma, FAO class 700, NK Syngenta Seeds S.p.A., Madignano (CR), Italy) were planted on March 13th, April 9th and May 14th, 2008 at a theoretical planting density of 74000 seeds/ha. Fertilizer was applied at the rate of 40 kg/ha of P_2O_5 and 55 kg/ha of K_2O immediately before planting. An additional 160 kg/ha of N was top-dressed at the six leaves stage. Irrigation was provided by spray irrigation system on July 17th, at a rate of 500 m^3 water/ha. Thirty whole-plants per plot were harvested and ensiled at two different stages of maturity, the first with milkline between 1/4 and 1/3 ML, the second stage ranging from 1/2 to 2/3 ML for both hybrids. The forage was chopped at theoretical chop length of 12 mm. The kernel milk line was measured on five plants per plot following Afuakwa and Crookston (1984). The chopped forage of each plot was sampled for the chemical analyses and ensiled in 30 l plastic jars

equipped with a lid that enables gas release only. The silos were conserved at 20°C and opened after 240 d. At silo opening, final silo weights were recorded, approximately 10 cm of silage from the top of each silo were discarded; the remaining silage was sampled for the analyses.

Table 7.1

Monthly and annual mean temperatures and accumulated precipitation for the study period and the long term average (1978-2008)

Month	Temperature (°C)		Rain (mm)	
	2008	mean ´78-´08	2008	mean ´78-´08
January	2.4	0.6	46	35
February	3.9	2.9	9	32
March	8.5	7.6	2	48
April	11.3	11.2	118	92
May	16.7	16.2	109	103
June	21.0	20.1	171	68
July	22.4	22.3	83	36
August	22.0	21.6	44	58
September	16.7	17.4	56	69
October	12.7	12.2	4	85
November	6.4	5.7	54	56
December	0.7	1.7	7	36
Annual	*12.1*	*11.6*	*703*	*719*

7.3.2. Sample preparation and chemical analyses

Samples were assayed in duplicate according to the AOAC (2000). The pre-ensiled material and the silage were oven-dried at 60°C for 72 h, and material was air equilibrated, weighed, and ground in a Cyclotec mill (Tecator, Herndon, VA, USA) to pass through a 1 mm screen. Samples were analyzed for dry matter (DM), crude protein (CP), according to the Kjeldhal method, for ash by ignition to 550°C, ether extract (EE) by ether extraction and starch concentration according to the methods 934.01, 954.01, 942.05, 920.39 and 920.40, respectively. The NDF, ADF and Lignin (sa) were determined with the ANKOM fibre analyser (Ankom Technology Corp., Fairport, NY, USA), following the procedure of Van Soest et al. (1991). The NDF was analyzed with the addition of sodium sulphite and heat stable amylase to the solution.

7.3.3. Animal and rumen fluid collection

Six rumen fistulated Merino sheep were used as donors of ruminal inoculum for the *in vitro* assays; animals had free choice access to alfalfa hay and water. A sample of ruminal contents was collected before the morning feeding in thermos flasks and taken to laboratory where it was strained through two layers of cheesecloth, kept at 39°C under CO_2 atmosphere and diluted (1:4 v/v) with a culture medium containing macro and micro mineral solutions, resazurin and a bicarbonate buffer solution, prepared as described by Menke and Steingass (1988). Oxygen was reduced by the addition of a solution containing cysteine hydrochloride and Na_2S.

Animal handling followed the recommendations of European Council Directive 86/609/EEC for protection of animals used for experimental

and other scientific purposes, and experimental procedures were approved by the University of León (Spain) Institutional Animal Care and Use Committee.

7.3.4. In vitro gas production

In vitro gas production measurements were conducted using a pressure transducer as described by Theodorou et al. (1994), in which 0.50 ± 0.01 g of sample was incubated in a 120 ml serum bottle containing 50 ml of diluted rumen fluid. Blanks were used to compensate for gas production in the absence of added substrate. Once filled, bottles were sealed with rubber stoppers and aluminium seals, shaken and placed in the incubator at 39°C. The head-space gas pressure released upon fermentation of feed was measured manually by inserting a sterile needle connected to the pressure transducer after incubation for 3, 6, 9, 12, 16, 21, 26, 31, 36, 48, 60, 72, 96, 120 and 144 h. Gas volume was estimated from pressure measurements using the equation proposed by López et al. (2007). Two incubation runs were performed in different weeks, using in each run two bottles per sample and three bottles containing only medium as blanks. The Ankom-Daisy procedure of continuous incubation for 144 h was also used to estimate the potential DM disappearance (D144). In order to assess the parameters of fermentation kinetics, the exponential model proposed by France et al. (2000) was fitted to gas production profiles:

$G = A\ [1 - e^{-c\,(t-L)}]$

where:

G (ml/g DM incubated) is the cumulative gas production at time t;

A (ml/g DM) the asymptotic gas production;

c (h^{-1}) the fractional rate of fermentation;

L (h) is the lag time.

Volume of gas (ml/g DM) produced after 24 h of incubation (G24) was used as an index of digestibility and energy feed value, as suggested by Menke and Steingass (1988). The extent of degradation in the rumen (ED), for a rate of passage (k) of 0.033 h^{-1} (characteristic of forage at maintenance level of intake), was estimated using the equation suggested by France et al. (2000):

$$ED = \frac{c\ D144\ e^{-kL}}{c+k}.$$

7.3.5. In vitro digestibility

In vitro dry matter digestibility (IVDMD) was determined using the Ankom-Daisy procedure following the approach proposed by Van Soest et al. (1966). Samples (0.25 ± 0.01 g) were weighed into F57 Ankom bags with a pore size of 25 µm, heat-sealed and then placed into an incubation jar. Each jar was a 5 l glass recipient with a plastic lid provided with a single-way valve, which prevents the accumulation of fermentation gases, and was filled with 2 l buffered rumen fluid in anaerobic conditions, then placed into the DaisyII Incubator (Ankom Technology Corp., Fairport, NY, USA). Heat (39°C) and agitation were maintained constant and uniform in the controlled chamber with continuous rotation. After 48 h of incubation the jars were emptied and the bags were gently rinsed and dried in an oven at 60°C. Bags were then washed with a neutral detergent solution at 100°C for 1 h and rinsed with distilled water into the fibre analyzer, so as to remove bacterial cell walls and other endogenous products. *In vitro* NDF degradation (IVNDFD)

was estimated from the amount of NDF incubated. Each sample was replicated 4 times in 4 incubation runs carried out in different weeks.

7.3.6. Statistical analysis

Chemical composition, kinetics of gas production, in vitro digestibility, estimated energy value and milk production data of pre-ensiled and post-ensiled whole plant maize were analyzed by ANOVA according to a split-split-plot design with the whole plots arranged in a randomized complete-block design, and involving three experimental factors (fixed effects) namely, planting date, maize variety and harvest date. Planting date was the whole-plot factor, maize variety the subplot factor (within each whole plot), and harvest date the sub-subplot factor (within each subplot). Random effects were planting date x block as the whole-plot error (to test planting date effects), planting date x variety x block as the subplot error (to test variety and variety x planting date effects), and the pooled residual error to test harvest date and its interactions with planting date and variety effects. Analyses were carried out with PROC MIXED of SAS (SAS Institute, 2004). Mean values of each parameter and standard error of the mean (SEM) are reported in the Tables.

7.4. Results

7.4.1. Whole plant maize productions

Whole plant maize as-fed and DM yield and grain:whole plant ratio are presented in Table 7.2. Hybrid influenced DM yield of whole plant maize, with Arma hybrid having higher as-fed and DM yield than Cisko hybrid, with a difference of approximately 40% in both cases. The planting date influenced DM yield with a slight increase from the first to the second planting date for Cisko hybrid, and a decrease on the third sowing date for both hybrids. Harvesting maturity of maize silage had an impact on as-fed yield per hectare, with a decrease as maturity advanced for both hybrids, with greater difference in the latter planting date for Cisko than for Arma.

Hybrid, sowing date and harvesting maturity affected grain:whole plant ratio. In the experiment, Cisko hybrid showed higher value than Arma hybrid, with higher differences between hybrids in the second and third planting dates. The contribution of the grain to the whole plant yield increased from 1/4<ML<1/3 to 1/2<ML<2/3, with greater differences between the two harvest date for the Cisko hybrid in the first planting.

Table 7.2
Yield and grain:plant ratio of whole plant maize

Hybrid	*Planting date*	*Maturity at harvest*	Yield (t/ha)		Grain/whole plant (g/g)
			As-fed	DM	
Cisko (FAO 300)	1st planting	1/4 < ML < 1/3	64.4	17.7	0.366
		1/2 < ML < 2/3	57.2	17.9	0.511
	2nd planting	1/4 < ML < 1/3	65.0	20.0	0.471
		1/2 < ML < 2/3	50.9	19.1	0.525
	3rd planting	1/4 < ML < 1/3	54.5	17.1	0.480
		1/2 < ML < 2/3	44.5	16.9	0.509
Arma (FAO 700)	1st planting	1/4 < ML < 1/3	88.7	25.3	0.380
		1/2 < ML < 2/3	80.8	27.0	0.450
	2nd planting	1/4 < ML < 1/3	92.7	26.7	0.395
		1/2 < ML < 2/3	86.3	26.0	0.447
	3rd planting	1/4 < ML < 1/3	73.1	23.2	0.406
		1/2 < ML < 2/3	71.5	23.1	0.486
Planting effect (P)			**	*	*
Hybrid effect (H)			***	***	***
PxH			*	NS	*
Maturity effect (M)			***	NS	***
PxM			NS	*	***
HxM			**	NS	NS
PxHxM			NS	NS	***
SEM					
P			0.99	0.34	0.0056
H			0.81	0.28	0.0045
M			0.68	0.23	0.0040
PxHxM			1.68	0.57	0.0097

ML, milk line.

7.4.2. Chemical composition of pre- and post-ensiled whole plant maize

The chemical composition of pre-ensiled whole plant maize is presented in Table 7.3. The DM concentration for both hybrids increased with the advancing stage of maturity. The ash content was constant across planting, hybrids and harvest dates. The CP, NDF, lignin and

starch concentrations of the pre-ensiled material were affected by hybrid, with Arma having higher values of CP, NDF and lignin, and lower values of starch than Cisko. Harvesting maturity had an effect on the CP and starch concentrations of the pre-ensiled whole plant maize, with lower CP values from the early (1/4<ML<2/3) to mid-late (1/2<ML<2/3) maturity, and a general opposite trend for starch values. The chemical composition of whole maize silage is presented in Table 7.4. All the silages were well fermented with pH values ranging from 3.58 to 4.02 and butyric acid was absent for all hybrids, sowing dates and harvesting maturity (data not shown). The concentration of NDF, with Arma having higher values than Cisko, and ADF declined, whereas the starch concentration increased from the early maturity (1/4<ML<1/3) to mid-late (1/2<ML<2/3), for both hybrids. Maize silages showed little differences from chemical composition of the pre-ensiled material, except for a slightly lower DM and higher starch concentrations.

Table 7.3
Chemical composition of pre-ensiled whole plant maize (g/kg DM)

Hybrid	*Planting date*	*Maturity at harvest*	DM, as fed	Ash	CP	EE	NDF	ADF	Lignin (sa)	Starch
Cisko (FAO 300)	1st planting	1/4 < ML < 1/3	278	44	64	29	455	252	33	296
		1/2 < ML < 2/3	316	39	57	29	434	240	31	318
	2nd planting	1/4 < ML < 1/3	311	43	61	28	457	249	39	298
		1/2 < ML < 2/3	374	39	59	29	433	239	34	316
	3rd planting	1/4 < ML < 1/3	315	39	61	28	437	237	39	319
		1/2 < ML < 2/3	378	40	60	27	446	244	39	313
Arma (FAO 700)	1st planting	1/4 < ML < 1/3	282	45	70	27	461	248	40	279
		1/2 < ML < 2/3	334	44	68	29	452	241	46	299
	2nd planting	1/4 < ML < 1/3	291	48	72	27	478	261	43	262
		1/2 < ML < 2/3	302	45	68	27	465	251	44	281
	3rd planting	1/4 < ML < 1/3	316	44	71	26	485	266	45	259
		1/2 < ML < 2/3	370	40	63	26	470	257	42	286
Planting effect (P)			**	NS	NS	NS	NS	NS	NS	NS
Hybrid effect (H)			*	NS	***	NS	*	NS	**	**
PxH			**	NS	NS	NS	NS	NS	NS	NS
Maturity effect (M)			***	NS	**	NS	NS	NS	NS	*
PxM			NS	NS	NS	NS	NS	NS	NS	NS
HxM			*	NS	NS	NS	NS	NS	NS	NS
PxHxM			**	NS	NS	NS	NS	NS	NS	NS
SEM										
P			3.5	1.6	0.9	0.8	11.3	6.7	1.5	6.8
H			2.9	1.2	0.8	0.6	8.8	5.7	1.2	6.1
M			2.8	1.2	0.8	0.6	8.7	5.6	1.2	6.1
PxHxM			6.5	2.9	1.8	1.2	17.3	10.3	2.9	11.1

ML, milk line; DM, dry matter; CP, crude protein; EE, ether extract; NDF, neutral detergent fibre; ADF, acid detergent fibre; Lignin (sa), lignin determined with sulphuric acid.

Table 7.4
Chemical composition of post-ensiled whole plant maize (g/kg DM)

Hybrid	*Planting date*	*Maturity at harvest*	DM, as fed	Ash	CP	EE	NDF	ADF	Lignin (sa)	Starch
Cisko (FAO 300)	1st planting	1/4 < ML < 1/3	300	51	80	26	471	276	32	298
		1/2 < ML < 2/3	336	46	75	28	443	265	31	344
	2nd planting	1/4 < ML < 1/3	331	39	72	29	426	250	25	351
		1/2 < ML < 2/3	398	40	69	28	422	248	22	339
	3rd planting	1/4 < ML < 1/3	342	37	67	26	450	274	27	338
		1/2 < ML < 2/3	400	38	69	29	412	244	26	366
Arma (FAO 700)	1st planting	1/4 < ML < 1/3	292	47	71	24	474	285	31	302
		1/2 < ML < 2/3	344	34	68	28	424	250	26	356
	2nd planting	1/4 < ML < 1/3	322	38	69	25	476	291	29	304
		1/2 < ML < 2/3	331	38	68	26	442	266	28	333
	3rd planting	1/4 < ML < 1/3	334	37	69	24	471	285	30	313
		1/2 < ML < 2/3	360	35	70	25	456	268	27	322
Planting effect (P)			**	NS	NS	NS	NS	NS	NS	NS
Hybrid effect (H)			**	NS	NS	NS	*	NS	NS	NS
PxH			*	NS	NS	NS	NS	NS	NS	NS
Maturity effect (M)			***	NS	NS	NS	**	**	NS	*
PxM			NS	NS	NS	NS	NS	NS	NS	NS
HxM			**	NS	NS	NS	NS	NS	NS	NS
PxHxM			**	NS	NS	NS	NS	NS	NS	NS
SEM										
P			5.3	2.1	2.1	1.0	10.8	7.7	1.5	8.0
H			4.7	1.8	1.7	0.9	9.3	7.3	1.2	7.3
M			4.7	1.6	1.6	0.9	9.1	7.2	1.0	7.1
PxHxM			7.4	4.1	4.2	1.5	15.0	11.2	2.7	13.9

ML, milk line; DM, dry matter; CP, crude protein; EE, ether extract; NDF, neutral detergent fibre; ADF, acid detergent fibre; Lignin (sa), lignin determined with sulphuric acid.

7.4.3. In vitro digestibility and parameters of gas production kinetics

Treatments (hybrids, planting and sowing dates) and interaction effects on gas production kinetics parameters and *in vitro* digestibility data for pre-ensiled and post-ensiled whole plant materials are presented in Tables 7.5 and 7.6, respectively.

In pre-ensiled material all parameters of gas production estimated were significantly affected by harvesting maturity, with significant differences between hybrids in parameters *c* and *L*, but not in G24 (volume of gas produced after 24h of incubation) and *A*. Planting date only affected G24 values. Cisko hybrid showed higher asymptotic gas production than Arma hybrid, suggesting that Cisko is potentially more degradable than Arma in the second and in the third planting dates. Values of fractional rate (*c*) were lower and lag time was longer for Cisko than for Arma, resulting in faster fermentation rate for Arma compared with Cisko. This was confirmed in the degradability coefficient (extent of degradation, ED) that was higher for Arma, thus more degradable in the rumen than Cisko. Fractional rate of gas production and G24 tended to increase as the sowing date was postponed in the season and harvesting maturity advanced, with greater gas production volumes in whole plant maize seeded in May or harvested at 1/2<ML<2/3. Estimated IVDMD and IVNDFD at 48h of incubation showed an average value of 0.782 g/g DM and 0.522 g/g DM, respectively, and they were not affected by any of the studied treatments, but statistical differences were only observed for hybrid x planting interaction.

Smaller differences were observed for kinetic gas production parameters in silages, except for a significant effect of hybrid on G24 and A parameters, with Cisko showing higher value than Arma, and of harvesting maturity on L value. There was significant interaction between planting date and maturity at harvest for the lag time only, that increase with increasing harvest maturity in the first and third planting date. Significant differences were observed for hybrid effect on IVDMD and D144, with Arma hybrid having the lower values than Cisko hybrid.

Table 7.5
Kinetics of gas production (ml/g DM) and *in vitro* digestibility (g/g DM) of pre-ensiled whole plant maize

Hybrid	*Planting date*	*Maturity at harvest*	Kinetics of gas production				*In vitro* digestibility			
			G24	*A*	*c* (h^{-1})	*L* (h)	IVDMD	IVNDFD	D144	ED
Cisko (FAO 300)	1st planting	1/4 < ML < 1/3	223	352	0.050	3.617	0.781	0.519	0.809	0.432
		1/2 < ML < 2/3	222	356	0.049	4.142	0.772	0.472	0.819	0.428
	2nd planting	1/4 < ML < 1/3	227	359	0.050	3.950	0.791	0.543	0.823	0.435
		1/2 < ML < 2/3	230	363	0.050	3.904	0.811	0.562	0.824	0.436
	3rd planting	1/4 < ML < 1/3	233	360	0.052	3.958	0.787	0.512	0.831	0.447
		1/2 < ML < 2/3	237	359	0.054	3.815	0.772	0.487	0.827	0.451
Arma (FAO 700)	1st planting	1/4 < ML < 1/3	226	350	0.051	3.591	0.793	0.551	0.819	0.442
		1/2 < ML < 2/3	232	353	0.054	4.083	0.786	0.526	0.834	0.452
	2nd planting	1/4 < ML < 1/3	216	335	0.050	3.331	0.770	0.511	0.808	0.436
		1/2 < ML < 2/3	229	343	0.054	3.655	0.768	0.506	0.818	0.451
	3rd planting	1/4 < ML < 1/3	233	348	0.053	3.081	0.778	0.542	0.821	0.458
		1/2 < ML < 2/3	248	364	0.056	3.456	0.780	0.532	0.820	0.460
Planting effect (P)			*	NS	NS	NS	NS	NS	NS	NS
Hybrid effect (H)			NS	**	*	**	NS	NS	NS	*
PxH			NS	*	NS	NS	*	*	NS	NS
Maturity effect (M)			*	*	*	**	NS	NS	NS	NS
PxM			NS	NS	NS	NS	NS	NS	NS	NS
HxM			NS	NS	NS	NS	NS	NS	NS	NS
PxHxM			NS	NS	NS	NS	NS	NS	NS	NS
SEM										
P			2.7	2.3	0.0007	0.0981	0.0052	0.0090	0.0062	0.0044
H			2.3	2.0	0.0006	0.0694	0.0047	0.0069	0.0050	0.0038
M			2.3	2.0	0.0006	0.0694	0.0047	0.0067	0.0050	0.0038
PxHxM			4.8	4.2	0.0013	0.1553	0.0089	0.0159	0.0090	0.0071

ML, milk line; G24, cumulative gas production at 24 h of incubation; *A*, asymptotic gas production; *c*, fractional rate of gas production; *L*, lag time; IVDMD, *in vitro* dry matter digestibility; IVNDFD, *in vitro* neutral detergent fibre degradability; D144, dry matter disappearance after 144 h of incubation; ED, extent of degradation.

Table 7.6
Kinetics of gas production (ml/g DM) and *in vitro* digestibility (g/g DM) of post-ensiled whole plant maize

Hybrid	*Planting date*	*Maturity at harvest*	Kinetics of gas production				*In vitro* digestibility			
			G24	*A*	*c* (h^{-1})	*L* (h)	IVDMD	IVNDFD	D144	ED
Cisko (FAO 300)	1st planting	1/4 < ML < 1/3	229	341	0.051	2.120	0.767	0.508	0.786	0.445
		1/2 < ML < 2/3	247	357	0.056	2.877	0.790	0.528	0.821	0.470
	2nd planting	1/4 < ML < 1/3	234	351	0.052	2.680	0.783	0.488	0.810	0.454
		1/2 < ML < 2/3	240	355	0.052	2.068	0.794	0.543	0.815	0.464
	3rd planting	1/4 < ML < 1/3	240	353	0.053	2.331	0.784	0.508	0.801	0.458
		1/2 < ML < 2/3	237	350	0.053	2.575	0.780	0.465	0.793	0.449
Arma (FAO 700)	1st planting	1/4 < ML < 1/3	229	335	0.053	2.015	0.780	0.537	0.797	0.458
		1/2 < ML < 2/3	229	338	0.054	2.957	0.782	0.490	0.788	0.444
	2nd planting	1/4 < ML < 1/3	231	333	0.055	2.178	0.765	0.512	0.774	0.448
		1/2 < ML < 2/3	223	329	0.052	2.266	0.765	0.461	0.783	0.446
	3rd planting	1/4 < ML < 1/3	229	334	0.054	2.342	0.751	0.470	0.785	0.451
		1/2 < ML < 2/3	229	336	0.054	2.784	0.773	0.506	0.794	0.450
Planting effect (P)			NS	NS	NS	NS	NS	NS	NS	NS
Hybrid effect (H)			*	***	NS	NS	*	NS	**	NS
PxH			NS	NS	NS	NS	NS	NS	NS	NS
Maturity effect (M)			NS	NS	NS	**	NS	NS	NS	NS
PxM			NS	NS	NS	**	NS	NS	NS	NS
HxM			NS	NS	NS	NS	NS	NS	NS	NS
PxHxM			NS	NS	NS	NS	NS	**	*	NS
SEM										
P			2.9	2.0	0.0011	0.1922	0.0055	0.0141	0.0070	0.0053
H			2.3	1.5	0.0010	0.1856	0.0051	0.0119	0.0066	0.0049
M			2.3	1.5	0.0010	0.1856	0.0051	0.0118	0.0066	0.0049
PxHxM			4.8	3.5	0.0016	0.2409	0.0080	0.0221	0.0099	0.0080

ML, milk line; G24, cumulative gas production at 24 h of incubation; *A*, asymptotic gas production; *c*, fractional rate of gas production; *L*, lag time; IVDMD, *in vitro* dry matter digestibility; IVNDFD, *in vitro* neutral detergent fibre degradability; D144, dry matter disappearance after 144 h of incubation; ED, extent of degradation.

7.4.4. Estimated energy value and milk production

The energy value and potential milk yield data of pre-ensiled and post-ensiled maize through Milk2006 model are reported in Table 7.7 and Table 7.8. The hybrid affected milk yield per hectare (kg/ha) of pre-ensiled material, with Arma showing 38% higher value than Cisko. The net energy for lactation and the potential milk production (kg/Mg DM) were similar for the two hybrids and across planting dates, and they were slightly higher for earlier harvesting maturity. The sowing date affected the milk production per hectare, with greater values in the first two planting periods.

The potential milk yield per hectare of the maize silage was affected both by hybrid and sowing date, with greater values for Arma and lower values for the third planting date, whereas the net energy for lactation and the potential milk production (kg/Mg DM) were not affected by any of the studied treatments.

Table 7.7
Estimated energy value and milk production of pre-ensiled whole plant maize (Milk 2006 model)

Hybrid	*Planting date*	*Maturity at harvest*	NE_{L-3x} MJ/kg DM	Milk kg/Mg DM	Milk kg/ha
Cisko (FAO 300)	1st planting	1/4 < ML < 1/3	6.18	1381	24446
		1/2 < ML < 2/3	6.21	1376	24652
	2rd planting	1/4 < ML < 1/3	6.23	1403	28106
		1/2 < ML < 2/3	6.04	1349	25823
	3rd planting	1/4 < ML < 1/3	6.31	1418	24302
		1/2 < ML < 2/3	5.81	1254	21159
Arma (FAO 700)	1st planting	1/4 < ML < 1/3	6.21	1399	35337
		1/2 < ML < 2/3	6.16	1376	37097
	2rd planting	1/4 < ML < 1/3	5.97	1312	34970
		1/2 < ML < 2/3	6.07	1341	34838
	3rd planting	1/4 < ML < 1/3	6.07	1352	31315
		1/2 < ML < 2/3	5.88	1290	29825
Planting effect (P)			NS	NS	*
Hybrid effect (H)			NS	NS	***
PxH			NS	NS	NS
Maturity effect (M)			*	*	NS
PxM			NS	NS	NS
HxM			NS	NS	NS
PxHxM			NS	NS	NS
SEM					
P			0.059	19.5	670.1
H			0.053	17.4	583.1
M			0.053	17.4	552.9
PxHxM			0.101	32.8	1106.5

ML, milk line; NE_{L-3x}, net energy of lactation at 3x of maintenance; DM, dry matter.

Table 7.8
Estimated energy value and milk production of post-ensiled whole plant maize (Milk 2006 model)

Hybrid	*Planting date*	*Maturity at harvest*	NE_{L-3x} MJ/kg DM	Milk kg/Mg DM	Milk kg/ha
Cisko (FAO 300)	1st planting	1/4 < ML < 1/3	5.9	1304	23072
		1/2 < ML < 2/3	6.1	1368	24433
	2nd planting	1/4 < ML < 1/3	6.3	1395	28003
		1/2 < ML < 2/3	6.0	1318	25343
	3rd planting	1/4 < ML < 1/3	6.2	1373	23658
		1/2 < ML < 2/3	5.8	1229	20725
Arma (FAO 700)	1st planting	1/4 < ML < 1/3	6.0	1330	34122
		1/2 < ML < 2/3	6.2	1376	37746
	2nd planting	1/4 < ML < 1/3	6.1	1340	35232
		1/2 < ML < 2/3	6.1	1346	34051
	3rd planting	1/4 < ML < 1/3	5.9	1287	30432
		1/2 < ML < 2/3	5.9	1304	30138
Planting effect (P)			NS	NS	**
Hybrid effect (H)			NS	NS	***
PxH			NS	NS	*
Maturity effect (M)			NS	NS	NS
PxM			NS	NS	*
HxM			*	*	*
PxHxM			NS	NS	NS
SEM					
P			0.11	39.3	1299.9
H			0.10	37.9	1279.9
M			0.09	36.2	1272.8
PxHxM			0.14	50.3	1486.7

ML, milk line; NE_{L-3x}, net energy of lactation at 3x of maintenance; DM, dry matter.

7.5. Discussion

7.5.1. Chemical composition of pre and post-ensiled whole plant maize

Changing management practices could be a method to influence the nutritive value of maize silage. In the current study, advancing the stage of maturity of whole-plant maize at harvest from 1/4<ML<1/3 to 1/2<ML<2/3 increased the DM concentration for both hybrids. These data are similar to those of Hunt et al. (1989), Xu et al. (1995) and Johnson et al. (2002a, 2003), where the whole-plant maize DM concentration increased as maturity advanced. The results could be related to kernel development (Argillier et al., 1995). Furthermore, harvesting maturity had an effect on the NDF and ADF concentrations, which declined as maturity advanced for both hybrids. Similar trends have been reported by Hunt et al. (1989) and Johnson et al. (2002a) who observed a decrease in NDF and ADF concentrations in maize silage from 1/3 ML to 2/3 ML maturity stages. This decrease was related to a higher proportion of grain in mature whole-plant maize, and the results are consistent with those of Argillier et al. (1995) who stated that although the fibre content of the stover increases with maturity, the fibre content of whole-plant maize decreases due to increasing proportion of grain. In this experiment the changes in concentration of starch due to maturity showed an opposite trend when compared with fibre. A similar trend, reported by Johnson et al. (2002a) and Bal et al. (1997), was related to changes in the proportion of grain in whole-plant maize, and

the same results were obtained in this experiment with a higher proportion of grain as maturity advanced.

7.5.2. In vitro digestibility and parameters of gas production kinetics

Digestibility of maize silage was determined by the conventional *in vitro* technique with Ankom procedure following the approach proposed by Van Soest et al. (1966). Rate and extent of ruminal degradation were estimated from gas production profiles derived from *in vitro* incubation of maize and maize silage samples in diluted rumen fluid (Theodorou et al., 1994). This method has been accepted as a sensitive and reliable tool in feed evaluation (López, 2005), because gas production is well correlated with *in vivo* and *in vitro* digestibility (Khazaal et al., 1993, López, 2005) and with microbial protein synthesis (Blümmel et al., 1997). This technique has already been used by DePeters et al. (2007) to compare maize hybrids and processing methods.

In the present study IVDMD and IVNDFD of pre-ensiled samples were not affected by hybrid, planting and maturity effects. These results do not confirm those obtained by other authors (Lewis et al., 2004; Johnson et al., 2002b; Johnson et al., 2003) where DM and/or NDF digestibility were lower at advanced maturity. A partial explanation for these differences can be found in the more advanced stage of maturity studied by these authors than those tested in our study. Different response of hybrids to planting date may be related to different climatic conditions, such as temperatures and growing degree days during the spring months. In silages, observed differences in DM disappearance can be partially

explained by concentration of NDF in the two hybrids. According to Bal et al. (2000) and Johnson et al. (2003) the DM disappearance in maize silage with low NDF was greater than in maize silage with high NDF amount due to increased starch disappearance. Differences in digestibility are primarily associated with the chemical composition of the samples, especially to their cell wall content (Ivan et al., 2005). The cell wall fraction may have a negative influence on digestibility as described in conventional feedstuffs by Van Soest (1994). Cell contents are readily and completely digested, whereas cell walls are slowly and only digested to a certain extent, depending on their degree of lignification.

7.5.3. Estimated energy value and milk production

Yield and nutrient analysis data of maize silage were used as inputs for Milk2006 (Shaver et al., 2006) to estimate the net energy of lactation at 3x maintenance ($NE_{L\text{-}3x}$), milk production per Mg of DM (kg/Mg) and per acre (kg/acre), the latter converted to the metric system (kg milk/ha) for presentation. In vitro NDF digestibility at 48 h of incubation was used as NDF digestibility value.

The 38% higher milk yield per hectare value observed for Arma is in agreement with the yield results, since this hybrid produced about 40% more Mg per hectare than Cisko. Concerning other parameters, energy value and potential milk yield per Mg DM were numerically greater for Cisko than for Arma, but differences were not statistically significant, meaning a substantial equivalence between the two studied hybrids across different planting date and harvesting maturity. Reduced values of

milk production per hectare for the latest planting date mainly reflected the differences observed in DM yield.

In this experiment, the optimum maturity stage for harvesting maize was 1/4<ML<1/3. At this stage, statistically higher values of NE_L and milk production (kg/Mg DM) were observed, but these differences were not reflected on the potential milk yield per hectare (kg/ha). In post-ensiling material, hybrid and planting factors maintained their effects only on milk production per hectare (kg/ha), with greater values for Arma hybrid than for Cisko and reduced values for the latest planting date, as observed in pre-ensiled maize silage.

7.6. Conclusions

In this experiment the late maturing maize hybrid (Arma FAO 700) had a higher yield than the early maturing maize hybrid (Cisko FAO 300). Their chemical compositions and *in vitro* digestibility did not show any significant differences in pre- and post-ensiling conditions. Results of estimated values calculated with Milk2006 emphasized that Arma maize silage allows higher milk yield per hectare than Cisko, but not per Mg of dry matter, the latter being higher for Cisko under pre-ensiling conditions. The stage of maturity has visible effects on pre-ensiled maize silage, but not in post-ensiling conditions, whereas it had increased its influence in the interaction with planting but especially with the hybrid effect. These results suggested that there is great flexibility in harvesting maize silage between the 1/4 to 2/3 milk line.

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8. Effects of inactivated and live cells of *Saccharomyces cerevisiae* on *in vitro* ruminal fermentation of diets with different forage:concentrate ratio

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8.1. Abstract

The effects of a commercial product containing inactivated cells of *S. cerevisiae* (Thepax) and a yeast culture with live cells of *S. cerevisiae* (Yea-sacc) on the ruminal fermentation of two different diets were investigated *in vitro*. Total mixed rations (TMR) having forage:concentrate ratios of 60:40 (medium-high forage diet) and 20:80 (low forage diet) were incubated in batch cultures of mixed ruminal microorganisms to which either inactivated (to reach concentrations of 500 and 250 mg product per l incubation medium) or live yeast (at a concentration of 150 mg product/l) were added directly as powder. To evaluate medium-term effects of the additive on ruminal microbial population, sheep used as donors of rumen fluid were allocated in three experimental groups, two of them receiving a diet with a different additive during 10 days and a control group. With both diets, yeast live cells decreased the ruminal pH compared to control, whereas inactivated yeast had no effect. Adding yeast additives to the high-fibre diet affected total volatile fatty acid (VFA) production and VFA composition, in general with a slight increase with inactivated yeast, and a significantly greater increase in response to the addition of live yeast product. Ammonia nitrogen ($P = 0.006$), total gas production ($P < 0.001$) and *in vitro* dry matter disappearance (IVD) ($P < 0.001$) showed the highest values with the live yeast supplement. Methane production was higher with Thepax inoculum, and increased even more with the Yea-sacc inoculum ($P < 0.001$). With the high-concentrate TMR no effects on total VFA concentration were observed when yeast additives were used.

Similar trends were shown for lactate and methane production and total gas production, where values tended to be higher in Yea-sacc inoculum (P values of 0.055, <0.001, 0.006 and <0.001, respectively). After 144 h of incubation differences were observed only with the high fibre diet in the cumulative gas production at 24 h of incubation and in the average fermentation rate, which were greater with live yeast, although the asymptotic gas production was not affected. These results indicate that live yeasts affect ruminal fermentation to a slightly greater extent than inactivated yeasts, although both products require a regular administration and some adaptation of the ruminal microbial population for the stimulatory effects to become apparent. Moreover the effects of yeasts on ruminal fermentation are diet dependent, being more noticeable with a high-fibre substrate, and subtle with a high concentrate diet.

Keywords: *Saccharomyces cerevisiae* inactivated cells; *Saccharomyces cerevisiae* live cells; Rumen fermentation; *In vitro* digestibility; Gas production technique.

Abbreviations: A, asymptotic gas production; ADFom, acid detergent fibre expressed exclusive of residual ash; c, fractional rate of gas production; G24, cumulative gas production at 24 h of incubation; IVDMD, *in vitro* dry matter digestibility; IVNDFD, *in vitro* neutral detergent fibre degradability; L, lag time; aNDFom, neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash; R, average fermentation rate; RSD, residual standard deviation; TMR, total mixed ration; VFA, volatile fatty acid.

8.2. Introduction

A yeast culture is a fermented feed additive that can contain either live or inactivated yeast cells, the culture media where the yeast cells were grown on, and the metabolic by-products produced by yeast during fermentation (Linn and Raeth-Knight, 2006). The most common yeast additive used in ruminant diets is obtained from cultures of *Saccharomyces cerevisiae*. This additive has been used for many years aiming at enhancing ruminal fermentation to reduce energy and nutrient losses and to improve production efficiency in ruminant production systems. In recent years, with increased consumer's concern about safety, quality of animal products and also environmental issues, antibiotics and synthetic chemical products have been banned as feed additives by the European Union (Anadón, 2006). Alternative additives such as yeasts are used not only to increase productivity, but also to decrease the risk of animal digestive transfer of potential human pathogens and to limit excretion of pollutants (Chaucheyras-Durand et al., 2008).

Some experiments have shown that supplementation of feed ration with yeast additives may improve feed intake and milk production in dairy cattle (Harris and Webb, 1990; Williams et al. 1991; Piva et al., 1993; Kung et al., 1997; Dann et al., 2000; Nocek et al., 2003). These responses are usually related to stimulation of cellulolytic bacteria by yeast cultures (Newbold et al., 1996) that enhance potential fibre digestion in the rumen, and to their potential to prevent a fall in rumen pH by decreasing lactic acid production and/or increasing utilization of lactic acid by some bacteria (Chaucheyras-Durand et al., 1996; Callaway and Martin, 1997).

S. cerevisiae yeasts also affect feed degradability, and the patterns of VFA production (Carro et al., 1992; Zelenak et al., 1994; Guedes et al., 2008). However, yeast responses were not always so consistent because several factors have influenced the controversial experimental results. (Arcos-García et al., 2000). Others authors, in fact, affirm that milk yield, milk composition, body weight gain and feed intake were not affected by the addition of *S. cerevisiae* to the diet (Chiquette, 1995; Kamalamma et al., 1996), with no noticeable effects of this yeast on bacterial counts or bacterial colonization of roughage in the rumen (Chiquette, 1995). Some of the possible causes for the inconsistency could be associated with the characteristic of different yeast strains (Newbold et al., 1995), differences between commercial additives (Mendoza et al., 1995), type of animals and diet composition (Carro et al., 1992; Wallace, 1994; Zeleňák et al., 1994).

Most of the above mentioned *in vivo* and *in vitro* research with yeast additives have been conduced with *S. cerevisiae* cultures that include the yeast and the medium where it was grown on, along with components such as vitamins and other fermentation products, potentially able to stabilize the rumen environment. Over the last few years, attention has turned towards the discrimination between the effects of culture products and live yeast cells on ruminal fermentation, that are processed to not include the whole culture medium and to maintain a high live cell count (Lynch and Martin, 2002). Some reports indicate that live yeast cells do not grow in the rumen but show some degree of viability (Dawson et al., 1990; Hession et al., 1992) and influence the course of rumen fermentation through interactions with ruminal microorganisms (Harrison et al., 1988; Martin et al., 1989; Wallace and Newbold, 1993).

The main effects that have been identified are improvement of rumen environment favouring microbial establishment, stabilization of ruminal pH and interactions with lactate-metabolising bacteria, and increase of fibre degradation and interaction with plant-cell wall degrading microorganisms (Chaucheyras-Duran et al., 2008).
In addition to live cells, recently research and development have provided inactivated cells of *S. cerevisiae* with potential use as alternative additives. According to manufacturers, these products can supply more benefits and advantages compared to live cells. Even though the mechanism of action of inactivated yeast cells are partly similar to those of live cells, they ensure a uniformity of action and a faster or immediate availability of the substances contained within the cells (vitamins or other grown factors) to autochthonous microbiota. However, experimental data regarding the use of this product are scarce (Piva et al., 1993; Mimosi et al., 2008; Fortina et al., 2009).

The objective of this study was therefore to investigate and compare the effects of a commercial product containing inactivated cells of *S. cerevisiae* and a yeast culture with live cells of *S. cerevisiae* on *in vitro* ruminal fermentation of diets with different forage:concentrate ratio. This study also aimed to test whether the potential positive effects of *S. cerevisiae* could be attributed to the yeast's viability or to its content in nutrients and essential microbial growth factors, and how the diet composition could influence the response.

8.3. Materials and methods

8.3.1. Experimental design

The additives tested were a commercial product containing inactivated cells of *S. cerevisiae* (Thepax 100 R, with declared composition of 5 x 10^9 inactivated cells/g of strain GSH351; Dox-Al Italia SpA, Sulbiate, Italy) and a extensively used yeast culture with live cells of *S. cerevisiae* (Yea-Sacc1026, with declared concentration of 1 x 10^9 CFU/g of strain CBS 493.94; Alltech Inc., Ireland). The experiment was carried out to evaluate two different doses of inactivated yeast (to reach concentrations of 500 and 250 mg product per l incubation medium) tested against the live yeast (at a concentration of 150 mg product/l), each of which were added directly as powder to *in vitro* batch cultures of mixed ruminal microorganisms (direct additive treatment (*T*) effect). To evaluate medium-term effects of the additive on ruminal microbial population, sheep used as donors of rumen fluid were allocated in three experimental groups each receiving a diet with a different additive. There was a control group (no additive), a group of sheep receiving Thepax 100 R and a group of sheep dosed Yea-Sacc^{1026}. Additives were administered for 10 days before the starting of the *in vitro* assay to test the effects of the additives on the fermentative activity of the rumen fluid used as inoculum in the *in vitro* trials (adapted rumen fluid or inoculum (*I*) effect). The diets incubated *in vitro* were total mixed rations (TMR's) characterized by medium-high (60:40) and low (20:80) forage:concentrate ratios. Composition and chemical characteristics of diets are reported in Table 8.1.

Table 8.1
Composition and nutrient content (g/kg DM) of experimental diets

Ingredient	High-forage TMR		High-concentrate TMR
Corn silage	423	Corn silage	250
Corn meal	221	Soybean meal	200
Soybean meal	179	Grass hay	200
Reygrass hay	141	Barley	163
Minerals and	17	Beet pulp	90
Buffer salt[b]	12	Beet molases	65
Sodium bicarbonate	7	Sodium bicarbonate	15
		Calcium carbonate	8.2
		Sodium chloride	4
		Wheat bran	1.8
		Dicalcium phosphate	1.7
		Calcium soap (blend)	1.3
Nutrient			
DM, as fed	0.481		0.892
Ash	0.091		0.101
CP	0.146		0.187
EE	0.035		0.026
NDF	0.416		0.254
ADF	0.242		0.156
ADL	0.027		0.021

[a] Containing (per 100 g): Beta carotene, 12 mg; vit. A, 400000 IU; vit. D3, 50000 IU; vit. E, 100 mg; vit. B1, 1.4 mg; vit. B2, 1.5 mg; vit. B6, 0.3 mg; vit. B12, 1 mg; vit. C, 700 mg; biotin, 0.15 mg; choline, 250 mg; Zn, 2 g; Mn, 1.2 g; Fe, 0.5 g; Cu, 0.25 g; I, 0.05 g; Co, 0.02 g; Se, 4 mg.
[b] Containing (per 100 g) : Ca, 20 g; Na, 2.8 g; Mg, 7 g.

8.3.2. In vitro experiments

Animal handling followed the recommendations of European Council Directive 86/609/EEC for protection of animals used for experimental and other scientific purposes, and experimental procedures were approved by the University of León (Spain) Institutional Animal Care and Use Committee. Twelve rumen-fistulated Assaf sheep were assigned randomly to the following treatments: four for the control group, four receiving 3.5 g Thepax 100 R/day per head and four receiving 1.5 g Yea-Sacc /day per head. Yeast products were dosed intraruminally through the cannula once daily at 08:00 h, just before feed was offered. The diet fed to the sheep consisted of alfalfa hay with free access to water. Sheep were fed *ad libitum* during the adaptation period of 10 days. Ruminal contents were collected individually for each donor sheep in thermos flasks before the morning feeding, and taken to laboratory, where ruminal contents from each sheep were strained through two layers of cheesecloth and kept at 39°C under a CO_2 atmosphere.

8.3.2.1. *In vitro* gas production

In vitro gas production measurements were conducted using a pressure transducer as described by Theodorou et al. (1994). Samples of the diet to be incubated (500 ± 10 mg) were weighed out in 120 ml serum bottles to which 50 ml of diluted rumen fluid were dispensed. Rumen fluid was previously diluted (1:4 v/v) with a culture medium containing macro- and micro-mineral solutions, a bicarbonate buffer solution and resazurin, prepared as described by Menke and Steingass (1988). The

medium was maintained at 39°C and saturated with CO_2; oxygen was reduced by the addition of a solution containing cysteine hydrochloride and Na_2S. Blanks (bottles without sample) were used to compensate for gas production in the absence of substrate. Once filled up, bottles were sealed with rubber stoppers and aluminium seals, shaken and placed in the incubator at 39°C. The head-space gas pressure released upon fermentation of feed was measured manually using a pressure transducer at 3, 6, 9, 12, 16, 21, 26, 31, 36, 48, 60, 72, 96, 120 and 144 h after inoculation time. Incubations were performed in two batches carried out in two consecutive weeks with different sources of inocula. Within each batch, 48 vials were incubated per substrate corresponding to 3 inocula (control, Thepax, and Yea-sacc) x 2 sheep per inoculum x 4 additive treatments (control, Thepax1, Thepax2 and Yea-sacc) x 2 serum bottles (duplicates). Gas volume was estimated from pressure measurements using the equation proposed by López et al. (2007). In order to estimate the fermentation kinetics parameters, gas production data were fitted to the exponential model proposed by France et al. (2000):

$$G = A\,[1 - \mathrm{e}^{-c(t-L)}]$$

where G (ml/g DM) is the cumulative gas production at time t; A (ml/g DM) the asymptotic gas production; c (h^{-1}) the fractional rate of fermentation, and L (h) is the lag time.

The average fermentation rate (R, ml gas/h) was defined as the average gas production rate between the start of the incubation and the time at which the cumulative gas production was half of its asymptotic value, and was calculated as:

$$R = Ac \,/\, [2\,(\ln 2 + cL)]$$

Volume of gas (ml/g DM) produced after 24 h of incubation (G24) was used as an index of digestibility and energy feed value, as suggested by Menke and Steingass (1988).

8.3.2.2. *In vitro* 24 h incubation

Samples (500 ± 10 mg) were incubated in serum bottles with diluted rumen fluid in a culture medium as described in the previous paragraph. Incubations were performed in two batches carried out in two consecutive weeks with different sources of inocula. Within each batch, 48 vials were incubated per substrate, corresponding to 3 inocula (control, Thepax, and Yea-sacc) x 2 sheep per inoculum x 4 additive treatments (control, Thepax1, Thepax2 and Yea-sacc) x 2 serum bottles (duplicates). At 24 h of incubation, gas pressure and volume were recorded using a pressure transducer and a graduated syringe, and a gas sample (10 ml) was taken from each bottle and analyzed for methane (CH_4). Then fermentation was immediately stopped by swirling the bottles in ice. Then bottles were open, pH was measured (using a pH-meter), and samples of supernatant were taken for ammonia, lactate and volatile fatty acid (VFA) determinations. Finally, the contents of each serum bottle were filtered using sintered glass crucibles (pore size No. 1) under vacuum and oven-dried at 100°C for 48 h to estimate the DM disappearance at 24 h of incubation. Methane was determined by gas chromatography. The volume of methane (*M*, ml) produced at the end of incubation was calculated from the volume of gas and the gas composition data, as proposed by López et al. (2007):

$M = (G + Vh)\ C$

where *G* is the volume (ml) of total gas produced at the end of incubation (24 h), *Vh* the volume (ml) of the headspace in the serum bottle, and *C* is the proportion of methane in the analysed sample. Samples of diluted rumen fluid, collected at 0 h (before incubation started) and 24 h of incubation, were processed. A 2 ml aliquot was acidified with 2 ml of 0.5 N HCl for ammonia-N (NH_3-N) and lactic acid determination, and a 0.8 ml sample of the supernatant was added to 0.5 ml of a deproteinizing solution (5 g metaphosphoric acid and 1 g crotonic acid in 250 ml of 0.5 N HCl) for VFA determination. Both samples were centrifuged at 14,500 x g for 15 min at 4°C, and supernatants were collected for subsequent analysis. Ammonia-N concentration was determined as described by Weatherburn (1967), VFAs were determined by gas chromatography using crotonic acid as internal standard (Ottenstein and Bartley, 1971) and lactate was determined using a colorimetric assay as described by Taylor (1996).

8.3.2.3. *In vitro* digestibility

In vitro dry matter digestibility (IVDMD) was determined using the Ankom-Daisy procedure following the procedure described by Robinson et al. (1999). Rumen fluid was diluted (1:4 v/v) into the medium, prepared as described by Menke and Steingass (1988). Two incubations were completed with one jar per inocula (control, Thepax and Yea-sacc) in each incubation, each of which was added with respective treatment where necessary (control without addition, Thepax 1 and Yea-sacc) to reach the optimal concentrations of 500 mg inactivated yeast product per l incubation medium and 150 mg/l for live yeast adapted inocula.

Samples of diets (250 ± 10 mg) were weighed into F57 Ankom bags with a pore size of 25 µm, heat-sealed and then placed into an incubation jar. Nine bags per substrate were used, corresponding to 3 treatments (control, Thepax, Yea-sacc) x 3 replications. Each jar was a 5 l glass recipient with a plastic lid provided with a single-way valve which avoids the accumulation of fermentation gases, and was filled with 2 l buffered rumen fluid under anaerobiosis and placed into the DaisyII Incubator (Ankom Technology Corp., Fairport, NY, USA). Temperature (39°C) and agitation were maintained stable and uniform in the controlled chamber with continuous rotation. After 48 h of incubation the jars were emptied and the bags were gently rinsed and dried in an oven at 60°C. Bags were then washed into the fibre analyzer with a neutral detergent solution at 100°C for 1 h and rinsed with distilled water, so as to remove bacterial cell walls and other endogenous products. *In vitro* NDF degradation (IVNDFD) was estimated from the amount of aNDFom incubated.

8.3.3. Chemical analysis

Samples of feed were oven dried at 60°C for 48 h, then ground in a Buhler mill to pass through a 1 mm screen, and assayed in duplicate according to the AOAC (2000) methods for dry matter (DM, method 934.01), ash (method 942.05), crude protein (CP, method 954.01) and ether extract (EE, method 920.39). Neutral detergent fibre (aNDFom), acid detergent fibre (ADFom) and lignin (sa) were determined with the Ankom fibre analyser (Ankom Technology Corp., 1997), following the procedure of Van Soest et al. (1991). Neutral detergent fibre was analyzed with the addition of sodium sulfite to the solution.

8.3.4. Statistical analysis

Data for each type of diet were subjected to ANOVA using GLM of the Statistical Package for Social Science (v 17.0, SPSS Inc., Chicago, Illinois, USA). Separate analyses were performed for each TMR used in the incubations. The statistical model used for gas production kinectis and *in vitro* 24 h fermentation data included the fixed effects of inocula (*I* effects, with levels Control, Thepax and Yea-sac), treatments (*T* effects, with levels Control, Thepax 0.5, Thepax 1 and Yea-sac) and their interaction, and random effects of donor sheep within each inoculum. *In vitro* digestibility data were analysed by one-way ANOVA with the fixed effect of additive treatment (*T* effects, with levels Control, Thepax 1 and Yea-sac) as the only source of variation. Multiple comparisons between treatment means were performed by the Tukey's HSD test, when a significant treatment effect ($P < 0.05$) was observed. The residual standard deviation (RSD) calculated as the square root of the residual mean square (RMS) was reported in the Tables.

8.4. Results

8.4.1. Fermentation parameters at 24 h of incubation

The results of the influence of different yeast cell supplements on *in vitro* fermentation parameters at 24 h of incubation for high-forage TMR are given in Table 8.2. Different adapted inocula (*I*) tested in the experiment affected some parameters, whereas treatments (*T*) added in the batch of fermentation and the interaction (*I x T*) did not show any significant effect ($P > 0.05$). There were no differences between control

and Thepax inocula in the ruminal pH, but values tended to be lower (P = 0.008) with the Yea-sacc inoculum. Ammonia nitrogen (P = 0.006), total gas production (P < 0.001) and *in vitro* dry matter disappearance (IVD) (P < 0.001) showed similar trends, where significant *I* effects were detected with feeding the live yeast supplement that showed the highest values. Methane production (mmol/g incubated matter) was higher with Thepax inoculum, and increased even more with the Yea-sacc inoculum (P < 0.001). When expressed on degraded substrate basis, the highest values of methane production (mmol/g degraded matter) were observed for Thepax inoculum (P = 0.007). Incubation in rumen fluid from sheep supplemented with live yeast (Yea-sac) led to increased total VFA production (P < 0.001), and similar trends were shown for acetate (P < 0.001), butyrate (P = 0.020), valerate (P = 0.002), and the sum of isobutyrate and isovalerate (P = 0.006) outputs, whereas the acetate:propionate ratio was increased with Thepax inoculum (P = 0.001). The production of propionate decreased in Thepax, but increased in Yea-sacc inoculum (P < 0.001). The molar proportions of individual VFAs were affected by yeast products, so that acetate decreased and valerate increased in the Yea-sacc inoculum (P = 0.046 and P = 0.039, respectively), whereas acetate increased (P = 0.046) and propionate decreased (P = 0.004) with Thepax inoculum. Table 8.3 shows the effects of different yeast cell products on *in vitro* fermentation when the high-concentrate TMR was incubated. There were significant differences only due to adapted inocula, whereas treatment and interaction *I x T* did not present differences. Similar trends were shown for lactate and methane production (either per g of incubated material or of degraded matter) and total gas production, where values tended to be higher when the high-

concentrate TMR was incubated in Yea-sacc inoculum (P values of 0.055, <0.001, 0.006 and <0.001, respectively). The pH showed an opposite trend, decreasing with the Yea-sacc inoculum (P = 0.033). In general, VFA production was not affected by the effects tested, with only propionate production being decreased slightly with Thepax and slightly increased with Yea-sacc inoculum (P = 0.003).

Table 8.2
Influence of different yeast cells products on *in vitro* fermentation of high-forage TMR (24 h, DM basis)

	Inoculum			Treatment				RSD	P value		
	Control	Thepax	Yea-sacc	Control	Thepax 0.5	Thepax 1	Yea-sacc		*I*	*T*	*I x T*
pH	6.65[b]	6.66[b]	6.61[a]	6.64	6.64	6.64	6.64	0.032	0.008	NS	NS
NH_3-N (mg/l)	276[a]	261[a]	339[b]	280	296	304	288	40.4	0.006	NS	NS
L-Lactate (mg/l)	10.78	11.18	11.39	10.00	11.18	11.18	12.10	1.809	NS	NS	NS
CH_4 (mmol/g)	1.41[a]	1.65[b]	1.83[c]	1.57	1.57	1.70	1.64	0.071	<0.001	NS	NS
CH_4 (mmol/g deg)	2.35[a]	2.66[b]	2.53[a,b]	2.46	2.46	2.62	2.51	0.152	0.007	NS	NS
CH_4 (mmol/mol gas tot)	233	241	227	232	244	230	231	19.1	NS	NS	NS
Gas tot (ml/g)	143[a]	153[a]	182[b]	155	157	163	161	8.9	<0.001	NS	NS
IVD (g/g)	0.610[a]	0.623[a]	0.729[b]	0.653	0.658	0.651	0.653	0.0316	<0.001	NS	NS
VFA (mmol/g)											
Acetate	2.802[a]	2.938[a]	3.388[b]	2.959	3.133	3.061	3.017	0.2074	<0.001	NS	NS
Propionate	1.119[b]	0.959[a]	1.258[c]	1.076	1.137	1.115	1.122	0.0316	<0.001	NS	NS
Butyrate	0.504[a]	0.582[a,b]	0.687[b]	0.570	0.607	0.600	0.587	0.1095	0.020	NS	NS
Valerate	0.064[a]	0.066[a]	0.090[b]	0.070	0.074	0.074	0.077	0.0129	0.002	NS	NS
Isobutyrate + isovalerate	0.092[a]	0.110[a]	0.169[b]	0.120	0.126	0.126	0.122	0.0447	0.006	NS	NS
Total VFA	4.617[a]	4.717[a]	5.600[b]	4.853	5.130	4.984	4.944	0.3194	<0.001	NS	NS
Acetate:propionate (mol:mol)	2.526[a]	3.072[b]	2.672[a]	2.741	2.789	2.766	2.732	0.2049	0.001	NS	NS
VFA (mmol:mmol)											
Acetate	0.610[a,b]	0.630[b]	0.599[a]	0.614	0.618	0.616	0.604	0.0316	0.046	NS	NS
Propionate	0.242[b]	0.206[a]	0.230[b]	0.226	0.224	0.224	0.231	0.0183	0.004	NS	NS
Butyrate	0.111	0.125	0.123	0.119	0.119	0.120	0.123	0.0158	NS	NS	NS
Valerate	0.014[a]	0.014[a,b]	0.016[b]	0.014	0.014	0.015	0.015	0.0018	0.039	NS	NS
Isobutyrate + isovalerate	0.021	0.024	0.030	0.025	0.024	0.025	0.026	0.0070	NS	NS	NS

I, inoculum effect; *T*, treatment effect; *I x T*, inoculum x treatment interaction; *VFA,* volatile fatty acid; IVD, *in vitro* dry matter digestibility; a-c within the same column means of each effect without a common letter are significantly different ($P < 0.05$); RSD, residual standard deviation.

Table 8.3
Influence of different yeast cells products on *in vitro* fermentation of high-concentrate TMR (24 h, DM basis)

	Inoculum			Treatment				RSD	P value		
	Control	Thepax	Yea-sacc	Control	Thepax 0.5	Thepax 1	Yea-sacc		*I*	*T*	*I x T*
pH	6.69[b]	6.69[a,b]	6.66[a]	6.68	6.68	6.68	6.68	0.020	0.033	NS	NS
NH_3-N (mg/l)	406	380	431	391	412	423	397	48.5	NS	NS	NS
L-Lactate (mg/l)	8.41[a]	9.33[a,b]	11.02[b]	8.81	9.89	9.94	9.70	1.942	0.055	NS	NS
CH_4 (mmol/g)	1.60[a]	1.67[a]	1.78[b]	1.66	1.69	1.68	1.71	0.063	<0.001	NS	NS
CH_4 (mmol/g deg)	2.05[a]	2.16[a,b]	2.26[b]	2.12	2.16	2.17	2.17	0.100	0.006	NS	NS
CH_4 (mmol/mol gas tot)	230	233	235	231	237	232	230	11.2	NS	NS	NS
Gas tot (ml/g)	152[a]	159[a]	169[b]	160	157	161	162	6.2	<0.001	NS	NS
IVD (g/g)	0.781	0.777	0.789	0.784	0.781	0.777	0.787	0.0129	NS	NS	NS
VFA (mmol/g)											
Acetate	3.138	3.180	3.384	3.250	3.200	3.277	3.208	0.1949	NS	NS	NS
Propionate	1.019[a,b]	0.942[a]	1.083[b]	1.007	1.005	1.025	1.022	0.0633	0.003	NS	NS
Butyrate	0.711	0.724	0.718	0.713	0.719	0.713	0.728	0.0949	NS	NS	NS
Valerate	0.105	0.102	0.104	0.101	0.103	0.108	0.103	0.0129	NS	NS	NS
Isobutyrate + isovalerate	0.211	0.207	0.215	0.209	0.218	0.214	0.202	0.0447	NS	NS	NS
Total VFA	5.181	5.164	5.513	5.267	5.257	5.364	5.256	0.3115	NS	NS	NS
Acetate:propionate (mol:mol)	3.092	3.387	3.133	3.259	3.192	3.207	3.159	0.2588	NS	NS	NS
VFA (mmol:mmol)											
Acetate	0.602	0.616	0.614	0.618	0.608	0.607	0.610	0.0158	NS	NS	NS
Propionate	0.199	0.182	0.197	0.191	0.191	0.194	0.195	0.0129	NS	NS	NS
Butyrate	0.135	0.140	0.130	0.134	0.137	0.134	0.135	0.0129	NS	NS	NS
Valerate	0.021	0.020	0.019	0.020	0.020	0.020	0.020	0.0018	NS	NS	NS
Isobutyrate + isovalerate	0.041	0.040	0.039	0.039	0.041	0.040	0.038	0.0053	NS	NS	NS

I, inoculum effect; *T*, treatment effect; *I x T*, inoculum x treatment interaction; *VFA,* volatile fatty acid; IVD, *in vitro* dry matter digestibility; a-b within the same column means of each effect without a common letter are significantly different ($P < 0.05$); RSD, residual standard deviation.

8.4.2. In vitro parameters of gas production kinetics and digestibility

The effect of *Saccharomyces cerevisiae* on *in vitro* gas production kinetic parameters at 144 h of incubation for the total mixed rations are presented in Table 8.4. The *A*, *c* and *L* parameters of high-forage diet showed no statistically significant differences due to inocula used in the experiment, treatment or *I x T* interaction. The Yea-sacc inoculum gave rise to a significantly (P = 0.010) higher cumulative gas production at 24 h of incubation (P = 0.010) and average fermentation rate (P = 0.014) than control and Thepax inocula, whereas *T* and *T x I* interaction effects were not significant (P > 0.05). In the high-concentrate TMR, none of the fermentation kinetics parameters were affected by inoculum, treatment and interaction effect. Data regarding the digestibility at 48 h of diets are presented in Table 8.5. Neither IVDMD nor IVNDFD for both TMRs were affected by the different yeast cells products used in the *in vitro* trial, showing values of IVDMD between 0.805 and 0.818 for high-forage TMR and between 0.860 and 0.871 g/g for high-concentrate diet, and values of IVNDFD between 0.530 and 0.561 for high-forage TMR and between 0.447 and 0.490 g/g for high-concentrate TMR.

Table 8.4
Influence of different yeast cells products on *in vitro* parameters of fermentation of diets (144 h, DM basis)

	Inoculum			Treatment				RSD	P value		
High-forage TMR	Control	Thepax	Yea-sacc	Control	Thepax 0.5	Thepax 1	Yea-sacc		*I*	*T*	*I x T*
A (ml/g)	334	340	342	336	340	343	336	15.5	NS	NS	NS
c (h^{-1})	0.045	0.044	0.049	0.046	0.045	0.046	0.046	0.0039	NS	NS	NS
L (h)	2.841	3.102	2.726	2.876	2.683	2.817	3.183	1.2386	NS	NS	NS
G24 (ml/g)	205[a]	203[a]	220[b]	209	209	214	207	10.0	0.010	NS	NS
R (ml/h)	9.12[a]	8.95[a]	10.08 [b]	9.37	9.35	9.61	9.21	0.690	0.014	NS	NS
High-concentrate TMR											
A (ml/g)	307	306	304	304	303	309	308	13.1	NS	NS	NS
c (h-1)	0.045	0.045	0.049	0.047	0.046	0.045	0.048	0.0030	NS	NS	NS
L (h)	1.096	0.786	1.023	0.930	1.036	0.977	0.930	0.7113	NS	NS	NS
G24 (ml/g)	199	199	205	204	196	200	206	12.6	NS	NS	NS
R (ml/h)	9.28	9.53	10.31	9.73	9.30	9.76	9.93	0.848	NS	NS	NS

I, inoculum effect; *T*, treatment effect; *I x T*, inoculum x treatment interaction; *A*, asymptotic gas production; *c*, fractional rate of gas production; *L*, lag time; G24, cumulative gas production at 24 h of incubation; R, average fermentation rate; a-b within the same column means of each effect without a common letter are significantly different ($P < 0.05$); RSD, residual standard deviation.

Table 8.5
Influence of different yeast cells products on *in vitro* digestibility of diets (48 h, DM basis)

	Treatment			RSD	P value
High-forage TMR	Control	Thepax 1	Yea-sacc		
IVDMD (g/g)	0.818	0.813	0.805	0.0141	NS
IVNDFD (g/g)	0.561	0.551	0.530	0.0316	NS
High-concentrate TMR					
IVDMD (g/g)	0.866	0.871	0.860	0.0060	NS
IVNDFD (g/g)	0.470	0.490	0.447	0.0316	NS

IVDMD, *in vitro* dry matter digestibility; IVNDFD, *in vitro* neutral detergent fibre degradability; RSD, residual standard deviation.

8.5. Discussion

The use of *Saccharomyces cerevisiae* in the ruminant diets has been shown to have affect ruminal fermentation, but it is not clear whether the effects could be attributed to interactions of active yeast with ruminal microorganisms (probiotic effect) or to the supply of essential nutrients contained in the yeast culture medium that stimulate the growth of certain rumen microorganisms (prebiotic effect). On the other hand, results are somewhat inconsistent throughout the literature, partially because effects are dependent upon the ration composition. Therefore, a study was designed to test two different doses of inactivated yeast supplement against a live yeast commercial product on *in vitro* ruminal fermentation of medium-high fibre and high concentrate rations. Doses of yeast products used in the assays were calculated on the basis of the amounts administered to the animals according to the manufacturer (3.5 g/day per sheep of Thepax 100 R and 1.5 g/day per sheep of Yea-Sacc^{1026}). The theoretical concentration of the products in the rumen was calculated assuming an average rumen volume, and the amounts added to the batch cultures were calculated to reach a similar concentration in vials and jars used in the *in vitro* assays. Live yeasts are viable microorganisms that become active in the rumen and may interact with other ruminal microorganisms (bacteria, protozoa or fungi) (Chaucheyras-Durand et al., 2008). The product will also supply the culture medium were the yeast was grown, containing some essential growth factors that may favour the ruminal microbes (Linn and Raeth-Knight, 2006). The inactivated yeast

will contain culture medium, but also whole yeast cells that have the potential to stimulate fermentation (Fortina et al., 2009). Several mechanisms have been suggested for this stimulatory effect. Thus, whole yeast cells are a source of nutrients (sterols and long chain fatty acids from the cell membrane, or amino acids, nucleotides, vitamins and minerals from the cell cytoplasm), as suggested by Choudhari et al. (2008). The yeast cell wall could play an important role, as it contains glucans and mannoproteins which have been considered as valuable prebiotics for ruminal microbes.

It is well-worthy to stress that no effects of either inactivated or live yeasts were observed when the yeast was added directly to batch cultures, with none of the diets used as fermentation substrate. Any significant differences observed in the present study were among the inocula used in the fermentation, obtained from animals that have received control or supplemented diets for a relatively extended period of time. This is an important observation and could explain the disparity of responses to live yeasts and yeast culture found in several *in vitro* studies reported in the literature. Our experiment was designed to test not only acute, immediate, responses to yeast additives, but also the effects on ruminal microorganisms and environment when the additive was administered regularly. The fact that the direct addition of yeast product to batch cultures (with 24-h or 144-h incubations) had no effect on *in vitro* ruminal fermentation, whereas some noticeable differences were observed among inocula from animals receiving no yeast or one of the yeast products tested, indicates that yeasts induce changes in the rumen microbial population, requiring some long-standing adaptation to and interaction with yeasts for changes in ruminal fermentation pattern to

become apparent. In contrast, yeast would not induce prompt changes in the ruminal microbial population, and thus short-term shifts in fermentation pattern cannot be expected in response to yeasts products.

With both diets, yeast live cells decreased the ruminal pH compared to control, whereas inactivated yeast had no effect. In all cases the pH values remained above 6.5, the physiological range of a healthy rumen. Inconsistent effects of *S. cerevisiae* on ruminal pH have been reported in numerous *in vivo* and *in vitro* studies. Our results could be partially consistent with those observed by Lynch and Martin (2002), where live cells decreased ruminal pH when alfalfa hay was incubated, with final values above 6.0. However, live yeast did not affect ruminal pH when a more concentrate substrate was fermented (Lynch and Martin, 2002). Adding inactivated yeast culture had no effect on pH values when total mixed rations with variable forage to concentrate ratios (ranging from 40:60 to 67:33) were fermented (Piva et al., 1993; Enjalbert et al., 1999; Erasmus et al., 2005) in agreement with our results. In contrast, other authors have reported a slight rise in ruminal pH in response to the addition of a live yeast supplement to the diet (Nocek et al., 2002; Bach et al., 2007; Thrune et al., 2009). Guedes et al. (2008) observed that when maize silage was fermented, the effect of the yeast culture on pH was consistent with the changes observed in ruminal lactate concentration. Increasing activity of lactate-utilizing bacteria and/or decreasing of activity of lactate producing bacteria will cause a decrease in lactate concentration giving rise to higher pH values in the rumen. Decreased lactate concentration has been reported in response to the addition of live yeast when forage or concentrates were fermented (Lila et al., 2004). In our study, lactate concentration was increased when yeast was added to a

high-concentrate diet in agreement with results reported by Lynch and Martin (2002) using ground corn as fermentation substrate. Therefore, these variations could be a consequence of the interaction between the *S. cerevisiae* and lactate-metabolising bacteria, such as *Streptococcus bovis*, *Megasphaera elsdenii* or *Selenomonas ruminantium*, when competing for the utilization of sugars, regarding yeast live cells, or through the supply of growth factors (such as amino acids, peptides, vitamins and organic acids) in the case of yeast culture. Ruminal ammonia nitrogen concentration was not affected by inactivated or live yeast supplements to a high-concentrate ration, consistently with other studies (Lila et al., 2004; Erasmus et al., 2005; Guedes et al., 2008) using different substrates in their experiments. However, with the high-fibre diet the addition of Yea-sacc resulted in higher ammonia concentrations. The unexpected increases of lactate and ammonia-N production observed upon the addition of yeast live cells, could be due to the level of yeast inclusion used in the experiment, as suggested by Newbold et al. (1995), who concluded that greater outputs of fermentation end-products detected in the Rusitec fermenters supplemented with yeast additives could represent a response to increased substrate supply rather than a shift in the fermentation pattern.

Supplementation of the high-fibre diet with yeast additives had effects on total VFA production and VFA composition, in general with a slight increase when inactivated yeast culture was added, and a significantly greater increase in response to the addition of live yeast (Yea-sacc) product. This result is consistent with the slight decline in the rumen pH discussed above, and with data obtained when yeast culture was added to maize silage (Guedes et al., 2008) or to a high forage diet (Enjalbert et

al., 1999), or when live yeast cells were added to a hay plus concentrate diet (Lila et al., 2004). No effects on total VFA concentration were observed when yeast additives were used with the high-concentrate TMR, in agreement with other published data (Piva et al., 1993; Doreau and Juoany, 1998). The increase in the molar proportion of acetate observed when inactivated yeast culture was added to a high-forage TMR is also in agreement with results reported by Mutsvangwa et al. (1992) testing a yeast culture. Increased acetate to propionate ratios in response to inactivated Thepax yeast supplementation occurred because acetate increased at the expense of propionate but, in general, acetate to propionate ratio measured in the batch cultures would be within the range of a good fibre digestion. In agreement with our results, Lila et al. (2004) also observed an increase in propionate molar proportion when live yeast was added to starchy substrates. The change in VFA concentration and/or molar proportion observed in our experiment can be explained by modification of bacterial population in response to yeast supplementation. Acetate formation is mainly due to structural carbohydrate fermentation by cellulolytic bacteria, whereas the fermentation of non-structural carbohydrate by amylolytic bacteria leads to a relatively greater production of propionate. Microbial changes that occur within the rumen in response to *Saccharomyces cerevisiae* addition to the diet was previously observed as increased (Newbold et al., 1996) or decreased (Mathieu et al., 1996) numbers of total viable bacteria, increased (Wiedmeier et al., 1987; El Hassan et al., 1996; Newbold et al., 1996), unchanged (Dawson et al., 1990; Erasmus et al., 1992; Yoon and Stern, 1996) or decreased (Mathieu et al., 1996) counts of cellulolytic bacteria, and no effects (Kumar et al., 1994; Yoon and Stern, 1996) in

amylolytic bacteria. These reported trends towards an increased ratio of cellulolytic to amylolytic bacteria could therefore lead to a change in VFA production and an increased acetate:propionate ratio.

In vitro DM and NDF digestibility were not affected by yeast products with both diets considered. A similar response in digestibility of bermudagrass hay was observed by Lynch and Martin (2002) when both yeast culture and live cells were added, and by Carro et al. (2002) with addition of yeast culture on 50 forage:50 concentrate ration. With both TMR's used in our study, total gas production was increased when live yeast was added, in agreement with Lila et al. (2004), whereas the inactivated product had no effect on fermentation gas. Both fermentation gas and propionate production were increased when substrates were fermented in rumen fluid obtained from animals supplemented with Yea-sacc, suggesting that propionate would derive from the succinate pathway explaining the higher total gas volume released, probably in the form of carbon dioxide amount (Wolin and Miller, 1988). As both gas production and substrate digestibility were increased with the high-forage diet when Yea-sacc inoculum was used, fermentation efficiency (mg DM degraded/ ml gas production) was not affected. Methane production was increased in response to the addition of inactivated yeast and, to a greater extent, in response to Yea-sacc. This increase is consistent with the higher acetate production, suggesting that fermentation may have been shifted to an acetogenic pathway.

The results obtained from 144 h incubations showed differences, only with the 60:40 forage:concentrate ration, in the cumulative gas production at 24 h of incubation and in the average fermentation rate, which were greater with live yeast, although the asymptotic gas

production was not affected. Dowson (1990) and Williams et al. (1991) suggested that ruminal microorganisms could be stimulated by yeasts at initial stages of fermentation, but these effects would become negligible in the medium-term with long-lasting fermentations.

8.6. Conclusions

Both inactivated and live yeast products tested in the trial may stimulate ruminal fermentation, although such effect requires a regular administration of the product and some adaptation of the ruminal microbial population for the stimulatory effects to become apparent. Based on our results, live yeasts would affect ruminal fermentation to a slightly greater extent than inactivated yeasts, but none of them showed immediate acute effects. On the other hand, effects of yeast on ruminal fermentation were diet dependent, being more noticeable with a high-fibre substrate, and subtle with a high concentrate diet.

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9. **General conclusions**

The assessment of the nutritive value of forages is important for an efficient management of ruminant nutrition. The contribution of forages to production system can vary according to their ability in supporting maintenance and/or production requirements due to their chemical composition, rumen degradability and digestibility. An adequate knowledge and a better use of forage sources would therefore play a major role in the economic strategy of farms. So estimative *in vitro* methods were applied in this thesis to obtain information on forage quality and animal response.

The nutritive value of representative forage sources from Piemonte region (N-W Italy), which differ by method of conservation, forage species, maturity stage or harvest season, was discriminated and classified testing different analytical methods, including *in vitro* systems and NIRS technique. Forage samples could be classified correctly according to the type of conservation only when chemical composition data were used in the PCA analysis. However, using absorbance data it was not possible to discriminate different clusters according to the botanical composition, and no categories could be identified within each method of conservation. The proper discrimination of botanical groups was not possible due to the high heterogeneity of the samples included in the study, which caused the overlapping of different clusters.

The management practices of maize silage were analyzed in experimental conditions to assess their influence on characteristics that affect the nutritive value and quality. Corn silage is widely used in ruminant nutrition due to its high yield and nutritive value. In the experiment, the comparison between two hybrids planted and harvested at different time showed some differences in chemical compositions and *in vitro* digestibility which influenced milk yield per hectare. The results also suggested that there is some flexibility in harvesting corn silage at the different maturity stages considered in this trial.

Regarding the animal performances, some improvements can be obtained through manipulation of rumen environment in order to prevent health disorders and to optimize performances. Some additives such as *Saccharomyces cerevisiae* yeast are widely used in ruminant nutrition, but responses on rumen fermentation have been variable according to several studies. The inactivated and live yeast products tested in the trial may stimulate ruminal fermentation, although such effect requires a regular administration of the product and some adaptation of the ruminal microbial population for the stimulatory effects to become apparent. Based on our results, live yeasts would affect ruminal fermentation to a slightly greater extent than inactivated yeasts, but none of them showed immediate acute effects. On the other hand, effects of yeast on ruminal fermentation were diet dependent, being more noticeable with a high-fibre substrate, and subtle with a high concentrate diet.

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